Chapter One

Introduction

1.1 Background to the Study

The abuse and misuse of drugs is a serious public health concern worldwide and has become one of the biggest societal issues in several nations of the world[1]. According to the World Drug Report 2021, published by the United Nations Office on Drugs and Crime (UNODC), an estimated 275 million people used drugs at least once in 2020, and over 36 million people suffered from drug use disorders, representing a 22 % increase in the last decade[2]. Different kinds of drugs have been reportedly abused, especially by college students, and young adults. The National Survey on Drug Use and Health (NSDUH) conducted in 2019 by the Substance Abuse, and Mental Health Services Administration (SAMHSA) estimated that 19.4 million adults (aged 18 or older) in the United States had a substance use disorder (SUD) in the past year[3]. In Africa and Sub-Saharan Africa, there is a report of drug abuse involving a large illicit drug trade. The report elaborated that illegal drug consumption in South Africa doubles world norms, with the largest illegal drug markets in Sub-Saharan Africa[4].

An estimated number as high as 3.6 million Nigerians were estimated to have drug abuse problems in 1996[5]. In 2018, a study steered by the National Bureau of Statistics (NBS) established the huge scale of Nigeria's drug challenge. The survey indicated that, in 2017, approximately 15 percent of the adult population in Nigeria (around 14.3 million people) reported a mandatorily significant level of use of psychotropic substances[5,6]. The drug data statement of the National Drug Law Enforcement Agency (NDLEA) showed that all categories of illicit drugs are widely abused in all the states of the federation, including the Federal Capital Territory (FCT)[4,7]

The indiscriminate use of contraceptives by young ladies has become worrisome in the last decade and has been of global concern. The prevalence of sexual experience among young girls is now alarmingly high, as they get introduced to sex as early as 12 to 15 years of age[8]. Factors such as early onset of puberty, peer pressures, exposure to pornographic movies and life scenes, lack of communication between parents and teenagers, adverse opinions on premarital sexual abstinence, social media, a low level of sexual knowledge, late marriage, and increased sexual partner concurrency have greatly contributed to increased sexual activities among young adults[9,10,11]. Many negative consequences attributed to unplanned pregnancy have led to the indiscriminate use, and abuse of emergency contraceptives (EC). The perceived societal stigmatization, alienation from families, perceived effect on education, stringent financial implications, and illegalization of abortion are some of the reasons many go to any length to prevent an unwanted or untimed pregnancy[12,13].

'Emergency contraception' is the use of a pill or device to prevent pregnancy after unprotected sexual intercourse[14,15]. Emergency contraceptive pills (ECPs) are hormonal oral contraceptives that a female can take within 72 hours after unprotected sexual intercourse. They are not recommended as a regular family planning method but are useful after unprotected sexual intercourse to reduce the chance of unwanted pregnancies, ensure optimum spacing among children, and reduce child and maternal mortality[15]. They are sometimes called "morning after" pills or postcoital contraceptives. They are used in situations of contraceptive failure, failure of barrier methods such as slippage and breakage of condoms, unpredicted sexual intercourse, coerced unprotected sex, or in cases of sexual assault[16].

Globally, hormonal contraceptives are used by the majority of sexually active females who are still ovulating and under their fertility phase or childbearing age. A high percentage of these

users are adolescents and young unmarried females[17]. Hormonal contraceptives, which are usually adopted as a method of preventing pregnancy because they have been proven to be effective when used between 1 and 3 days of sexual intercourse, work primarily by preventing or delaying the release of eggs from the ovaries (ovulation) and impairing luteal function. It also prevents pregnancy by inhibiting the fertilization of an egg by sperm cells, prevents sperm migration and function in the genital tract by thickening the cervical mucous, and/or prevents implantation by thinning the endometrium. The Ulipristal acetate (Yuzpe method) also delays ovulation and inhibits the follicular ruptures especially when taken prior to or immediately after the luteinizing hormone (LH) surge. Other action mechanisms include reducing the endometrial thickness, endometrial maturation delay, and alterations in the progesterone–dependent markers required for implantation. This action reduces the receptivity potential of the uterus to a trophoblast. Combined ECP work by inhibiting implantation of a fertilized egg. Other postulated mechanisms include delaying or suppressing ovulation, interfering with corpus luteum function and making changes in the endometrium that prevents implantation [18]. However, they do not work if a woman is already pregnant. The sooner a contraceptive is taken, the greater its efficiency[16,19].

Emergency contraceptives are formulated differently, have different brand names, and are taken at different doses and times. There are those containing progestin alone or progestin, and estrogen together. They are like the natural hormones, progesterone and estrogen, in a woman's body. ECP products formulated with levonorgestrel only (LNG), or estrogen and levonorgestrel combined (LNG-EE), or ulipristal acetate (UPA); Progestin-only pills with levonorgestrel or norgestrel; Combined oral contraceptives with estrogen, and progestin—levonorgestrel, norgestrel, or norethindrone (also called norethisterone) are taken in doses taken once, twice, or

within specified intervals. They are available for purchase over the counter and can be gotten without a doctor's prescription in many pharmacies, chemist stores, drug stores, or other superstores[16,18,19].

Available brands include Postinor 2, My Way, After Pill, E Contra, After A, Option 2, Post pill, PLAN B, One Step, Ella One, Take Action, and Morning After Pills. There are also those originally formulated for other purposes that are used as ECPs, such as misoprostol, mestrogen, and gynaecosid. Other birth control methods such as injectables, combined birth control pills, and Intrauterine devices (IUDs) are also used as regular contraception methods by sexually active females. Studies have also indicated the use of herbs, and concoctions, probably because of misinformation, ignorance, availability, accessibility, affordability, and ease of administration. There is no known social marketing campaign for these methods of contraception in this part of the world[16,20].

Among the several ECPs commercially available, high-dose progestogen (Levonorgestrel) is the most commonly known and used type[16]. There have been reports on the misuse, and abuse of ECPs, especially postinor-2, such as taking more than the required doses and even multiple times in order to increase their effectiveness or due to ignorance. Often times, information about contraceptives is transferred among young females or via the internet, as parents often fail to provide contraceptive information and sex education to their children due to religious and socio-cultural reasons[21]. It has been reported that many students who engage in sexual activities lack knowledge about contraceptives, while many others do not know how to use birth control pills appropriately[22,23]. This was contributed to by inadequate sex education, and reproductive health knowledge, even among medical practitioners who are supposed to educate the public. Additionally, proper consultation on the dosage and use of ECPs with medical practitioners is

not often done[24]. A study by Jolic and his colleagues[25], implicated poor knowledge of contraceptives among Pharmacists. This has motivated a recent legislative call to facilitate the involvement of Pharmacists in ensuring the discharge of adequate information to patients and prescribers on increasing knowledge and adherence as well as healthy options in contraceptive care[25]. Also, a lack of in-depth knowledge as well as misconceptions among Nursing, and midwives' student has been reported[26]. It was also observed that resident Doctors received very little EC education with Obstetrics/gynecology having more knowledge than their counterparts in family medicine[24].

There are also concerns over the indiscriminate or incessant use of the drug since such practice may have toxicological and chronic effects on the body system[19,27,28]. However, it has not been measured how long the hormonal contraceptives may be used before they cause toxicity in the female body or at what dosage; as toxicity is affected by several factors, including immunity, dosage, frequency of usage, duration of usage, and so on. The usage of hormonal contraceptives for a longer period or incessantly has the propensity to cause damage to vital internal organs of the female body, cause endocrine disruption, gonadal impairment, genetic alterations, which may lead to infertility through recurrent failed attachment of blastocysts to the uterus[29,30]. There is limited information on the possible contribution of indiscriminate contraceptive use to uterine fibroid development, recurrent implantation failure (RIF), failed *in vitro* fertilization procedures, and more generally, the health of the woman. There is a possibility that the usage of hormonal contraceptives for a longer period or incessantly has the propensity to cause damage to vital internal organs of the female body, which may lead to infertility by inducing recurrent spontaneous abortions and failed attachment of blastocysts to the uterus. It may also cause aberrations in non-reproductive organs such as the liver, and the kidney.

Generally, hormonal contraceptives are manufactured from synthetic hormones, which have been implicated in depression, mood swings, incessant headaches or dizziness, nausea, cancer, irregular menstrual cycles, acne, enlarged or tender breasts, enlarged hips, vaginal bleeding or spotting, and fatigue[21].

There have been a few conflicting reports on the effects of postinor-2 demonstrated in animal studies[27,28,29]. Studies have associated the use of Postinor-2 with an increased occurrence of ectopic pregnancy as well as altered tubal environments and impaired embryo-tubal transportation[31,32,33]. It was suggested in a study using Mongolian gerbils that LNG may have inhibitory effects on uterine growth via its direct progestational effects. Decreased stromal density, and atrophy of glandular epithelium have also been observed after LNG treatment in mice[35]. Cigarette smoking has been noted to exacerbate the toxic effects of ECPs, with high cardiovascular risk[36]. High dosages of ECPs reportedly cause chronic toxicity in the body that may directly lead to death or indirectly by causing a disease[37].

1.2 Statement of the Problem

More than ever before, there is an increase in unprotected sexual activities among youths, irrespective of their educational, religious, or socioeconomic background or exposure, has become a common practice in different parts of the country (Nigeria). Studies have ascertained that many females have knowledge of the existence of emergency contraceptives for use either before or after coitus and that a large population uses the pills often[16,18,27,30,32,35]. Studies have also indicated that a good percentage of these young ladies do not possess adequate knowledge about the proper use of these medications, as well as decreased compliance[45,46]. These may lead to misuse, abuse and indiscriminate use of contraceptive pills that is likely to have toxicological effects on the vital and reproductive organs, which in turn can adversely affect fertility or result

in chronic health issues later in life. Failure to conceive and bear children could be a devastating condition for couples and may lead to psychological distress, depression, low self-esteem, and domestic violence[47].

Therefore, more information is needed on the toxicological factors associated with the use of contraceptives among females, especially in developing countries with inadequate information, and low compliance, such as Nigeria[40]. A survey through the use of a questionnaire has shown that Postinor 2 is one of the most commonly used emergency contraceptives among unmarried female adults. There is limited scientific evidence on the effect of incessant use of Postinor 2 on the menstrual cycle, reproductive and key metabolic organs[42,48,49]. This study was therefore designed to assess the possible toxicological effects of Postinor-2 on selected vital and reproductive organs, using female Wistar rats as experimental models.

1.3 Justification of the Study

A number of adverse effects have been associated with the use of Postinor-2 as an emergency contraceptive method. They include weight gain, nausea, variations in menstrual flow, breast changes such as tenderness, discomfort, or swelling, depression or mood disturbances, decreased sexual desire or response, and acne. Rare but serious potential effects include cardiovascular diseases, such as stroke, and an increased risk for breast cancer, liver tumors, and gallbladder disease[32,35]. There is a paucity of information, particularly in Nigeria and sub-Saharan Africa, on the local effect and impact of incessant use of postinor-2 on vital and reproductive organs, as well as possible hormonal modulations that may have a negative impact on fertilization, implantation, and invariably infertility, which is a major public health concern in Nigeria, and the world at large. Several studies have been inconclusive on the causes and contributions of different biological and environmental factors to unexplained infertility and recurrent

implantation failure (RIF)[47, 48, 49]. Infertility has been shown to affect about 15 % of the world's reproductive-age population[34]. The World Health Organization (WHO) forecasts that in the 21st century, infertility will probably become one of the three major diseases affecting human health, next to cancer and cardiovascular diseases[43]. It is therefore imperative to evaluate the incessant use of Postinor 2 as a probable contributing factor to hormonal imbalance, implantation failure, and other metabolic disoders that may ultimately result in reproductive complexities.

1.4 Aim and Objective of the Study

The study sought to assess the possible toxicological effects of Postinor-2 in selected reproductive and vital organs using female Wistar rats as experimental models.

1.5 Specific Objectives

The specific objectives are to:

i. evaluate the awareness, knowledge, and use of emergency contraceptives among selected students of Lead City University, Ibadan, using a semi-structured questionnaire
ii. to determine the prevalent method of contraception, the frequency of use of the prevalent contraceptive, and the adverse effects observed with the use of the contraceptive method among the students
iii. determine the effect of Postinor 2 intake on the behavior of rats by observation before and after treatment
iv. evaluate the effect of incessant use of postinor-2 on the menstrual (estrus) cycle through physical evaluation, and vaginal cytological evaluation of the estrus cycle of the Wistar rats

v. investigate the effects of Postinor-2 intake on body weights and organ weights by measuring the weights of the rats before and after treatment and the relative organ weights

vi. investigate the effects of Postinor-2 intake on selected organs namely uterus, ovary, liver, and kidney through histopathological examination of the organs

vii. estimate hormonal changes (sex hormones) associated with the use of Postinor-2 by measuring the serum levels of progesterone (P4), estradiol (E2), luteinizing hormone (LH), and follicle stimulating hormone (FSH) using the ELISA method.

viii. evaluate the effect of postinor-2 on fertilization and implantation by measuring endometrial proteins such as, progesterone associated endometrial protein (PAEP), leukemia inhibitory factor (LIF), prolactin (PRL), and sex hormone - binding globulin (SHBG) in the serum using an enzyme linked immunosorbent assay (ELISA).

ix. assess some markers of inflammatory tissue damage by determining the levels of Nuclear Factor Kappa B (NF-κB), and Tumor Necrosis Factor α (TNF-α) in the serum, and reproductive tissues using the ELISA method.

x. investigate the hepatotoxic potential of the indiscriminate use of Postinor-2 by measuring aspartate transaminase (AST), alanine amino transaminase (ALT), the AST:ALT ratio, alkaline phosphatase (ALP), and total protein in the serum and liver using standard methods.

xi. assess the nephrotoxic effect of LNG by measuring cystatin c, urea, creatinine, gamma glutamyl transferase (GGT), BUN, and the BUN-creatinine ratio in the serum, and tissues using standard procedures.

xii. assess the level of oxidative damage associated with the use of Postinor-2 by measuring selected oxidative stress biomarkers and the activities of some enzymatic antioxidants such as, catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD) and Lipid peroxidation (LPO) in selected tissues using standard procedures.

1.6 Significance of the Study

This study was designed to assess the toxicological effects of postinor-2 on vital, and reproductive organs. The outcome of this study will therefore provide important information to the public, especially, young female adults, about the potential danger of incessant use of Postinor-2 (if any) as it relates to female reproductive health, and other vital organs such as the liver and kidney.

1.7 Scope of the Study

This is an animal-based, toxicological, experimental study carried out on female rats of the Wistar strain within the Lead City University animal house using the emergency contraceptive pill, Postinor 2. The study was carried out between October 2021 and August 2023 through the administration of questionnaires, acclimatization and treatment of animals, sacrifice, homogenization, and centrifugation, laboratory analyses, and statistical analysis. Questionnaires were used to identify the prevalent ECP used among female students, which was evaluated in this study to determine its effects on vital and reproductive organs in the body. The study accessed seven categories of biochemical assays: (i) the hormonal assays which investigated any modulatory effects or irregularities the levels of sex hormones due to incessant use of Postinor 2, (ii) the reproductive assays which studied the effects of Postinor 2 on fertility, (iii) the liver-

function, and kidney-function assays to determine the effects of Postinor 2 on liver, and renal health hepatotoxic effects, the nephrotoxic effects, (iv) histopathological examination of the organs of interest to determine the effects of Postinor 2 on their morphology as anatomical evidence of toxicity (if any), (v) the estrous cycle monitoring for 2 cycles to assess any effect of Postinor 2 on its regularity, (vi) oxidative stress assays to evaluate the effects of Postinor 2 on the oxidant-antioxidant balance in th e tissues of interest, and (vii) markers of inflammatory assay. These laboratory analyses were done within and outside the university laboratories using different methodologies and equipment.

1.8 Limitations of the Study

i. During administration of questionnaire, some students were unresponsive, and reluctant to reveal information about their sexuality

ii. This research was self-funded. Hence, more of the genes expressed in unexplained infertility, recurrent abortions, or implantation failure could not be studied because of a lack of funding.

1.9 Operational Definition of Terms

i. **Contraception:** This is the use of a drug, device or surgery to prevent pregnancy

ii. **Emergency Contraceptives:** These refers to several contraceptive options that can be used within a few days after unprotected or under protected intercourse or sexual assault to reduce the risk of pregnancy.

iii. **Levonorgestrel:** Levonorgestrel is a synthetic progestogen similar to progesterone used in contraception, and hormone therapy that works by preventing ovulation and fertilization by altering tubal transport of the ova and sperm. It is the active ingredient of popularly used pills such as Postinor-2, Plan B, and After pill

iv. **Ethinyl Estradiol:** It is an ECP containing estrogen but with greater oral bioavailability, and effectiveness. There is a substitution of an ethinyl group at carbon 17 of estradiol which makes it structurally different. However, this difference in structure leads to a significant increase in estrogenic activity of the drug. It was granted FDA approval on 25 June 1943.

v. **Ulipristal Acetate:** Ulipristal acetate is a selective progesterone receptor modulator that is approved by the U.S. Food and Drug Administration (FDA) for emergency contraception. This can still be used up to 5 days after the sexual intercourse

vi. **Combined Oral Contraceptive Pills:** The combined oral contraceptives (COC) pills are commercially available formulations taken in two divided doses with each dose containing synthetic estrogen (usually 100 – 120 mcg ethinylestradiol), and progestin (either 0.50 – 0.60 mg levonorgestrel or 1.0–1.2 mg norgestrel)

vii. **Fertilization:** This is the fusion of male and female gametes to produce a new individual or offspring and initiate its development

viii. **Implantation:** This comprises of a unique biological phenomenon, by which the developing embryo known as blastocyst becomes intimately connected to the maternal endometrial surface to form the placenta that will provide an interface between the growing fetus, and the maternal circulation

ix. **Ovulation:** Ovulation occurs in females when a matured egg (follicles) from an ovary is released. It plays an important role in fertility

x. **Infertility:** Infertility is a disease of the reproductive system characterized by inability to achieve pregnancy after 12 or more months of regular unprotected sexual intercourse.

xi. **Morbidity:** This is the rate of an individual suffering from a disease or medical condition

xii. **Mortality:** The condition of being subject to death in a given area or period, or from a particular cause

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Chapter Two

Literature Review

2.1 Increased Sexual Activities among Young Adults

More than ever before drug misuse, and abuse particularly among adolescents, and/or young adults for different reasons is on drastic increase. The increasing exposure of adolescents to sexual activities is becoming alarming, and has contributed immensely to the incessant use of contraceptives to prevent unwanted pregnancy, and unsafe abortion[1]. It was reported by United State Centre for Disease Control[2] from their survey among U.S. high school students that 38 % has had sexual intercourse, 9 % had four or more sexual partners, 7 % had been physically forced to have sex, 27 % had had sexual intercourse within the previous 3 months, and, of these 46 % was unprotected last time they had sex, 12 % did not use any method of contraception, 21 % had drunk alcohol or used drugs before last sexual intercourse, less than 10 % of all students have been tested for sexually transmitted diseases during the past year. Data from the survey on sexual activities contraceptives use among teenagers in the US between 2015 – 2017 by National

Survey of Family Growth showed that, 42 % of female teenagers aged 15 – 19, and 38 % of male teenagers had had sexual intercourse[3]. In Nigeria, reduced age at sexual debut, and multiple sexual partners have been reported to have contributed immensely to increased sexual activities. In a study involving 60, 611 women, 45.4 % reported experiencing first intercourse before reaching 15 years, whereas 47.2 % had 2 - 3 sexual partners while 47.6 % had more than 3 sexual partners[4].

Several factors have been noted to cause increased exposure to sexual activities among adolescents (age 13 - 18) such as decreasing age of menarche, and coitarche, peer pressure, social influences, access to pornographic sites, nudity displayed on social media, and movies, poor sex education, emotional well-being, family structure, body image, socio-economic status, participation in sport, and family support among others[1,5]. Consequently, there has been a geometric progression in the incidence of unwanted pregnancy, and abortion all over the world. Out of the estimated 210 million pregnancies that occur annually, 46 million (22 %) are reported to be unwanted, and more than 90 % of the unwanted pregnancies usually end in induced-abortion with its attendant complications[6]. The situation is worse in low, and middle-income countries like Nigeria where an estimated 89 million unwanted pregnancies occur annually. Fifty percent (50 %) of the unintended pregnancies ends in abortion, 38 % results in unplanned births, and others results in miscarriages, and maternal deaths. Abortion in many African countries is prohibited, and unsafe, contributing 9 % of all maternal deaths, leading to contraceptives being identified as one of the four pillars of safe motherhood program[7].

2.2 Impact of Unwanted Pregnancy and Unsafe Abortion

The reproductive, emotional, and psychological impact of unintended pregnancies, and unsafe abortion could be damaging. Unsafe abortion with its associated complications is a major public

health concern due to its immense contribution to women infertility, morbidity, and mortality[8]. In developing countries like Nigeria where abortion is not legalized, several crude, and unsafe methods are used to terminate, and prevent pregnancies, thus making unwanted pregnancy a major cause for concern. The potential of emergency contraceptives to play a major role in the prevention of unwanted pregnancy is now well known, and popular[9]. In Nigeria, and several countries in Africa, this awareness is apparently substantial. However, abuse of these contraceptives is on the increase, probably due to poor sex education or lack of adequate knowledge about the use of contraceptives.

2.3 Emergency Contraceptive Pills (ECPs)

Emergency contraceptives are substances, either devices or pills that can be used to prevent unwanted pregnancy when used within the first few days of sexual intercourse usually 1 - 3 days. Emergency contraceptive pills (ECPs) are pills or oral tablets also known as post-coital contraceptives, emergency birth control or "morning–after" pills used after unprotected sex to prevent pregnancy[10]. There are several myths or wrong beliefs about the use of emergency contraceptives. These myths among others include the following: (i) They can only be used the next morning after sex (ii) They are the same as abortifacients (iii) There are no side effects associated with their use. (iv) They protect against sexually transmitted diseases (STDs) (v) The higher the dosage, the greater the efficiency[9]. Geographical location is believed to influence the use of EC as women from urban areas use EC more than their counterparts in rural areas. The rural resident females tend to prefer the use of the traditional or local methods, probably due to lack of knowledge, fear of side effects, and lack of access[11].

Several regimens are currently available globally as emergency methods of contraception. However, the recommended methods in clinical practice include the progesterone-only pills (levonorgestrel), the combined estrogen-progesterone pills, otherwise known as the Yuzpe regimen, Ulipristal acetate (UPA), and Copper-T intrauterine contraceptive device (IUD). The oral pills are called emergency contraceptive pills (ECPs). The IUD must be inserted within five days after unprotected sexual intercourse which must be removed after the next menstrual cycle when it is certain that the individual is not pregnant[8,12]. Other methods not commonly used in clinical practice include: The progesterone antagonist, mifepristone, high dose estrogen regimens, synthetic, androgen-Danazol, and the luteinizing hormone releasing hormone analogue (Buserelin). Low dose mifepristone is available only in countries like Armenia, China, Russia, and Vietnam[13].

EC has been in existence for over 40 years, since the 1970s' and since then several changes have been made in dosage, and there are several brands of ECPs in pharmacies worldwide. There have been advocacies to move them from prescription-only to over-the-counter medicine which increased accessibility to them[7]. Emergency contraceptive method should not replace normal contraceptive method, and should be avoided or monitored in patients with health conditions such as pelvic inflammatory diseases, hepatic impairment, cardiovascular disorders, diabetes mellitus, seizures, mental depression, migraine, and high blood pressure[14]. The World Health Organization (WHO) recommended the use of any of the following drugs for emergency contraception:

i. ECPs containing UPA taken as a single dose of 30 mg;
ii. ECPs containing levonorgestrel (LNG) taken as a single dose of 1.5 mg, or alternatively, LNG taken in 2 doses of 0.75 mg each, 12 hours apart.

iii. ECPs as Combined oral contraceptives (COCs) containing LNG, and Ethinyl estradiol (EE), taken as a split dose, one dose of 100 µg of EE plus 0.50 mg of LNG, followed by a second dose of 100 µg of EE plus 0.50 mg of LNG 12 hours later.

2.3.1 Brief History of ECPs

The use of emergency contraceptives is as far back as 1920s when researchers first observed that estrogenic ovarian extracts could interfere with pregnancy in mammals. Veterinarians were the first to apply this finding by administering estrogens to dogs, and horses that had mated when their owner had not wanted them to. The first documented cases were not published until the mid-1960s despite scattered reports of clinical use of post-coital estrogens in humans as early as the 1940s when physicians in the Netherlands applied the veterinary practice of post-coital estrogen administration to a 13-year-old girl who had been raped at midcycle[15].

At around the same time, U.S. researchers were investigating the efficacy of high-dose estrogens, and in 1960, the first contraceptive pill was marketed in the USA, and in a few years, the contraceptive method spread throughout the world. Women typically received conjugated estrogens, the steroidal estrogen ethinyl estradiol or the non-steroidal estrogen diethylstilbestrol (DES)[16]. Later, high-dose estrogens were administered in the so-called 5 by 5 regimen which involves 5 mg of ethinyl estradiol per day for five days. In the early 1970s, the high-dose estrogen regimens gave way to a combined estrogen-progestin standard. Canadian physician Albert Yuzpe, and his colleagues in 1972 formulated a combined regimen, guided by their observation that a single dose of 100 µg of estrogen coupled with 1mg of the progestin Norgestrel induces endometrial changes that prevented implantation. The "Yuzpe method," later named after the inventor, replaced high-dose estrogen formulations, chiefly because it offered a lower incidence of side effects more effective, and also because the commonly used DES at the

time was linked to vaginal cancer in the daughters of women who had taken it to prevent miscarriages[15]. The regimen then extended to 72 hours after unprotected intercourse, and typically contains 200 µg of ethinyl estradiol, and 1mg of levonorgestrel[17].

Over the years, many drugs were formulated such as danazol, and mifepristone. Danazol, a synthetic progestin, and antigonadotropin, was first used as an emergency contraceptive in the early 1980s. Mifepristone, more commonly known as RU-486, is potent anti-progesterone registered in four countries as an abortifacient. As seen in recent years, the acceleration in the production of EC was caused by modernization, urbanization, greater presence of women in the labor market, increased female education, and the boom of the multinational pharmaceutical industry. The market for ECPs developed rapidly, despite restrictions imposed by the Criminal Misdemeanors Act of 1941 – which prohibited announcing the process, substance or object intended to cause abortion or prevent pregnancy such as contraceptives or abortifacients as a result of social, and religious norms that ratified both pro-Natalist, and conservative patterns of sexual morality, and the intense controversies surrounding the issue of the concept of family planning[16].

The spread of this innovative fertility control technology in recent times has had important social effects, sparking networks of interactions among stakeholders such as doctors, scientists, government authorities, researchers, political groups, religious representatives, private family planning entities, pharmacists, pharmaceutical industry, media, and, of course, women consumers. Between 1970s up until now, ECPs are already stabilized having a large contingent of women using the different available methods[18]. Since then, studies have explored the trajectory of ECPs, discussing different aspects: the experiences of women using ECPs, the development of new reproductive norms, and fertility management practices, and the effects of

this technology on gender, the role of pharmacy attendants, as "moral eyes" on users[18,19]. The fast assimilation of ECPs in the daily practices of medical practitioners, and their relationship with the formation of a modernizing style of medical thinking, the dissemination of knowledge about ECPs, and the transfer of the knowledge to upcoming generations[20]. Today, several regimens are available, and scientists are constantly studying about their awareness, use, effectiveness as well as side effects.

2.3.2. Lack of Adequate Information on ECPs

Inadequate, and wrong information on EPCs is a major problem connected to its use by a large chunk of the African populace. This may be because people shy away, and seldom discuss issues about sexuality as a result of religion, and social beliefs. Financial capacity, education, and cultural values are also important factors in reproductive behavior[21]. Confusion, and misconceptions exist among studied population about ECPs, and how they work. The misunderstandings reflect poor quality sex education, and public information[10]. Many researchers have called for urgent need to educate Nigerian young people about emergency contraception, emphasizing available methods, and correct timing of use[11, 21]. Evidence has shown from many countries that comprehensive programs on sex education, and awareness lead to a safer sexual behaviors as low levels of sexual knowledge contribute to risk behaviors among adolescents[21,22]. Increased availability of this drug over the counter has greatly increased its repetitive use, and abuse as it was reported that young women now chew Postinor-2 drug 'as chocolate in Kenya, and as candy in India[23].

A German survey explained that the use of oral contraceptives can be controlled, and the women properly educated on their proper use, if the drugs are made available only in pharmacies (over-the-counter, OTC) though without a prescription. This way Pharmacists will be able to dispense

their professional expertise in giving advice, instructions, directions to the consumers. They will also be able to intervene in situations of unsafe sex, sexually transmitted diseases, suspicion of assault/ abuse, acute health impairment or chronic diseases, potential drug/drug interaction, and refer to gynecologists if need be. Pharmacists' safety concerns when dispensing emergency contraceptives will ensure a reduction in the abuse of these pills[24].

There have been conflicting reports from developed, and developing countries about the knowledge, use, contraindications, and toxicity of EC. Knowledge of the appropriate use at correct time, and dose reportedly vary from 83 % in developed countries to less than 60 % in developing countries[8]. One of the lowest percentages (10 %) was observed in a study done in Ethiopia at the Addis Ababa University, and Unity University College, Ethiopia on the knowledge, attitudes, and practices affecting the use of EC[25]. Another study examined the awareness, and usage of Emergency Contraceptives (ECs) among students of the University of Ghana. They showed that majority (87.9 %) were aware of some regular contraceptives, and ECs. Despite this, majority (70.4 %) claimed they had never used any form of contraceptives[8].

Knowledge, and information about EC methods among Resident Doctors (n = 689) showed that Residents Doctors received very little EC education[26]. Additional training to ensure that all medical practitioners are knowledgeable about the effectiveness, and different types of EC methods is necessary to decrease unintended pregnancies as well as myths surrounding EC use[26]. Approximately a third of pharmacy students at the University of Arkansas's College of Pharmacy were reported to lack the knowledge of the mechanism of action of levonorgestrel, and only 4 % knew the appropriate time frame for effectiveness. Only 26.7 % agreed that they felt competent instructing patients on the appropriate use of emergency contraception[27].

Particularly, several studies have observed the awareness, and perception of students to EC, and observed varying level of knowledge about the correct use, and timing of the drugs in different parts of Nigeria. About 75 % of sexually active (13 - 19) teenage girls in the south-west geopolitical zone of Nigeria use some form of perceived contraceptive devices such as laxatives, local potash, "white quinine", and mestrogen pills. Many women preferred the traditional methods of contraception to the more effective modern methods for the fear of side effects[11].

A questionnaire based cross-sectional survey using randomly selected 700 female undergraduates of Imo State University, Owerri reported two-third (67 %) of the students to be sexually active. 85.1 % was observed to have awareness of emergency contraception which is very high but only 34.6 % could correctly identify the appropriate time interval for its effectiveness. The awareness was also observed to be significantly higher amongst students in health-related faculties than in the non-health related faculties. The main sources of information about ECPs were via friends (43.1 %), and lectures (22.1 %). High dose progestogen (postinor-2) was the most commonly known type of emergency contraception (70.8 %). In their conclusion, they pointed out that although the awareness of emergency contraception was high, the attitude, and practice are still poor[28].

In a cross-sectional observational study, 675 female non-medical undergraduates in South Eastern part of Nigeria were interviewed using pretested semi-structured questionnaire to assess their knowledge, and experience with emergency contraception. 51.6 % of the respondents reported knowledge of emergency contraception which was significantly influenced by being sexually active, use of regular family planning methods, and having fear for unintended pregnancy. Only 45.7 % was reported to know the correct methods (Postinor-2, combined oral contraceptive, and intra-uterine contraceptive device)[29]. In the Northern part of Nigeria, another

study determined factors associated with knowledge, and use of EC by female nursing, and midwifery students. Out of the 317 students examined, 94.3 % indicated awareness of EC. Of the respondents, and 24.1 % were not aware of the correct timing for the use of hormonal EC following unprotected sexual intercourse. In addition, 25.8 %, 13.4 %, and 9.4 % of the participants incorrectly identified menstrogen, quinine, and ampicillin, respectively as forms of EC. They concluded that although a large number of the student nurses, and midwives surveyed had adequate general knowledge of EC, but many lacked the knowledge with some having misconceptions about the forms of EC[30]. An empirical study among female students of Ahmadu Bello University, Zaria investigated the issues surrounding emergency contraceptives, and problems that characterized the use of EC among female students. They explored the knowledge of female students about EC, out of the 500 female students randomly selected for the study, (58.0 %) affirmed knowledge of EC, (38.0 %) have never heard while only (4.0 %) did not respond to this question. They revealed that the knowledge of female students about ECs was not adequate because they are ignorant about what to use, and do not know the correct usage in cases of unprotected sex with their partners[31].

These studies collectively show that there is misconception, gap in knowledge among the users of ECPs, and those who are supposed to educate the populace on the correct use, and timing of the drug. Education on emergency contraception is therefore needed to improve the knowledge of the public on EPCs as well as the confidence, and counseling skills of medical, and paramedical practitioners[32]. It means the health sector has quite a number of intervention steps/work to do in terms of seminars/workshops, enlightenment, orientation, and educating the students, and women on the use of contraceptives, its various options, their side effects, and management[11].

2.3.3 When to Use ECPs

Emergency contraception can be used in different situations following sexual intercourse which can be unplanned, untimed or unprotected. These include:

i. When no contraception method has been used.

ii. Sexual assault when the woman was not protected by an effective contraceptive method such as rape, assault or incest

iii. When a woman is concerned about possible contraceptive failure, from improper or incorrect use, such as:

iv. A situation of condom breakage, slippage, or incorrect use

v. Three or more consecutively missed combined oral contraceptive pills or 3 days late during the first week of the cycle

vi. More than 3 hours late from the usual time of intake of the progestogen-only pill (minipill), or more than 27 hours after the previous pill

vii. More than 12 hours late from the usual time of intake of the desogestrel-containing pill (0.75 mg) or more than 36 hours after the previous pill

viii. More than 2 weeks late for the norethisterone - enanthate (NET-EN) progestogen-only injection

ix. More than 4 weeks late for the depot-medroxyprogesterone acetate (DMPA) progestogen-only self-injection

x. More than 7 days late for the combined injectable contraceptive (CIC)

xi. Dislodgement, tearing, breakage, or early removal of a diaphragm

xii. Failed coitus interjectus (withdrawal) leading to ejaculation in the vagina or on external genitalia

xiii. Failure of a spermicide tablet or film to melt before intercourse

xiv. Miscalculation of the free period, or failure to use a barrier method on the fertile days of the cycle when using fertility awareness- based methods

xv. Misplacement or expulsion of an intrauterine contraceptive device (IUD) or hormonal contraceptive implant[12].

2.3.4 Effectiveness of ECPs

ECPs should be taken as early as possible after unprotected intercourse, within 3 days. ECPs with UPA can still be effective up to 5 days after unprotected intercourse than other ECPs while LNG are most effective within 8 hours of unprotected sex[24]. The earlier the pills are taken, the more the effectiveness[7].

Analysis showed that women who used ECPs with UPA had a pregnancy rate of 1.2 %. Studies have shown that ECPs with LNG had a pregnancy rate of 1.2 % to 2.1 %. Efficacy studies of the COCs also known as the Yuzpe method have yielded greatly varying results, in part because the definition of efficacy is slightly different for a post-coital method than for a conventional method. In one approach, researchers observe women using EC in a given cycle, note the number of pregnancies that occur, and divide that number by the number of women who took the drug. When studied in this fashion, the failure rate of the Yuzpe method ranges from about 0.2 % - 2 %[12].

It has also shown that the probability of observed pregnancy was zero whenever LNG EC was administered at any time before the day of ovulation. Analysis has shown a direct relationship between potential mechanisms of action of LNG EC, and the observed clinical effectiveness. If ovulation disruption is taken to be the only significant mechanism of action, the total

effectiveness could be not much higher than 50 % if EC is administered immediately after intercourse; with delays, it would be substantially less than 50 %. Similarly, if LNG EC given before the day of ovulation were able to completely prevent fertilization by any mechanism, if there were no post-fertilization effects, and if there were a 24-hour delay in administration, the highest possible effectiveness would be about 60 %[12]. The marked decline in effectiveness of LNG with increasing delay between the time of intercourse, and administration is attributable to the increasing proportion of women who are within the fecundity window at the time of intercourse, and subsequently have already ovulated by the time of EC use[33].

If EC is administered within 24 hours after intercourse, it has 95 % effectiveness, if administered 25 to 48 hours after intercourse, it was reported to be 85 % effective, and if administered 49 to 72 hours after intercourse, it was observed to be 58 % effective. Similarly, in a study of 1356 women receiving LNG EC, it was estimated to be 79 % effective when administered up to 72 hours after intercourse, and 60 % effective when administered between 72, and 120 hours after intercourse. However, in another study of 1021 women receiving LNG EC, the overall effectiveness was estimated to be 64 %, and there was no evidence for a trend of reduced effectiveness with increasing delay between intercourse, and administration of ECPs although the results become unexplainable if disruption of ovulation plays a major role[12]. In a Chinese study of 2018 women receiving two different formulas of LNG EC, the proportion of observed pregnancies was higher when EC was administered after a 72-hour delay, but the difference was not significant, and it was not presented in the form of effectiveness[34].

There discrepancies observed in the results of the findings may be due to the fact that the effectiveness rates estimated in trials of LNG could be inflated; the actual effectiveness of LNG may be substantially lower than what has been estimated from clinical trials. Recent analyses of

the procedures used to estimate effectiveness of LNG have suggested that the overall effectiveness has indeed been overestimated by approximately 10 % (absolute difference).

Figure 2.1: Effectiveness of Emergency Contraceptive Pills (ECPs)

Source [35]

2.3.5 Mode of Action of ECPs

ECPs have been shown to have an effect on fertilization as well as disrupting ovulation. They can delay ovulation for less than 5 days, inhibit sperm migration, and function in the genital tract, or reduce sperm capability for fertilization of the egg (although this potential effect has not been supported in physiologic studies [36]. In addition, they work by thickening the cervical mucus, and by thinning the lining of the endometrium. If the lining is thinner, it is difficult for a fertilized egg to implant in it, which will prevent a pregnancy. Studies have shown that levonorgestrel works by preventing or delaying ovulation, and impairing luteal function. Similar to the hormone progesterone, it promotes the closure of the cervix to prevent the entrance of sperm[37,38].

Ulipristal acetate is thought to inhibit or delay ovulation. A clinical trial showed that it could delay ovulation for 24 – 48 hours even on the day of the luteinizing hormone (LH) peak. When ulipristal was given before or immediately after the LH surge, it inhibited 100 % of the follicular ruptures. Other mechanisms include reducing the endometrial thickness, delayed endometrial maturation, and alterations in the progesterone–dependent markers required for implantation. These effects may subsequently inhibit implantation because the uterus will be less receptive to the trophoblast. Combined ECP work by inhibiting the implantation of a fertilized egg. Other postulated mechanisms include delaying or suppressing ovulation, interfering with corpus luteum function, and making changes in the endometrium that prevents implantation[9].

2.3.6 Potential Toxicity of ECPs

There is a low level of research regarding the toxic levels, and effects of ECPs in humans. While there could be toxicity seen in patients with liver disease, there is not enough research to support this, and more human trial studies will be necessary[36]. Significant decrease in white blood cell counts have been reported to be associated with the use of ECPs[19]. Studies have also been done to evaluate the adverse effects of the use of EC on the health of women. Menstrual disturbances were associated with a number of users that may be of concern in women who uses 1.5 mg of Levonorgestrel[10]. They also observed that menstruation was late in 21 % of women by more than a week, while 69 % had early or timely return of menstruation. Normal vaginal bleeding occurred in 57 % of the studied women while there was intermenstrual bleeding or spotting as well as premenstrual bleeding or menorrhagia in others. Exposure to EC (Levonorgesrel only (LNG), and Estrogen-Levonorgestrel (EE-EC) combination has been shown to have effects on well-established cardiovascular risk factors[39].

The efficacy, safety, and acceptability of pericoital oral contraception using 1.5 mg LNG in fertile women observed that there were reported severe adverse events, and other mild adverse events such as headaches, nausea, abdominal, and pelvic pain. They also found a case of severe anaemia, vaginal bleeding patterns showed a slight decrease in volume while the number of bleeding free days increased over time. Although the method was considered acceptable, a number of pregnancies were recorded both in typical, and sole use[40, 41].

Very few studies have accessed the toxicological impact of EC in animal models. The research work to determine the histo-toxic effects of the drug Postinor- 2 on the liver of female wistar rats revealed vacoulation, necrosis of the hepatocytes, Inflammatory cells, and presence of perinuclear hallow around the recorded hepatocyte. They inferred that excess use of Postinor was associated with cellular, and tissue damage of the liver[42].

The effects of LNG on concentration of reproductive hormones in the serum, and their receptor mRNA expression in the ovary, and uterus of Mongolian gerbils was studied by[43]. After treatment with LNG, the results showed that serum follicle-stimulating hormone (FSH), and luteinizing hormone (LH) increased, while serum estradiol (E2), and progesterone (P4) decreased markedly. LNG down-regulated the mRNA expression of follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), estrogen receptor (ER) β, and progesterone receptor (PR) in the ovary, and ERα, and PR in the uterus of Mongolian gerbils. The down-regulated effects were reported to be time-dependent, and dose-dependent. They suggested that LNG impairs reproductive hormone receptor expression at the molecular level in Mongolian gerbils[43]. When given LNG orally, rats were also observed to have significant decrease in white blood cell counts[44]. LNG, combined with a super-physiological level of progesterone, has been shown to decrease the tubal ciliary beat frequency, and thus lead to embryo retention in the fallopian tube without changing tubal receptivity causing ectopic pregnancy[45].

2.3.7 Types of ECPs

2.3.7.1 Levonorgestrel

Levonorgestrel (LNG—17alpha-ethynyl-18-methylestr-4-en-17beta-ol-3-one) or synthetic progestogen is the active component of the racemic mixture of norgestrel. Levonorgestrel is the levorotatory form of norgestrel, and synthetic progestogen with progestational, and, androgenic activity. It is the active ingredient of progestin only ECPs. Levonorgestrel is a 17-beta-hydroxy steroid, a 3-oxo-Delta (4) steroid, and a terminal acetylenic compound. It has a role as a contraceptive drug, a progestin, a synthetic oral contraceptive, and a female contraceptive drug. It derives from a norgestrel. It is an enantiomer of a dexonorgestrel[46].

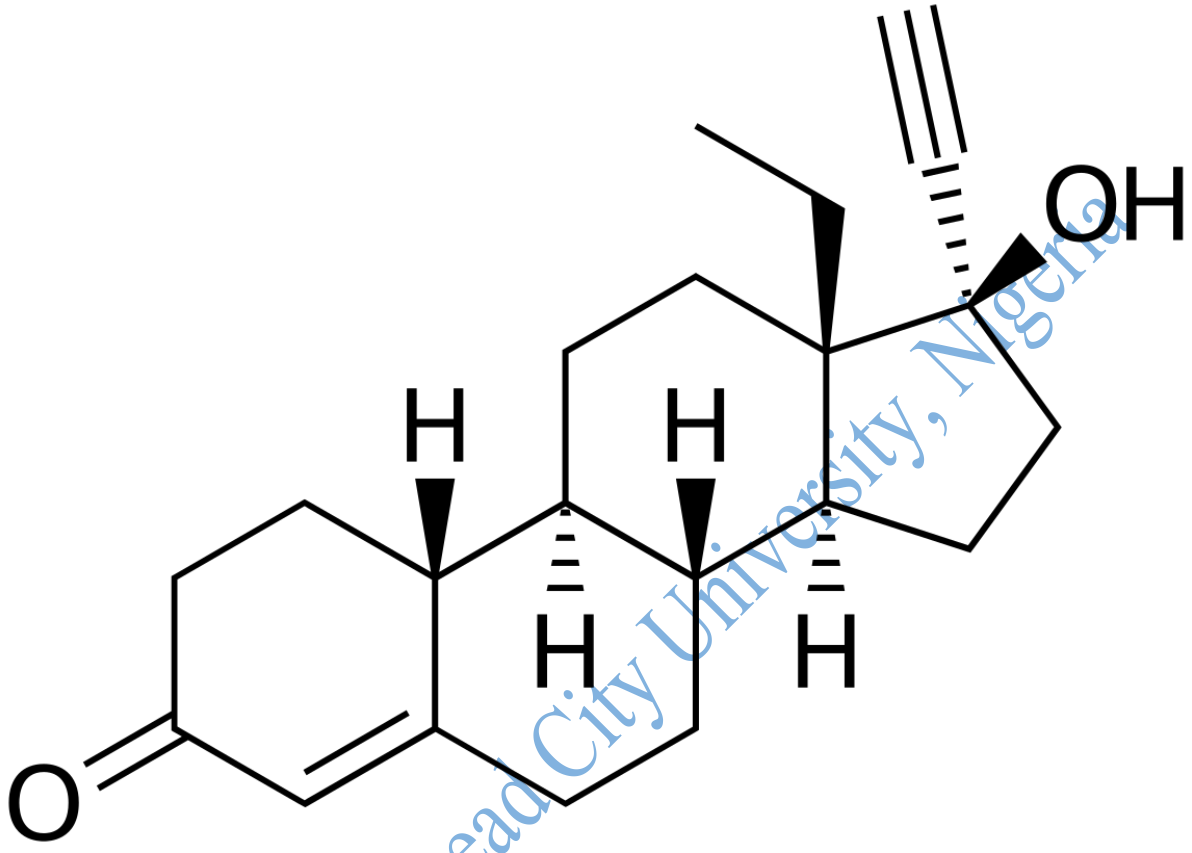

Figure 2.2: Chemical Structure of Levonorgestrel

Source [46]

2.3.7.2 Mechanism of Action of LNG

Levonorgestrel is a progesterone agonist which binds to the progesterone receptor in the nucleus of target cells, thereby stimulating the resulting hormone-receptor complex, initiating transcription, and increasing the synthesis of certain proteins. This results in a suppression of luteinizing hormone (LH) activity, and an inhibition of ovulation, as well as an alteration in the cervical mucus, and endometrium[46]. The mechanisms of LNG include actions affecting follicular development, ovulation, corpus luteum formation, sperm motility, fertilization, blastocyst implantation, and endometrial function. The activity of the substance varies with species, the dose given, and the route of administration[26].

Levonorgestrel is a synthetic progestogen similar to Progesterone used in contraception, and hormone therapy. It is the active ingredient of popularly used pills such as Postinor-2, Plan B, after pill, used as a single agent in emergency contraception, and as a hormonal contraceptive released from an intrauterine device, commonly referred to as an IUD[34]. Some of these devices are known as Jaydess, Kyleena, and Mirena. A subdermal implant of levonorgestrel that slowly releases the hormone over a long-term period is also available. In addition to the above uses, levonorgestrel is used as a component of long-term combination contraceptives[33]. Globally, levonorgestrel is the most commonly used emergency contraceptive. It was initially granted Food, and Drug Administration (FDA) approval in 1982, and was the first emergency contraceptive containing only progesterone, showing high levels of efficacy, and a lack of estrogenic adverse effects when compared to older emergency contraceptive regimens[26].

LNG is widely used alone or combined with estradiol as a long-term option for birth control, and is available as implants or transdermal patches. There is a levonorgestrel-releasing intrauterine device considered to be a "low maintenance" birth control option that is efficacious for up to five years. It has also been used to treat endometrial hyperplasia, menorrhagia, endometriosis, and menopausal hormone therapy. It is approved by both World Health Organization (WHO), and US FDA to be used to prevent pregnancy within 72 hours of unprotected sex[36]. LNG are sold under different brand names either as a 2-dose 0.75 mg or 1-dose 1.5 mg. The 0.75 mg oral tablet is given with a second 0.75 mg dose 24 hours later. A 3 mg oral levonorgestrel is available also for patients concomitantly taking a CYP3A4 cytochrome p450 liver enzyme-inducing drug, e.g., rifampicin, St. John's wort, carbamazepine, or phenobarbital, which are agents capable of increasing hepatic clearance of levonorgestrel. If vomiting occurs within two hours of administration, the patient would need to repeat the initial dose taken[47].

For the long-term birth control options, the levonorgestrel intrauterine T-shaped device has 52 mg of levonorgestrel covered by a rate-controlling membrane that regulates the rate of release of hormones. The combined oral contraceptive pill with ethinylestradiol comes in a 21-pill pack per month with 0.1 mg of levonorgestrel, and 0.02 mg of ethinylestradiol[47]. Side effects reported from the use of LNG include, menstrual abnormalities, amenorrhea, dysmenorrhea, oligomenorrhea, headaches, weight gain, spotting, acne, nausea, and vomiting[33].

2.3.7.3 Postinor 2 (Levonorgestrel 0.75 mg and 1.5 mg)

Postinor is a brand name of levonorgestrel, contraceptive tablet manufactured by a Hungarian firm called Gedeon Richter Plc, Budapest. Postinor is marketed as a post-coital drug for the prevention of conception. It has been shown to be effective for the prevention of conception. It is estimated that Postinor-2 (figure 2.1) will prevent 95 % of expected pregnancies if taken

within the first 24 hours, declining to 85 % and 58 % if taken between 48 hours, and 72 hours after unprotected intercourse respectively[45]. There are different doses of Postinor product available, there is a ten tablets called Postinor 1 used immediately after sex, the other types of postinor brand are Postinor 0.75 mg (2 tablets) taken either one immediately after sex and the other one taken after 12 hours or both at once after sex and 1.5 mg which is currently sold in packet of 1 tablet, and taken immediately after sexual intercourse. Both brands are available in pharmacies in urban centers across the counter.

Postinor-2 is claimed to be the same progesterone used in regular contraceptive pills, but used in higher concentration. Postinor-2 contains the following inactive ingredients: potato starch, maize starch, silica colloidal anhydrous, magnesium stearate, talc, and lactose monohydrate[48]. It works primarily by preventing ovulation, and fertilization, if one had unprotected sex in the first two weeks of the month before ovulation has taken place, which is when likelihood of fertilization is the highest. It also helps to reduce the chances of implantation by preventing development, and thickening of the endometrium (lining of the uterus) that is needed for implantation of the fertilized egg, so that the fertilized egg would not be able to implant, and grow. If fertilization, and implantation has already taken place, taking Postinor-2 will no longer be effective. Generally, it is recommended that postinor-2 be taken no later than three (3) days of having unprotected sex. However, it has proven to be more effective in cases where the treatment was started closer to the time of the sexual encounter, the earlier the better[42]. Figures 2.3a and b as well as figure 2.4a and b shows some commercially available products with levonorgestrel as their active ingredient.

Figure 2.3a: Postinor 2 Tablet (Source [49])

Figure 2.3b: Plan B Levonorgestrel Tablet (Source [50])

Figure 2.4a: Postpill Tablet (1 tablet of Levonorgestrel 1.5 mg) (Source [52])

Figure 2.4b: Afterpill Tablet (Source [53])

2.3.7.4 Pharmacology and Pharmacokinetics of LNG

Levonorgestrel does not undergo "first–pass" metabolism, and has 100 % bioavailability. This makes it effective when administered orally as a tablet. After a single dose administration of levonorgestrel, maximum plasma concentrations of 19.1 ng/mL were reached at a median of 1.7 hours (range 1–4 hours). No studies have evaluated the effect of food on the rate, and the extent of levonorgestrel absorption following single oral administration[9]. Studies have shown that the apparent volume of distribution (Vd) of levonorgestrel is approximately 1.8 L/kg. The elimination half-life of levonorgestrel after single dose administration (0.75 mg) was 27.5 + 5.6 hours. These pharmacokinetic parameters facilitate the single, and 12-hour dosing of LNG. Levonorgestrel is highly protein bound (97.5 to 99 %), mainly to the sex hormone binding globulin (SHBG), and, to a lesser extent, serum albumin. Any displacement of LNG to the bound protein could potentially cause side effects, hence the need to monitor concurrent medications[9,51].

2.3.7.5 Metabolism of LNG

Levonorgestrel is well absorbed orally, and bioavailability is about 85 – 100 %. It is distributed in to the target sites in a protein bound form. It undergoes partial metabolism in liver, and is therefore subject to impairment in patients with liver dysfunction therefore monitoring liver function tests at the time of administration may be beneficial. Also, drugs containing CYP3A4 cytochrome p450 liver-enzyme inducing properties require close vigilance when a patient takes ECP. For instance, patients may need to consider a different emergency contraceptive method to avoid drug-drug interactions. These liver-enzyme-inducing drugs can cause rapid metabolism, and decrease the efficacy of LNG when there is concomitant use[54]. Simultaneous administration of certain anticonvulsant drugs (phenobarbital, phenytoin, primidone, carbamazepine, certain antibiotics such as rifabutin, rifamycin, and griseofulvin) can reduce or suppress its effectiveness.

Levonorgestrel is conjugated and forms many sulfate conjugates after the oral emergency contraceptive preparation is absorbed. It also forms glucuronide conjugates, which have been identified in the plasma. High levels of conjugated and unconjugated 3α, 5β-tetrahydrolevonorgestrel are found in the plasma. The whole metabolic pathway for levonorgestrel has not been studied; however, 16β-hydroxylation is one pathway that has been identified. Small quantities of 16βhydroxylevonorgestrel and 3α, 5α¬ tetrahydrolevonorgestrel are also formed. No active metabolites have been identified. The rate of metabolism may vary greatly depending on the patient and could account for a wide range in levonorgestrel clearance. Liver CYP3A4 and CYP3A5 hepatic enzymes are reportedly involved in LNG metabolism About 45 % of levonorgestrel, and its metabolites are excreted in the urine, and while about 32 % are excreted in feces, mostly as glucuronide conjugates[9]. Studies have shown that levonorgestrel is not subject to significant first-pass metabolism. Levonorgestrel undergoes metabolism via hydroxylation, conjugation, and reduction in the liver[55].

2.3.7.6 Distribution Volume of Levonorgestrel

According to one pharmacokinetic research, the mean steady-state volume of distribution of 1.5 mg of levonorgestrel was found to be 162.2 L in individuals with a normal body mass index and to be between 404.7 and 466.4 L in patients who were obese and had a body mass index of at least 30.6. Another pharmacokinetic research had 16 participants who received 0.75 mg of levonorgestrel; the mean volume of distribution was 260 L. An apparent volume of distribution of 1.8 L/kg is reported on the FDA label for the Plan B one-step.

2.3.7.7 Binding of Levonorgestrel to Protein

Levonorgestrel binds to proteins with a 97.5 – 99 % affinity, primarily to sex hormone-binding globulin (SHBG). Albumin and levonorgestrel are also linked. According to the prescribing

advice for implanted levonorgestrel, the drug's levels decrease within a few days of its administration due to a decrease in the concentration of SHBG.

2.3.7.8 Drug-Drug Interactions and Contraindications of LNG

The therapeutic effect of LNG may be reduced by drugs such as acitretin, anticoagulants, antidiabetic agents, barbiturates, carbamazepine, fosphenytoin, griseofulvin, mifepristone, phenytoin, primidone, retinoic acid derivatives, and St. John's wort. Its serum concentration may also be reduced by aprepitant; artemether; bile acid sequestrants, brigatinib; bexarotene; bosentan; clobazam, CYP3A4 inducers, dabrafenib; saquinavir; lamotrigine; darunavir; encorafenib, eslicarbazepine, exenatide; fosaprepitant; ixazomib; lixisenatide; lopinavir; lumacaftor, felbamate; metreleptin; mycophenolate; lesinur, and; nelfinavir, nevirapine; lorlatinib; oxcarbazepine; efavirenz; perampanel; rifamycin derivatives; sugammadex, and topiramate. The serum concentration of progestins may increase with atazanavir, cobicistat, tipranavir, and voriconazole while C1-inhibitors, and carfilzomib may enhance the thrombogenic effect of progestins[36]. Contraindications for LNG includes allergy, hypersensitivity, severe liver disease, pregnancy, and drug-drug interactions with liver enzyme-inducing drugs[33].

2.3.7.9 Route of Levonorgestrel Elimination

Roughly 45 % of a dose of LNG taken orally dose and its conjugated or sulfate metabolites are discovered to be excreted in the urine while about 32 % of an oral dose is found excreted in feces, primarily in the form of glucuronide conjugates of levonorgestrel. A 0.75 mg dosage of 1.5 mg levonorgestrel has an estimated elimination half-life between 20- and 60-hours post-administration. Body mass index (BMI) has been found to affect the elimination rate of LNG. Women with a normal BMI and those with a BMI over showed an elimination half-life of 29.7 hours and 41.0 - 46.4 hours, respectively, in a pharmacokinetic investigation[36].

2.3.8 Other Forms of ECPs

2.3.8.1 Ullipristal Acetate (UPA)

In August 2010, UPA (Ella, Laboratoire HRA Pharma/Watson Laboratories) was the first agent in the novel selective progesterone receptor modulator class to gain the FDA's approval for use as an oral EC tablet in the U.S. On August 13, 2010, HRA Pharma obtained approval for the use of ulipristal acetate (Ella) 30 mg tablets for the prevention of pregnancy following unprotected intercourse or a known or suspected contraceptive failure [56]. As ulipristal acetate was unavailable in any drug product in the US, it obtained approval as a new molecular entity. Ella potentially extends the availability of FDA-approved EC from 3 days (72 hours) after unprotected intercourse for currently approved levonorgestrel-based EC products to 5 days (120 hours)[57].

Under the proprietary name of ellaOne, ulipristal gained approval from the European Medicines Agency (EMA) in May 2009, and is currently marketed in over 20 countries. The recommended dosage is a single 30 mg oral tablet, taken after unprotected intercourse or contraceptive failure. The drug should be taken as soon as possible, and within five days (120 hours) regardless of food intake or time of menstrual cycle. If vomiting occurs within three hours after administration, it is advised to take a second tablet [57]. The drug is a progesterone receptor modulator, and also classified as a Pregnancy Category X which is why it is contraindicated in pregnant women or those unsure of their pregnancy status. Although it is not known whether the drug induces fetal damage when taken during pregnancy, women are still warned of possible fetal harm. Because of the unknown fetal effects, the drug is advised not to be taken by nursing mothers[56].

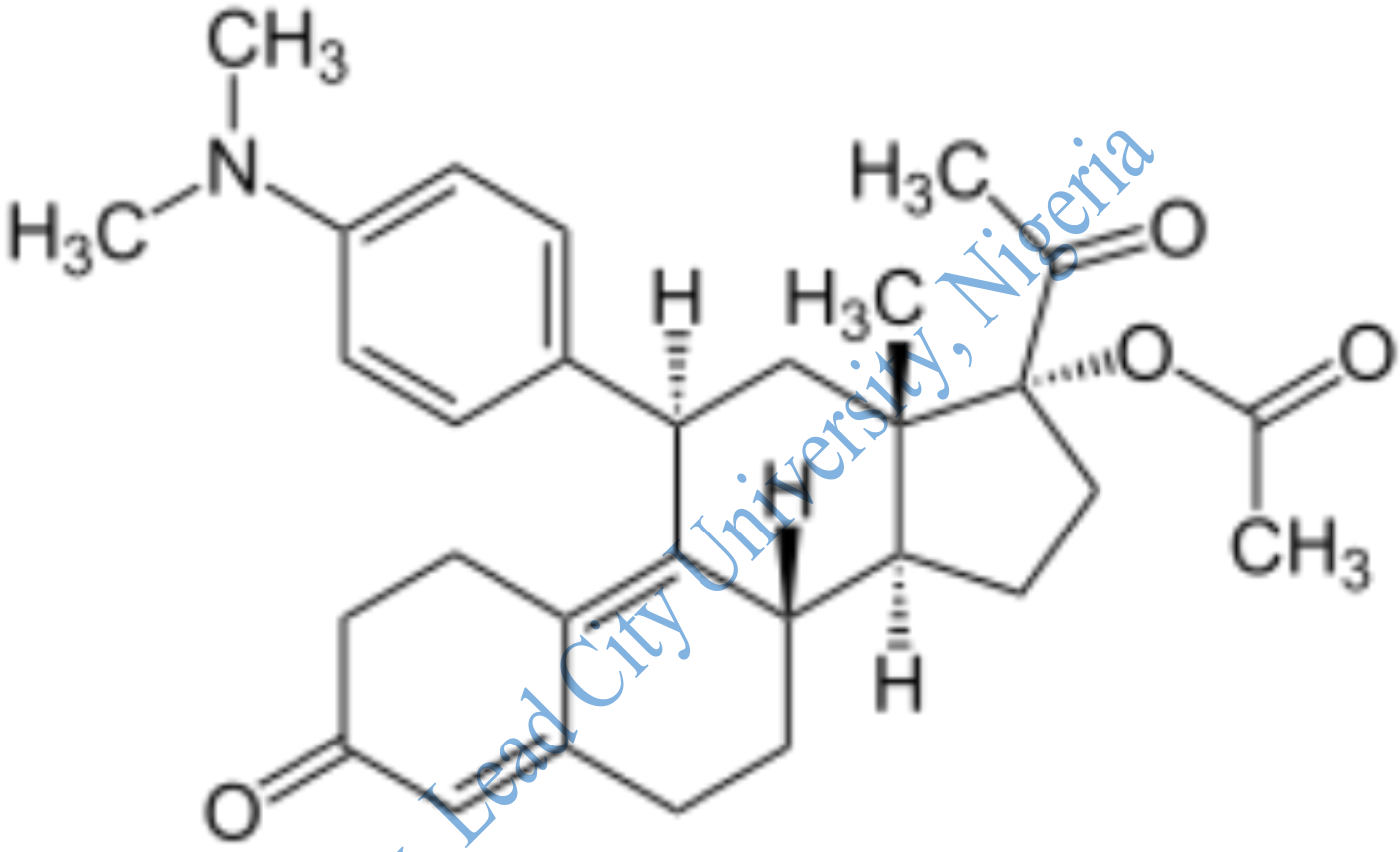

Figure 2.5: Chemical Structure of Ullipristal Acetate (Source [46])

It is advised that hormonal contraceptives such as implants, vaginal rings, birth control pills, skin patches, and injectables, should not be used at least 5 days after taking UPA as they interfere with each other, and reduces efficacy. Although UPA has been studied for use in women as young as 16 years of age, it is currently approved for use in patients 18 years of age, and older. It is not indicated for use before menarche or in postmenopausal women[58]. The other brand name includes Esmya, a 50 mg drug that was first approved, and then had its approval revoked for its implication in severe liver injuries. On September 4th 2020, a further review by EMA-PRAC (Pharmacovigilance Risk Assessment Committee) confirmed that UPA 5 mg can cause liver injury, including the need for liver transplantation. Since it was not possible to identify which patients were most at risk or measures that could reduce the risk, the PRAC concluded that the risks outweighed its benefits, and Esmya should no longer be marketed in the EU (EMA/455818/2020). The strict post-marketing surveillance made it possible to link Esmya administration to side-effects. The time from the first intake of Esmya to hepatic failure ranged from a few days to 6 months[57].

2.3.8.1.1 Pharmacokinetics and Pharmacodynamics of UPA

Ulipristal acetate (UPA, 17α-acetoxy-11β-(4-N, N-dimethylaminophenyl-19-norpregna-4,9-diene-3,20-dione)), a second-generation SPRM derivative of 19-norprogesterone previously known as CDB-2914. In the last 10 years, UPA has been introduced as an EC pill (30 mg) in more than 50 countries, and it is currently recommended as a first-line hormonal treatment for EC due to its higher effectiveness, and similar rate of side effects compared to LNG[59]. The half-life after oral intake is 32 hours when it binds to plasma proteins for 97 – 99 %, and metabolized by the cytochrome P450. Depending on the location, the presence of coactivators or co-inhibitors of gene expression, and the serum levels of progesterone, UPA may exert actions of an agonist or

antagonist of progesterone. The binding capacity to glucocorticoid, and, androgen receptors is lower than its antiprogestin activity: UPA shows in vivo antiglucocorticoid, and anti, androgen activity only at doses 50-fold higher than those needed for antiprogestin effect[58].

Ulipristal acetate exerts its pharmacological activity by binding to the body's progesterone receptors to produce an anti-progesterone contraceptive effect on the ovary (by suppressing or delaying ovulation), interferes with oocyte or zygote transport through fallopian tube, and on the endometrium receptivity, and embryo implantation (by decreasing endometrial thickness). These effects vary according to the timing of drug administration during the menstrual cycle[13]. Ulipristal acetate binds to the progesterone receptor, and blocks the hormone's effects. To achieve improved specificity for the progesterone receptor, UPA was developed to be a derivative of 19-norprogesterone. It possesses the ability to inhibit or delay ovulation, ultimately preventing pregnancy within 120 hours of unprotected intercourse or suspected contraceptive failure. Administering UPA before ovulation causes delayed follicle development, and release, probably a result of suppression of estradiol levels. If the EC drug is taken during the LH peak, follicular rupture, and ovum release may also be delayed[60]. During the latter part of the menstrual cycle, UPA effect may be attributed to its ability to decrease endometrial thickness, and modulate progesterone receptors[13]. The figure shows the binding of UPA to pituitary gland, endometrium, and uterine fibroids elicit its actions via modulations of several markers that regulate different cell functions such as proliferation, apoptosis, extracellular matrix deposition, and angiogenesis.

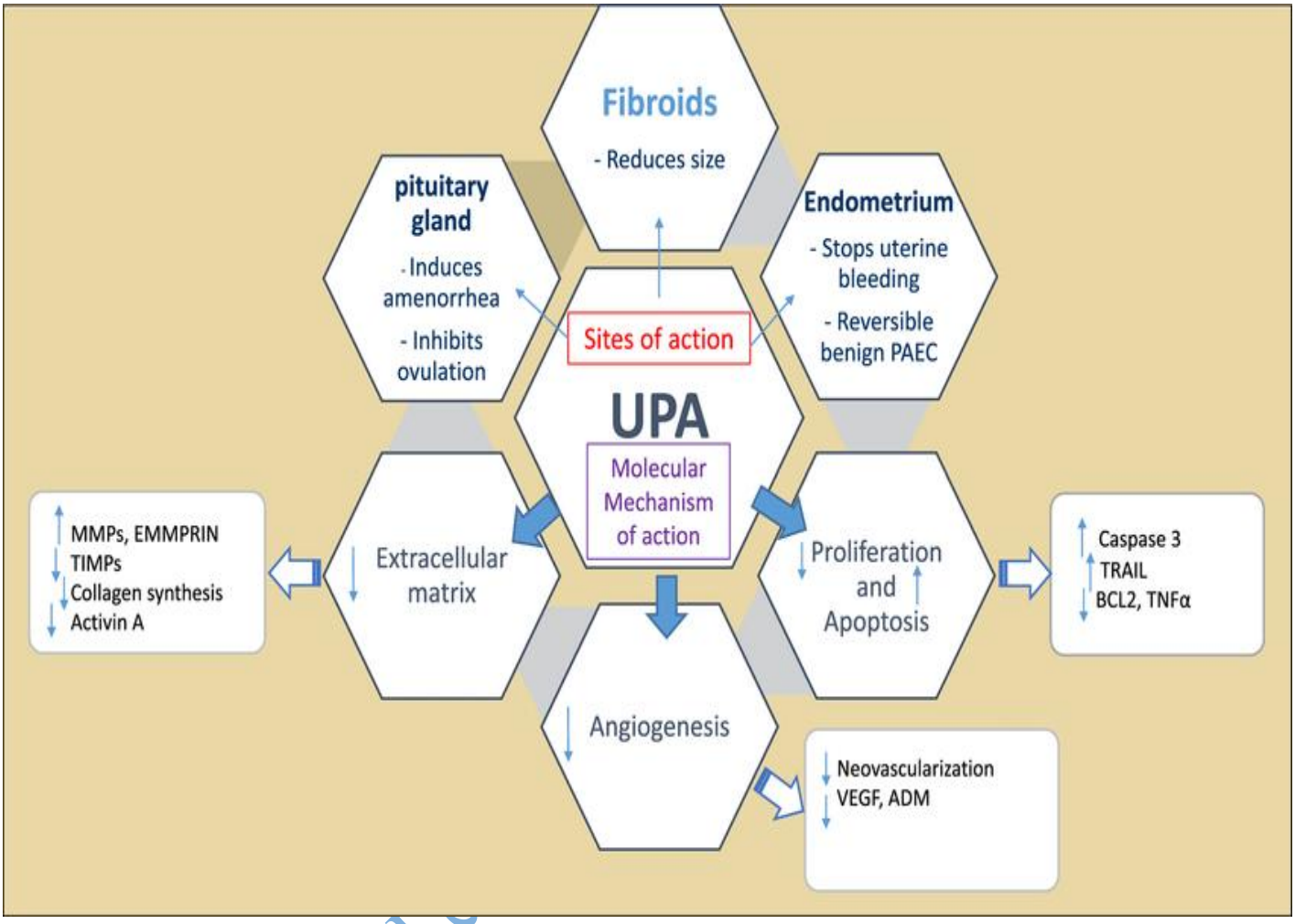

Figure 2.6: Mechanism of Action of Ulipristal Acetate (UPA) (Source [61])

Abbreviations: PAEC - Progesterone Receptor Modulators associated Endometrial Changes; UPA - Ulipristal Acetate; TRAIL - TNF-related apoptosis-inducing ligand; BCL 2 - B-cell lymphoma 2; TNF - Tumor Necrosis Factor Alpha; VEGF - Vascular Endothelial Growth Factor; ADM - Adrenomedullin; MMPs - Matrix Metalloproteinases; EMMPRIN - Matrix Metalloproteinase Inducer; TIMP - Tissue Inhibitor of Metalloproteinases

In addition to its effects on human progesterone receptors, UPA binds to glucocorticoid, and, androgen receptors as shown in figure 2.6. However, its capacity as an antagonist in binding to these receptors is markedly lower than its anti-progestational activity. Anti-glucocorticoid, and anti-, androgen activities were observed at doses 50-fold greater than that necessary for an anti-progestin effect, indicating that UPA is a progesterone receptor modulator with reduced anti-glucocorticoid activity[61].

2.3.8.1.2 Effect of UPA on the Reproductive Organs

Studies have also indicated the effect of UPA on the fallopian tube which plays an important role in conception. The ampullary region of the tube is where fertilization takes place usually within 24hours after ovulation, and zygote are transported by muscular, and cilia activity. Too slow or too rapid tubal transport could cause desynchronization between the fallopian tube, and the zygote, and/or the blastocyst, and the endometrium[13]. It was demonstrated that UPA inhibits CBF, and Muscular contraction in the fallopian tubes through an agonistic effect on the tubal progesterone receptor (PR) even at pharmacological dose[45]. They postulated that firstly, UPA exerts an agonistic effect on PR in the fallopian tube on the PR-A isoform but only a partial agonist/antagonist activity on the PR-B isoform. Animal model was also used to show that UPA has an agonist action in the fallopian tube as against the antagonist action at the ovary[62]. Secondly, they postulated that UPA upregulates mRNA expression of the PR (both generic, and specific to PR-B), and thirdly that it downregulates, andrenomedullin in the fallopian tube, which plays an important role in enhancing the CBF, and muscle contraction tone, frequency, and amplitude. Finally, they postulated that UPA upregulates the expression levels of estrogen receptor (ER), and the PR in the fallopian tubes while they are down regulated by progesterone[62].

Figure 2.7: EllaOne, a 30mg UPA Tablet. (Source [63])

When administered by mouth at a dose of 30 mg (figure 2.7), ulipristal acetate is rapidly absorbed. The maximum or peak mean serum concentration (Cmax) ± the standard deviation (SD) of 176 ± 89 ng/mL is observed at approximately one hour. Its active metabolite, mono-demethyl-ulipristal acetate, has a Cmax of 69 ± 26 ng/mL, which is also exhibited at about one hour. Taken after a high-fat meal, the drug's Cmax is decreased by 40 % to 45 %, the time to maximum concentration (Tmax) is delayed by three hours, and the area-under the-drug concentration (AUC) curve is increased by 20 % to 25 %, compared with administration during the fasting state[61]. Differences in absorption rates, however, are not expected to be clinically relevant; thus, ulipristal acetate can be taken with food or without it. The drug exhibits high plasma protein (> 98 %) binding including albumin, alpha1-acid glycoprotein, and high-density lipoprotein-cholesterol (HDL-C). Its effects in patients with altered plasma protein levels is unknown[59].

2.3.8.1.3 Metabolism of UPA

UPA is metabolized predominantly by the cytochrome P450 (CYP) 3A4 hepatic isoenzyme, and, to a lesser extent, by CYP 1A2. It is metabolized into two major metabolites namely: mono-demethylated, and di-demethylated metabolites, although the mono-demethylated metabolite is pharmacologically active, the di-demethylated metabolite has no clinical activity. The half-life of the drug in the plasma, following a single dose of 30 mg, is approximately 32.4 ± 6.3 hours. In pharmacokinetic, and population studies, UPA has not shown any significant variations in women of different races. The drug is not recommended for breast-feeding or postmenopausal women. The concomitant administration of medicinal products that tend to increase gastric pH may also decrease the drug's efficacy via drug-drug interaction[59].

Table 2.1: Comparison of Adverse Effects of LNG and UPA

	LNG	UPA
Adverse Reactions Reported		
Abdominal pain	27	31
Bleeding	1	1
Breast tenderness	15	16
Diarrhea	11	12
Dizziness	18	20
Fatigue	37	37
Headache	29	29
Nausea	24	29
Spotting	6	4
Vomiting	2	2

$p < 0.05$.

Source [64].

2.3.8.1.4 Biotransformation/Elimination of UPA

Ulipristal acetate is readily converted to its mono-N-demethylated, and subsequently to its di-N-demethylated metabolites. In vitro data indicate that this is predominantly mediated by the cytochrome P450 3A4 isoform (CYP3A4). The main route of elimination is through feces, and less than 10 % is excreted in the urine. The terminal half-life of ulipristal acetate in plasma following a single dose of 5 or 10 mg is estimated to be about 38 hours, with a mean oral clearance (CL/F) of about 100 l hour. In vitro data indicate that ulipristal acetate, and its active metabolite do not inhibit CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A4, or induce CYP1A2 at clinically relevant concentrations.

2.3.8.2 Ethinylestradiol (EE)

17α-ethinyl estradiol was first synthesized in 1938 by Hans Herloff Inhoffen, and Walter Hohlweg at Schering. It was developed in an effort to create an ECP containing estrogen but with greater oral bioavailability, and effectiveness. This was achieved by the substitution of an ethinyl group at carbon 17 of estradiol which makes it structurally different (figure 2.8a). However, this difference in structure leads to a significant increase in estrogenic activity of the drug [65]. It was granted FDA approval on 25 June 1943 (Figure 2. 8b). Ethinylestradiol (EE, 100 µg) has been widely used as contraception. It is most frequently used as the estrogenic component alongside levonorgestrel in combined oral contraceptives [66]. It is also used for the treatment of menopausal, and post-menopausal symptoms especially the vasomotor effects, female hypogonadism, and as a palliative treatment in malignant neoplasm of breast, and prostate. It has also found usefulness in the treatment of some women with acne, and in Turner's syndrome [59].

2.3.8.2.1 Pharmacology and Pharmacokinetics of Ethinyl Estradiol

Ethinylestradiol decreases luteinizing hormone by decreasing endometrial vascularization, and decreases gonadotrophic hormone to prevent ovulation. It has a long duration of action as it is taken once in a day, and a wide range of therapeutic index as overdoses are generally not associated with serious adverse effects. Patients should however be counselled regarding the risks of thrombotic events[65,66].

Ethinyl estradiol has the ability to bind to the estrogen receptor complex, enter the nucleus, and activate DNA transcription of genes involved in estrogenic cellular responses. It also inhibits 5-alpha reductase in epididymal tissue, which lowers testosterone levels, and may delay progression of prostatic cancer. In addition to its antineoplastic effects, ethinyl estradiol has been shown to protect against osteoporosis. In animal models, short-term therapy with this pill has been shown to provide long-term protection against breast cancer, mimicking the antitumor effects of pregnancy[67]. Ethinylestradiol is rapidly, and completely absorbed from the gastrointestinal tract. The ethinyl substitution in the C17 position inhibits first-pass metabolism. Bioavailability is reported at 40%. It is extensively plasma protein bound, mainly to albumin. Unbound molecules distribute widely in the tissues due to their lipophilic nature. Peak plasma concentrations occur initially at 2 to 3 hours after oral ingestion[65]. A second, 12-hour peak, is thought to represent extensive enterohepatic circulation. Biological half-life is approximately 7.7 hours following a single oral therapeutic dose. Elimination phase half -life is reported between 13, and 27 hours. Compared to other estrogens, metabolism of EE is slow. Primary route of biotransformation is through 2-hydroxylation, and of 2-, and 3-methyl ethers formation. First-pass metabolism occurs primarily in the gut wall. Some enterohepatic circulation of sulfate, and glucuronide metabolites does occur hence some are excreted via the feces or via the kidneys[69,70].

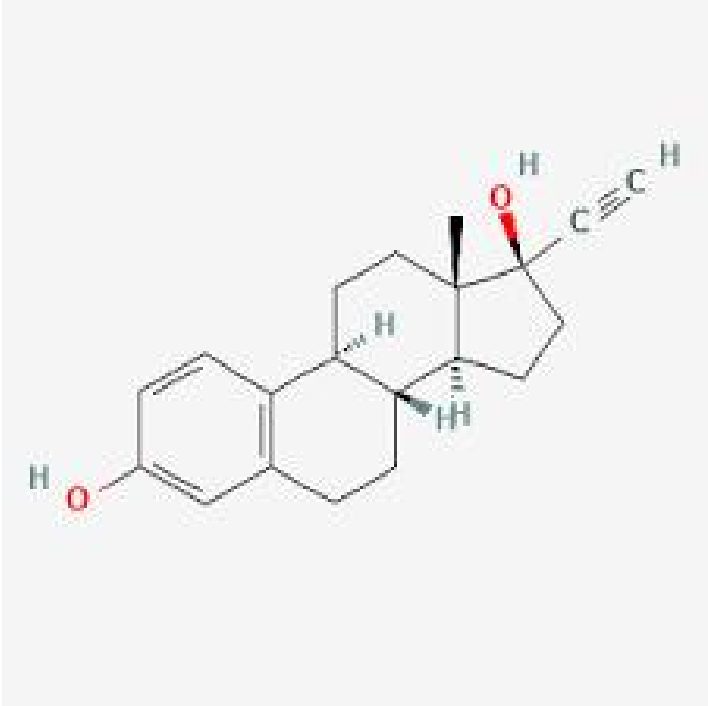

Figure 2.8a: The Chemical Structure of Ethinylestradiol (Source [46])

Figure 2.8b: Ethinyl Estradiol 0.05mg Tablet (Source [68])

2.3.8.2.4 Toxicity of Ethinyl Estradiol

Ethinyl estradiol has been associated with acute poisoning usually involving the gastrointestinal tract which has mild, and self-limiting effects which may include nausea, vomiting, and occasional vaginal bleeding. Chronic toxicity has also been reported to increase the risk of cardiovascular disease such as myocardial infarction, cerebrovascular disease, thromboembolic disease, gallbladder disease, and different types of cancers in some people[65]. Jaundice, hypertension, nasal congestion, headache, dizziness, and fluid retention has also been reported. Endometrial, breast, and certain liver cancers may occur at a higher incidence in continuous use than in the general population[67].

Data has reported a 2 - 15 folds increase in the risk of endometrial carcinoma with its use. The higher the dose, and the longer the length of therapy the greater the risk. However, the addition of progestogen to estrogen replacement therapy was observed to be protective. More research is needed to study whether EE therapy, and other estrogens, increase the risk of breast carcinoma. It was also suggested that there is a moderate increase in the risk of breast carcinoma, but this occurs only after 5 years of therapy[67]. A report also suggested a link between fetal exposure to female sex hormones, and congenital abnormalities which include heart defects, and limb defects. Other estrogens, namely diethylstilbestrol (DEB), have been associated with the development of vaginal, and cervical adenocarcinoma in female offspring of mothers who had taken this drug during the first trimester. Diethylstilbestrol ingestion during pregnancy is also associated with a number of other abnormalities in male offspring, including, smaller testes, and urogenital abnormalities. Although no studies relating EE directly to these findings were identified, the pharmacological similarities in this class of compounds suggest caution should be used[70].

The main adverse effects of EE given in therapeutic dose are directly related to its estrogenic, and metabolic effects. They include water, and sodium retention, which may result in edema, weight gain, and tender breast enlargement. Changes in libido, and withdrawal vaginal bleeding is also reported. Liver function impairment, jaundice, and gallstones may occur. Headache, depression, dizziness, glucose intolerance, and a sensitivity to contact lenses are described. Large doses may produce hypercalcemia when used in the treatment of metastatic carcinoma. Nausea, vomiting, and diarrhea are not uncommon[69]. Dermatological effects include chloasma, melasma, rashes, and urticaria. Erythema multiforme, and erythema nodosum occur. Hypertension, and thromboembolic disease are reported. Acute poisoning such as Nausea, vomiting, and break through vaginal bleeding have been reported following oral contraceptive overdose. Also, nasal congestion, visual disturbances, headache, and hypertension have also been reported in association with estrogen overdose. A correlation between the prolonged use of oral contraceptives, and the development of liver cancer has also been demonstrated in rats[70].

2.3.8.3 Combined Oral Contraceptives (COCs)

Also known as the Yuzpe method, named after the Canadian physician who first described the regimen, COCs contain estrogen combined with levonorgestrel. The combined oral contraceptives (COC) pills are commercially available formulations taken in two divided doses with each dose containing synthetic estrogen (usually 100 –120 mcg ethinylestradiol), and progestin (either 0.50–0.60 mg levonorgestrel or 1.0 – 1.2 mg norgestrel)[37]. Depending on the br, and available, and the concentrations of the estrogen/progestin, each dose consists of 4, 5, or 6 pills. The regimen is one dose taken not more than 5 days after unprotected sex, followed by a second dose 12 hours later[9]. The Yuzpe regimen is reported to be 97 % to 98 % effective in preventing pregnancy. WHO conducted a double-blinded randomized trial of levonorgestrel with

the Yuzpe combination. The levonorgestrel regimen was found to be more effective at preventing pregnancy[14].

In addition to its contraceptive effects, constant administration of COC is also being explored as a therapeutic intervention against polycystic ovary syndrome (PCOS), and dysmenorrhea. However, the use of COC has been linked with disruption in body weight regulation, arterial blood pressure, glucose metabolism, and general homeostatic regulations in females. Endocrinologists, and researchers in the field of reproductive health suggest that continuous prescription of COC should be revised with respect to these adverse effects[37].

2.4 The Female Reproductive System

The female system (figure 2.9) consists of the internal, and the external sex organs that functions in reproduction. The genitals system is made up of internal, and external organs. The external sex organs are organs of the vulva including the labia, clitoris, and vaginal opening. The internal sex organs are the vagina, uterus, Fallopian tubes, and ovaries. The vagina allows for sexual intercourse, and birth, and is connected to the uterus at the cervix. The uterus or womb accommodates the embryo which develops into the foetus[71].

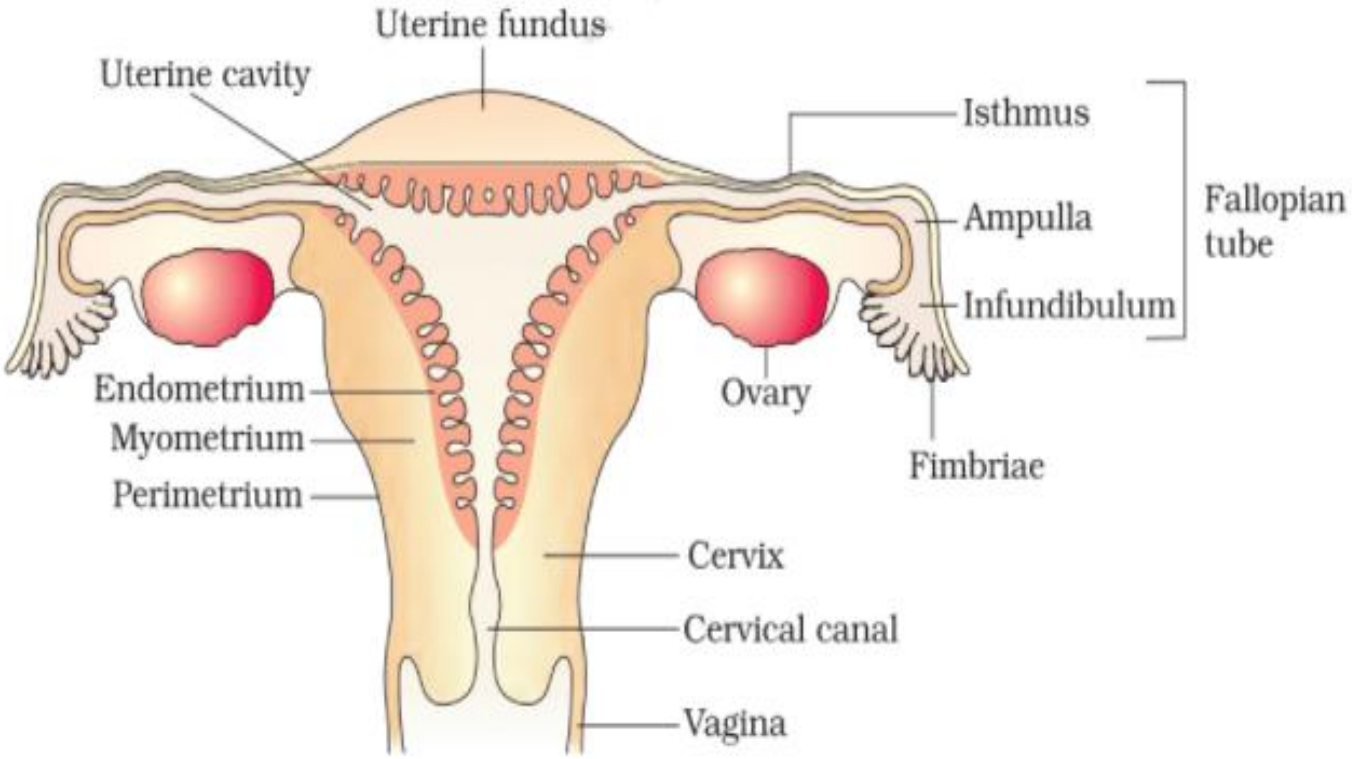

Figure 2.9: The Female Reproductive Organ (Source [71])

2.4.1 The Ovaries

The ovaries are paired pelvic organs that rest on both sides of the uterus, close to the lateral wall of the pelvis, and anterior to the rectum. The ovaries are highly vascular, round with an irregular, nodular surface giving them a red appearance. Each ovary is contained within a bursa, and is located several millimeters caudal to the kidney about 1 cm lateral to the midline. The ovaries are attached at their hilus to the dorsal abdominal wall by the mesovarium, and suspensory ligaments. Each ovary is about 5 mm in largest dimension, and weighs approximately 60 mg although these measurements vary considerably depending on age, and reproductive status[72]. The ovary is covered externally by a layer of modified mesothelium often called the ovarian surface epithelium. Although the ovary is described as having a cortex, and medulla, the division between them is not always distinct. Generally, the medullary region has more prominent blood vessels, lymphatics, connective tissue stroma, and aggregates of pale-staining polygonal interstitial cells, while the cortex has more follicles, and corpora lutea[73]. The histologic appearance of the normal ovary varies with the stage of the ovarian cycle, and age of the animal. Normally there is a complex array of developing follicles with varying degrees of atresia. Following ovulation, the granulosa cells of each follicle form a corpus luteum, which soon becomes well vascularized. Without the cervical stimulation of mating, large corpora lutea of pregnancy are not seen in rats from standard toxicity/carcinogenicity studies, but in addition to the recently formed corpora lutea of the current cycle, corpora lutea formed from subsequent cycles are present in various stages of regression[72].

2.4.1.1 The Menstrual Cycle

The menstrual cycle also known as the endometrial cycle or the ovarian cycle is a well-organized serial event which occurs monthly in a woman between puberty (menarche) to menopause, and

primarily influenced by hormones secreted by pituitary gland (FSH, and LH), and ovary (progesterone, and estrogen). It is divided into three different phases namely; Menstrual phase, proliferative or follicular phase, and the luteal or secretory phase[73]. The menstrual phase is characterized by discharge of blood, connective tissues, and mucus due to cast off of epithelial lining of endometrium wall. It lasts for 3-5 days, and the ovum is unfertilized. During this period, level of estrogen, and progesterone is very low in blood resulting in breaking of endometrium wall of uterus. The normality of menstrual cycle is needed for a successful embryonic implantation [73,74].

The proliferative phase is characterized by rapid proliferation, and repair of damaged endometrium wall, and lasts for 9-10 days (5th – 14th days). FSH is released from the anterior pituitary gland which in turn stimulates development, and maturation of graffian follicle hence the name follicular Phase. Mature grafian follicle secretes estrogen, its level gradually increases, and optimum on 12th day. Estrogen stimulates endometrium repair, proliferation, and ovulation to about 2-3 mm thick, and highly vascular. Luteal phase is the last phase, and lasts for 12-14 days (14th – 28th day). It is characterized by the release of ovum from mature graffian follicle which is stimulated by the secretion of luteinizing hormone (LH) from the anterior pituitary gland. Mature graffian follicle releases ovum, and the rapture follicular cell form corpus luteum. Corpus luteum secretes progesterone which inhibit maturation of any other follicles at high concentration, and stimulate thickening of endometrium wall[73].

When ovum remain unfertilized, corpus luteum degenerate; level of both hormone (progesterone, and oestrogen) decreases, causing breaking of endometrium wall continuing the menstrual phase. At the start of the menstrual cycle, both estrogen, and progesterone levels are low. This signals the pituitary gland to make follicle stimulating hormone (FSH). When FSH levels increase,

around 10-20 follicles begin to grow, and their eggs start to mature. The follicles produce estrogen, which start to prepare the uterus (womb) for pregnancy. The rising estrogen levels signal the pituitary gland to release luteinizing hormone (LH), which in turn, signal the ovaries to produce more, and more estrogen. This forms a positive feedback loop. This sharp increase in LH causes a mature egg to be released from the ovarian follicle. Only one egg generally reaches maturity, and is released at about midway through the menstrual cycle. This is called ovulation. When the egg is released, the positive feedback loop ends, and estrogen, and LH levels fall[74].

After the egg is released, the remains of the ovarian follicle form a structure called the corpus luteum. This structure releases progesterone, and estrogen to continue preparing the uterus (womb) for pregnancy. In most cases, fertilization does not occur, and the corpus luteum decomposes, and stops producing progesterone. Without the progesterone, the uterine lining breaks down, and is discarded with the egg, and other cells, mucus, and fluid during menstruation (or a period)[73].

2.4.1.2 Effects of LNG on the Menstrual Cycle

Levonorgestrel can alter the body's natural hormone levels which can affect the menstrual cycle, leading to an earlier or delayed period as well as heavier or lighter bleeding. Levonorgestrel activity, and efficiency as an emergency contraception was believed to be by delaying the LH surge, and interfering with ovulation which is greater in the days of the cycle with less probability of pregnancy, and lower in the phase with greater probabilities. The major pre-ovulatory mechanism of contraceptive activity of LNG is at the level of the hypothalamic-pituitary-ovarian axis affecting the release of pituitary LH leading to either inhibition or delay of ovulation. It may also disrupt corpus luteum formation causing premature luteinization of unruptured follicles[75].

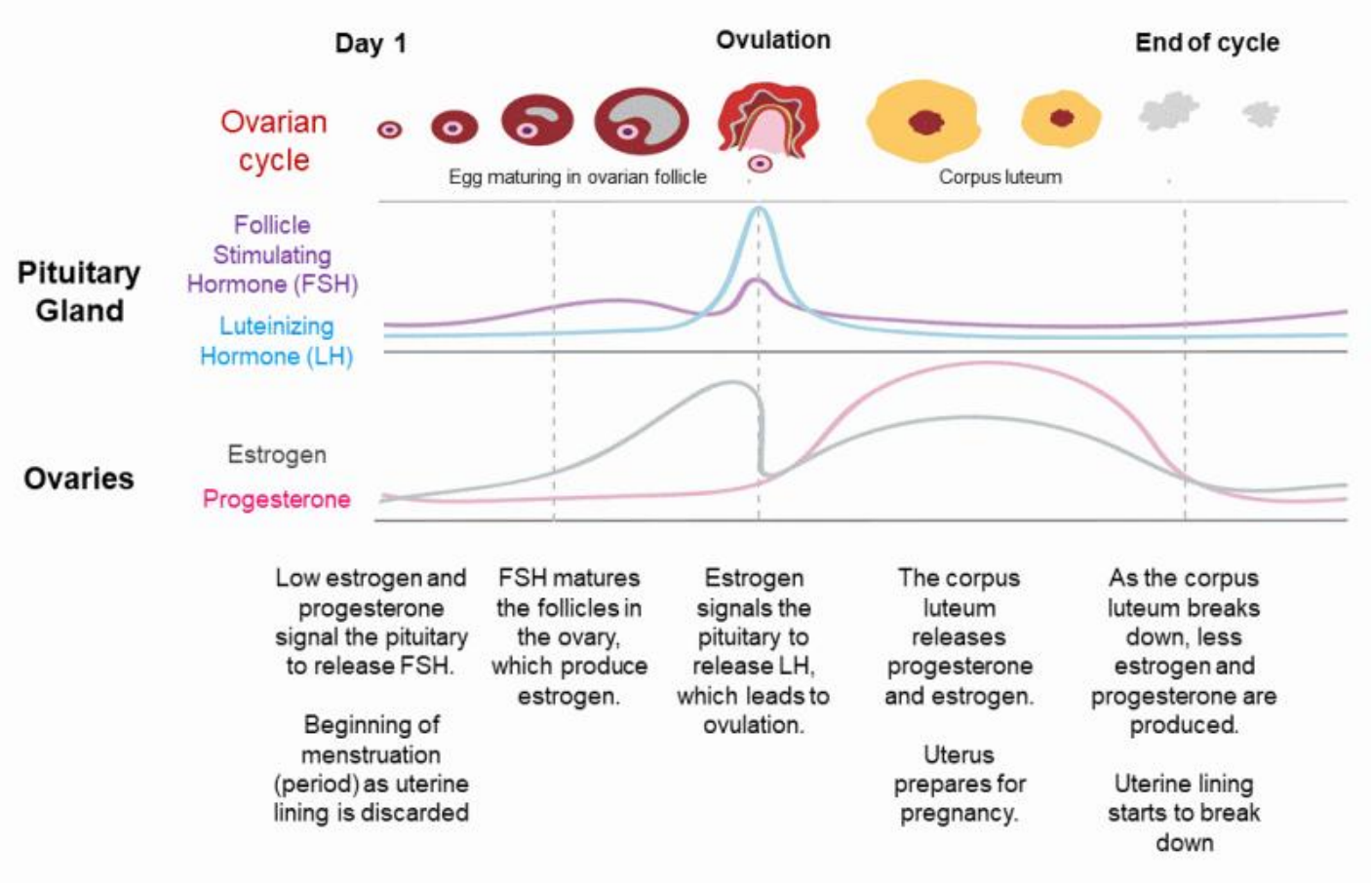

Figure 2.10: The Menstrual Cycle

Source [73]

2.4.2 The Fallopian Tubes

The fallopian tubes are 2 tubular structures connected to the uterus on each side with its fimbriated ends floating freely beside the ovaries on each side in the pelvis[76]. The fallopian tube is also known as the uterine tube, or oviduct, which serves a dual purpose of providing a channel for ejaculated sperm to swim up into the peritoneal cavity, and to bring a fertilized or unfertilized egg down to the uterus, and eventually out of the body at parturition or menstruation. The oviductal environment is crucial for sperm migration, egg pick-up from the ovarian surface, and transport to the uterus, fertilization, preimplantation embryo development, and embryo transport to the uterus, where the embryo will reside for the remainder of pregnancy. If the environment becomes hostile within the oviduct, it can lead to early embryo death, and disrupt normal progression to the blastocyst stage[77].

It is about 10cm long, and enclosed in the broad ligament of the uterus. It is divided into the 4 parts: the intramural part which opens into the uterine wall; the Isthmus which is the narrowest part where the embryo undergoes several cleavage divisions; the Ampulla which is the widest part, and site of fertilization;, and the Infundibulum which is the funneled-shaped end with finger-like fimbriae that relates with the ovaries[77,78]. The fallopian tube is made up of multiple cell types, including stromal cells (fibroblasts), ciliated epithelial cells, secretory epithelial cells, endothelial cells, and smooth muscle cells. These cells are tightly regulated, and controlled by ovarian steroid hormones. The epithelial cells of the oviducts directly interact with sperm, eggs,

and embryos. Furthermore, co-culturing gametes, and embryos with oviduct epithelial cells promotes oocyte maturation, prolongs sperm motility, and improves fertilization rate, and embryo quality in several mammalian species[78]. These findings highlight that gamete/embryo interactions with the oviduct epithelium are crucial for pregnancy establishment. Dysregulation of estrogen (E2) signaling, which may be caused by EC intake, in the oviductal epithelial cells alters the inflammatory response, and ultimately leads to preimplantation embryo death in mice [79].

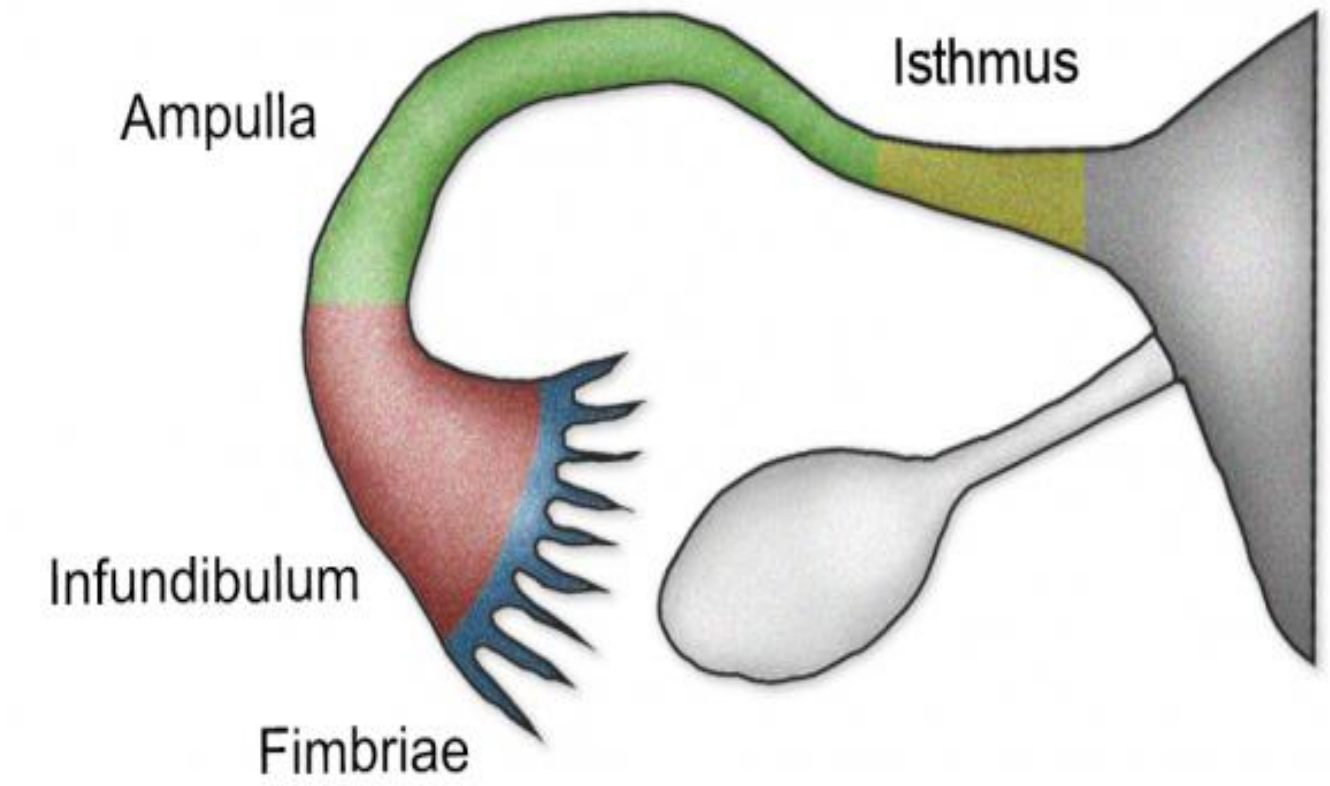

Figure 2.11: The Parts of the Uterine Tube

Source [80]

2.4.2.1 Effects of LNG on the Fallopian Tubes

An increased risk of ectopic pregnancy (EP) has been indicated following LNG contraceptive failure. The use of LNG-EC has also been shown to increase more than nine times the odds of ectopic pregnancy compared to other contraceptive methods[81]. Tubal pregnancy following LNG-EC failure was found to be associated with lower rates of *Chlamydia trachomatis* infection, fallopian tubal inflammation, and/or fibrosis compared with general tubal pregnancy[45]. It was hypothesized that LNG might influence fallopian tube physiology rather than tubal morphology or salpingitis. This is because the transport of embryos in the fallopian tubes is believed to be facilitated through the fallopian tube physiology, which involves ciliary activity, and muscular contractions[81]. Previous studies have reported that progesterone can suppress the epithelial ciliary beat frequency in human fallopian tubes by 40–50 %. Treatment with progesterone also decreases the contractions of the longitudinal muscular layer of the human fallopian tubes compared with the baseline value. Although studies have investigated the physiological effects of progesterone on tubal ciliary beats, and smooth muscle contractions, the effects of the physiological P4 levels induced by LNG-EC is not well understood[72]. Although LNG is a synthetic analogue of progesterone, its structure, and pharmacological properties, including its effective dose, its metabolism, pharmacokinetics, bioavailability, and binding affinity to serum binding proteins, differs from those of progesterone, and as such may elicit a different reaction.

Furthermore, there is no reliable information regarding the influence of LNG on fallopian tubal receptivity, embryo-tubal transportation, and/or implantation[77].

A dose-dependent effect of LNG on ciliary motility was found by[45] which shows an increase in the concentration of LNG resulted in decrease in the tubal ciliary beat frequency, which ultimately led to embryo retention in the fallopian tubes of mice. With regard to the mechanism of EP, it is believed that an altered tubal environment, and impaired embryo-tubal transportation allow implantation of an embryo in the fallopian tube. In an EP, alterations to the fallopian tubal receptivity might be a response to the sequence of factors or cytokines that alter various physiological functions, including the promotion of embryo implantation[74].

2.4.3 The Uterus

The uterus, a hollow, pear-shaped organ, is responsible for different functions such as pregnancy (gestation), menstruation, labor, and delivery. It principally hosts, and nourishes the developing embryo, and fetus up until delivery[76]. It is located in the female pelvis immediately posterior to the bladder, and anterior to the rectum. The uterus also produces secretions that help the movement of sperm to the Fallopian tubes, where sperm fertilizes the egg cells (ova) which are produced by the ovaries[82]. The uterus is divided into three segments: the fundus (the part above the uterine tubes), the lower uterine segment or body (the part from the level of the uterine tube to the level of the isthmus), and the cervix uteri (the part below the isthmus). The uterus is made up of an external layer of smooth muscle called the myometrium, and an internal layer called the endometrium[83]. The endometrium is subdivided into three layers: stratum compactum, stratum spongiosum (which comprises of the stratum functionalis), and stratum basalis. The Stratum compactum, and stratum spongialis develop into the stratum functionalis during the first half of the menstrual cycle (proliferative phase). In the proliferative phase, facilitated by FSH, the

endometrium thickens, connective tissue is renewed, along with glandular structures, and ehlicrine arteries. Estrogen causes the endometrial stroma to become deep, and richly vascularised. Simple tubular glands in the stratum functionalis open oto the surface, and the endometrium thickens[86].

In the secretory phase, facilitated by LH, the endometrial glands become cork-screw shaped, and filled with glycogen. They secrete a glycogen rich fluid during the secretory phase (after ovulation). Decreased levels of LH, and progesterone result in the menstrual phase or menstruation. During menstruation, (shedding of the uterine lining, which occurs when the egg is not fertilized), the spiral arterioles in the stratum functionalis layer contract, resulting in ischemia, and degeneration of the functionalis layer. The arteries rupture, and the rapid blood flow dislodges the necrotic functional layer, which is lost[85].

The functions of the uterine endometrium (uterine lining) include preparation for implantation, maintenance of pregnancy if implantation occurs (progesterone-primed endometrium), and menstruation in the absence of pregnancy (low progesterone). The endometrium thus plays a pivotal role in reproduction, and continuation of our species. Implantation of an embryo occurs during a relatively short window of implantation (WOI) in the menstrual cycle[83]. It is becoming clear that overcoming the current bottleneck in improvements to assisted reproductive techniques, and infertility, a closer look at the interface between uterus, and embryo will be needed as successful embryo implantation requires a cross talk between a healthy embryo with a receptive endometrium[86,87].

2.4.3.1 Effect of LNG on the Uterus

Levonorgestrel can cause changes in the cervix making it harder for sperm to reach the uterus, and harder for a fertilized egg to attach to the uterus. It is also believed that spermatozoa are unable to fertilize the oocyte by possible alteration of the process of capacitation which occurs within the female genital tract to prepare the sperm for fertilization through a series of physiological, and functional changes. Although, it was observed by[88] that LNG has no effect on spermatozoa in an in vitro study, the possibility cannot be entirely ruled out, and more studies will be required. As regard LNG impact on endometrium, focus is on glycodelin, an endometrial protein, its expression, and serum concentrations both during peri-ovulatory midcycle, and at the time of LH surge[88].

Glycodelin, also known as pregnancy-associated α2-globulin, placental protein 14, or progesterone associated endometrial protein, is the major progesterone-regulated glycoprotein that is secreted into uterine luminal cavity by secretory/ decidualized endometrial glands. It is expressed in secretory endometrium, pregnancy deciduas, and amniotic fluid originally, which is vital for the maintenance of normal human reproductive activities. With its distinct glycans, and characteristic carbohydrate structure, glycodelin mediates various biological activities in human reproduction, and fetomaternal immunity. It has four different isoforms which exert their distinctive biological functions based on the protein backbone as well as the glycosylation. They are differentiated based on their differences in glycosylation namely glycodelin A (GdA) mainly from amniotic fluid, and pregnancy decidua, glycodelin S (GdS) from seminal plasma, glycodelin F (GdF) from ovarian follicles, and glycodelin C (GdC) from cumulus oophorus [89].

The absence of glycodelin in the uterus during periovulatory midcycle is consistent with an open "fertile window". So, human endometrium contains no detectable glycodelin during the peri-ovulatory midcycle; the first appearance of glycodelin in endometrium is observed three days

after the LH surge, and its significant increase five-six days after LH surge, at the opening of the implantation window. Glycodelin inhibits NK cell activity, monocytic cell chemotaxis, T-cell proliferation, and it induces T cell apoptosis at the concentrations present in endometrial tissue, and uterine fluid[89]. Glycodelin induced by local or systemic administration of progestogens may potentially reduce the fertilizing capacity of sperm in any phase of the menstrual cycle: it is the first endogenous glycoprotein that was found to potentially, and dose-dependently inhibit binding of human sperm to the zona pellucida[88]. Serum glycodelin concentrations, and endometrial expression during the luteal phase following oral administration of levonorgestrel (LNG) at different stages of the ovarian cycle has been observed. The result showed that hormonal EC with LNG, taken before the LH surge, alters the secretion of endometrial glycodelin in two important phases of the cycle: firstly, in the fertile window when an early increase of glycodelin secretion is significant for its antifertility activity; secondly is in the phase of uterine receptivity in which reduced glycodelin expression may be a reflection of weakened immunosuppressive microenvironment within the uterus at the time of implantation. In another study, thirty women with normal ovarian function were treated with LNG during the pre-ovulatory phase approximately two days before the LH surge. It was observed that LNG did not modify follicle rupture in 20 out of 30 women, and serum glycodelin concentrations significantly increased during early, and mid-luteal phases[85,88].

LNG also shows effect on considerable number of markers of endometrial receptivity in women after LNG oral or vaginal administration. There is either minor or no alterations in markers of endometrial receptivity[37]. Glycodelin suppresses the monocyte chemotaxis, and facilitates its apoptosis, and reduces TNF-α secretion by macrophages, which further influences innate immunity. It also impairs the maturation of DCs, and inhibits their immunogenic T-cell

stimulatory capacity. Glycodelin inhibits the proliferation, IgM secretion, and MHC class II expression of stimulated B cells to regulate humoral immunity, and also decreases the cytotoxicity of NK cells, and upregulates its secretion of IL-6, IL-13, and GM-CSF which results in attenuated lethal effect against target cells. The polarization of naive CD4+ T cells toward T helper type 2 (Th2) is skewed by glycodelin but not Th1 subsets, and subdues the cytotoxic effects of CD8+ T cells. (−) Inhibition; (+) promotion. The expression of glycodelin in females is related to some reproductive system diseases such as premature ovarian failure, recurrent spontaneous abortion, and unexplained infertility. Up till recent times, more studies have reported that glycodelin is expressed in various cancers from female-specific malignancies, such as endometrial cancer, ovarian cancer, and breast cancer, to non-gender specific cancers including lung cancer, and colon cancer[84,85].

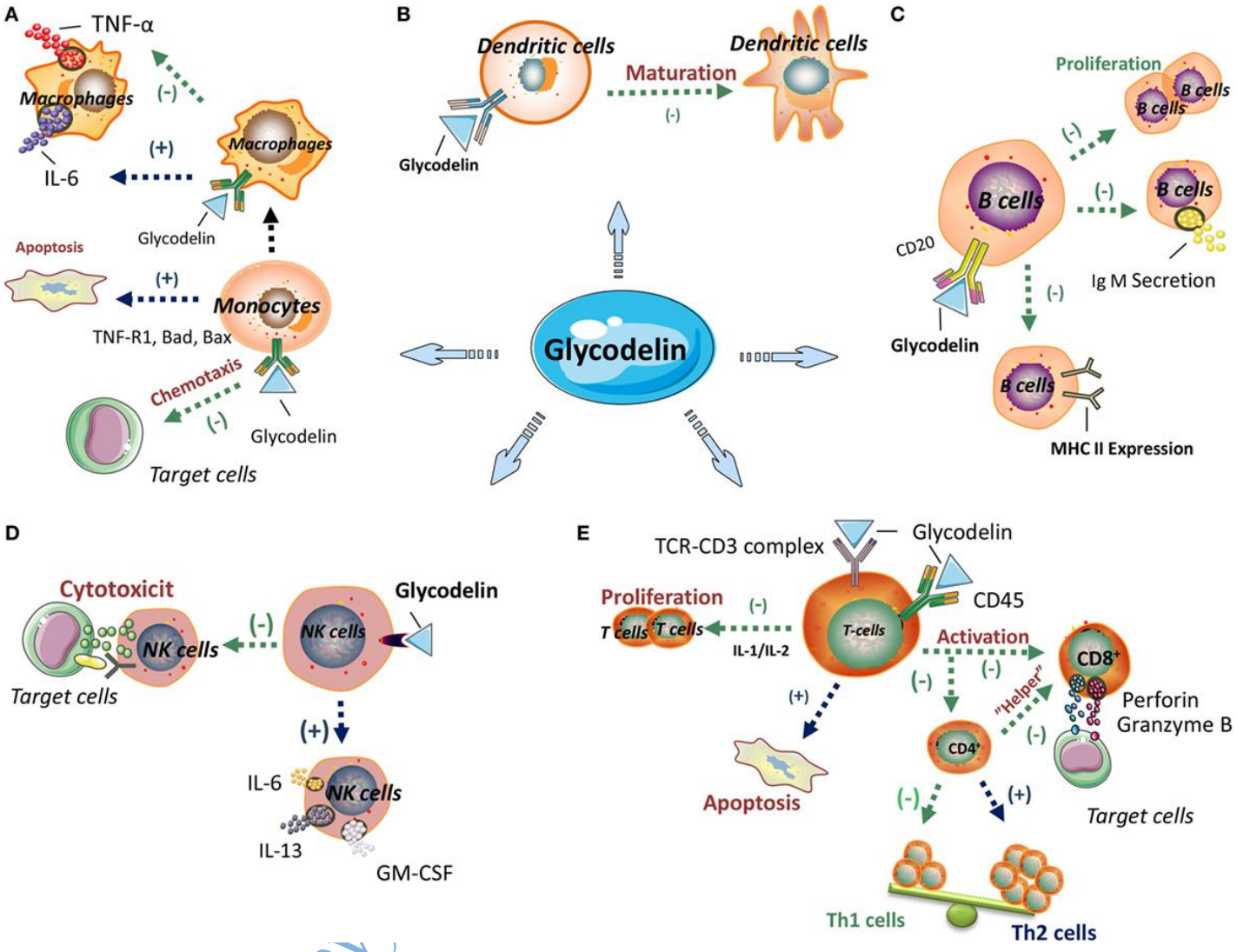

Figure 2.12: The Immunoregulatory Effects of Glycodelin on Multiple Immune Cells Including Monocyte-Macrophages, Dendritic Cells (Dcs), B Cells, Natural Killer (NK) Cells, and T Cells.

Source [89]

2.5 Implantation

Implantation is a highly complex biological process by which the developing embryo moving as a blastocyst attaches to the endometrial surface of the uterus, and invades the epithelium, and

then the maternal circulation to form the placenta Implantation is described as a highly complex biological process in which a developing embryo, moving as a blastocyst through the uterus, makes contact with the uterine wall, and remains attached to it until birth. Before the initiation of implantation, however, both embryo, and endometrium should embark on an elaborated process in a time-, and location-specific manner. The crosstalk between a receptive uterus, and a competent blastocyst can only occur during a limited time span, known as the "window of implantation"[90].

2.5.1 The Process of Implantation

Embryo implantation is the most critical step of the reproductive process in many organisms including humans. It comprises of a unique biological phenomenon, by which the blastocyst becomes intimately connected to the maternal endometrial surface to form the placenta that will provide an interface between the growing fetus, and the maternal circulation[83]. Successful implantation requires a receptive endometrium, a normal, and functional embryo at the blastocyst developmental stage, and a synchronized dialogue between maternal, and embryonic tissues. There must be a coordination between a healthy embryo, and a receptive endometrium to ensure a successful implantation. The process is initiated via fertilization, where the female reproductive tract serves as a natural selection system to guarantee that the most viable, and the best quality sperm reaches, and fertilizes the matured oocyte. After fertilization is done, the fertilized oocyte forms the zygote which travels through the fallopian tube, and develops into a blastocyst stage in the uterine cavity before the implantation process. This process can take up to three days, during which the embryo, and the endometrium relates through secreted, and cell surface factors to prepare for the initial adhesion, and attachment[91].

A single cell embryo divides through several rounds of mitotic division into a morula, and then produces the blastocyst, which comprises of differentiated tissues. The blastocyst in most mammals is made up of a layer of trophectoderm cells that will give rise to the placenta, and the inner cells mass (ICM) which forms the embryo. The blastocyst becomes implantation competent after shedding the zona pellucida. Once the trophectoderm, the outer layer of the embryo, firmly attaches to the endometrial luminal epithelium, it initiates implantation. The endometrium prepares for the attachment of the blastocyst via several internal changes. Without these changes, implantation will not occur, and the embryo is removed during menstruation[90].

The process of implantation may be classified into three stages: apposition, adhesion, and invasion. During blastocyst apposition, trophoblast cells adhere to the receptive endometrial epithelium. The blastocyst will subsequently anchor to the endometrial basal lamina, and stromal extracellular matrix (ECM). At this point, the achieved embryo–endometrial linkage can no longer be dislocated by uterine flushing. This is followed by the invasive blastocyst penetration through the luminal epithelium[90]. Even though the blastocyst can implant in different human tissues, surprisingly in the endometrium, this phenomenon can only occur during a self-limited period spanning between days 20, and 24 of a regular menstrual cycle (day LH+7 to LH+11). At the endometrium is primed for blastocyst attachment, given that it has acquired an accurate morphological, and functional state initiated by ovarian steroid hormones[83].

Implantation involves a complex sequence of signalling events that are crucial to the establishment of pregnancy. A large number of molecular mediators, under the influence of ovarian hormones, have been postulated to be involved in this early feto-maternal interaction. These mediators embrace a large variety of inter-related molecules including adhesion molecules, cytokines, growth factors, lipids, and others. Endometrial receptivity consists in the acquisition

of adhesion ligands together with the loss of inhibitory components that may act as a barrier to an attaching embryo[84]. The relative inefficiency of the implantation process is paradoxical in view of the fact that reproduction is critical to species survival. Implantation failure occurs when there is a failure of firm adhesion to the endometrium. Embryo implantation failure is considered a leading cause of infertility, and a significant bottleneck for in vitro fertilization (IVF). Implantation failure remains an unsolved problem in reproductive medicine, and is considered as a major cause of infertility in otherwise healthy women. Inadequate uterine receptivity is responsible for approximately two-thirds of implantation failures, whereas the embryo itself is responsible for only one-third of these failures[92].

Uterine receptivity is defined as a restricted time-related period when the uterus is receptive to blastocyst attachment, and implantation. The establishment of this endometrial transition, which supports embryo implantation, is primarily coordinated by ovarian hormones, estrogen, and progesterone which modulate uterine events in a spatiotemporal manner. Although numerous molecules involved in implantation have been identified in rodents, and in humans, microarray analysis of the human receptive phase endometrium has only provided an insight into the role a select few molecules. The molecular mechanisms which regulate endometrial-blastocyst interaction remain poorly defined[92].

The window of endometrial receptivity is restricted to days 16-22 of a 28-day normal menstrual cycle, 5-10 days after the luteinizing hormone (LH) surge[90]. The uterus then continues into the non- receptive period for the remaining cycle as the late luteal phase until menstruation ensues. It is not surprising that early pregnancy loss, which occurs during the peri-implantation period before pregnancy is confirmed clinically common in humans. The risk of spontaneous abortion significantly increases with implantation beyond this window. The synchronized molecular

dialogue mediated by cytokines, a variety of growth factors, prostaglandins, matrix degrading enzymes, and their inhibitors, and adhesion molecules is required for successful implantation. Infertility, the inability to conceive within a year of unprotected intercourse, affects one in seven reproductive-aged couples in Korea[93]. Investigating the hierarchical pathways that orchestrate the implantation process will help to develop solutions for implantation failure, and to raise fertility rates in women[91].

2.5.2 Stages of Implantation

The process of implantation occurs in three stages namely: Apposition, when the blastocyst contacts the implantation site of the endometrium; Adhesion, when trophoblast cells of the blastocyst attach to the receptive endometrial epithelium, and Invasion, when invasive trophoblast cells cross the endometrial epithelial basement membrane, and invade the endometrial stroma[94].

2.5.2.1 Apposition and Adhesion

As shown in figure 2.13, implantation begins with apposition of the blastocyst at the uterine epithelium, generally about 2-4 days after the morula enters the uterine cavity. The implantation site in the human uterus is usually in the upper, and posterior wall in the midsagittal plane. Implantation is considered a pro-inflammatory reaction in which endometrial vascular permeability is markedly increased at the attachment site, mediated by Cyclooxygenase (Cox)-derived prostaglandins. Prostaglandin E2 is increased in the luminal epithelium, and the underlying stroma at the both of mice, and human implantation site, thus indicating its role in attachment, and localized endometrial vascular permeability[92,94].

Adhesion of the blastocyst, trophectoderm, and endometrial luminal epithelial cells of the uterus is mediated by specialized cell adhesion molecules which includes integrins, cadherins, selectins, and immunoglobulins. These molecules are expressed on the surface of invasive trophoblast, and they interact with ligands expressed by the extra-cellular matrix of the decidua in a temporal, and spatial way. Integrins are a family of transmembrane glycoproteins that act as cell surface receptors formed by various combinations of two different, non-covalently linked α, and β subunits. Menstrual cycle-specific integrins are up-regulated in the mid-luteal phase of human endometrium, and have been considered as markers of the window of implantation. It has been suggested that a lack of integrin expression during the window of implantation can contribute to unexplained infertile women. The trophoblast also expresses integrins at the time of implantation, and at a site of outgrowing trophoblast cells[94, 95].

2.5.2.2 Invasion

Invasion takes place when fetal trophoblast cells invade, and migrate into the maternal decidua. By this time, the trophoblasts at the site of implantation have formed masses of cytotrophoblasts, and syncytiotrophoblasts. The trophoblast cells eventually destroy the wall of the maternal spiral arteries, changing them from muscular vessels into flaccid sinusoidal sacs lined with endovascular trophoblast. The aim of the process of invasion is to reconstruct the maternal spiral arteries, which will maintain a high blood flow between the fetus, and the mother, replacing small, high-resistance vessels with large, low-resistance vessels. The success, and extent of trophoblastic invasion invariably determines placental efficiency, and fetal viability in late gestation. Deficiencies or unsuccessful trophoblastic invasion results in adverse pregnancy outcomes such as intrauterine growth restriction (IUGR), and preeclampsia[95, 96].

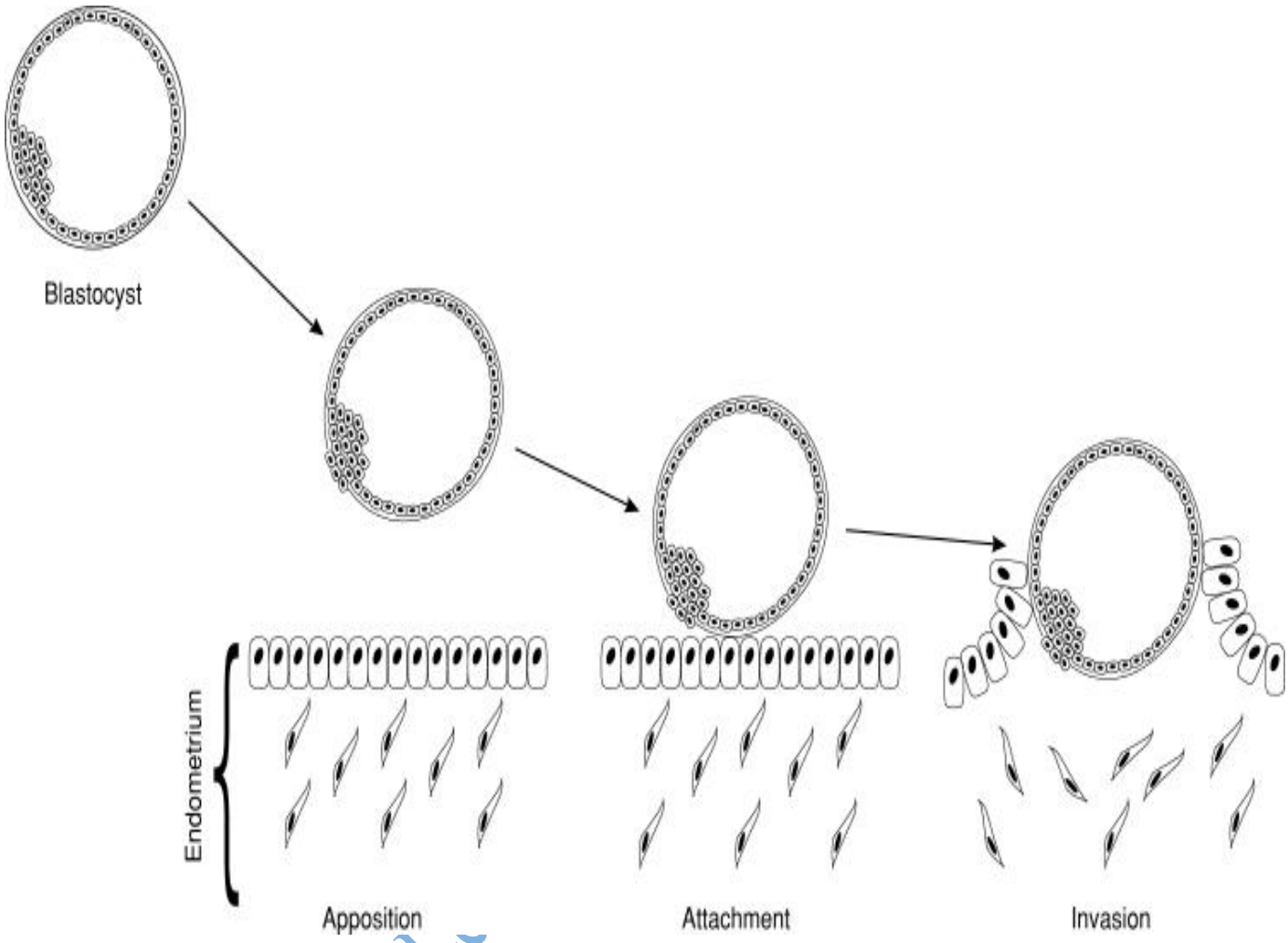

Figure 2.13: Stages of Blastocyst Implantation (Source [96])

2.5.3 Biological Factors that Mediates Implantation

Implantation is mediated by specific biochemical factors, including hormones, growth factors, enzymes, integrins, and cytokines. Any alteration in the secretion, expression or binding of any of these can result in unsuccessful process.

2.5.3.1 Hormones

To achieve successful implantation, the uterus should undergo structural, and functional remodeling which are regulated by a number of hormones. Estrogen, and progesterone are the master hormones mediating these changes. Their action involves their binding firstly to their respective nuclear receptors. The progesterone receptors are present in two isoforms in the human endometrium as PR-A, and PR-B, and the estrogen receptor also exists in two isoforms, as ERα, and ERβ[97]. PR-B levels are tightly regulated in the endometrium during the human cycle, and therefore believed to have more biological importance (In recent years, genetically engineered mouse models have provided information to understand the roles of ovarian steroid hormones during embryo implantation. PR-A is essential for implantation as mice lacking both PR-A, and PR-B are infertile, whereas mice lacking only PR-B have normal fertility. Likewise, ERα is the primary mediator of estrogen action because the uterus of ERα knockout mouse is hypoplastic, and they are infertile, while ERβ knockout mice are fertile[77].

2.5.3.1.1 Estrogen

Estrogen is a class of hormone that includes estradiol (E2), estriol (E3) (which is increased in pregnancy), and estrone (E1) the main form of estrogen after menopause. Estrogen is the primary sex hormone that promotes the growth, development, and regulation of the female reproductive system, and secondary sex characteristics such as the menstrual cycle, breast development, fertility, and pregnancy. In females, estrogens, the most important, are mainly produced in the

female ovaries. Also, estrogens are produced by fat cells, and adrenalgland[98,99]. Estrogen is also important in keeping bones healthy as it prevents the breakdown of bone, and helps the absorption of calcium from the gut that is why a very small amounts of estrogens are also produced in the male body. Estrogens are the main hormone that is produced during the puberty in the female body that is responsible for the development of all the secondary female organs – breast, hips, pubic hairs, armpit hairs. Menstruation in females is regulated by estrogens as it helps in the building of the uterus wall, and decreases on the very low level at the time of menstruation, and thus the uterus wall sheds[100]. Estradiol, which is the main female hormone in women prior to menopause), is released by the pre-ovulatory follicle, and its concentration steadily increases as a result of the growing follicle (s) called Graafian follicle). Once a threshold is reached, an LH surge from the pituitary gland is triggered which decreases further secretion of estrogens, and increases the secretion of progesterone. This ultimately results in an inflammatory process that causes the follicle to rupture, and ovulation to take place. This is the first phase of the female reproductive cycle called the follicular phase characterized by high concentrations of estradiol[99].

The second phase of the ovarian cycle is the Luteal phase which follows the follicular phase. Once the egg is released, the follicle transforms into the corpus luteum (CL) which is a temporary endocrine structure that secrets large amount of progesterone. Also, during pregnancy placenta produces estrogens specifically estriol. Post pregnancy estrogens control lactation, and changes in the breast. Therefore, the intake of hormonal contraceptives which consist of estrogens can result in several health problems, and can also cause estrogens toxicity in the female body on regular intake which may lead to death. Estriol is a metabolic product of the other 2, and all 3 estrogens may be found in the urine of mature females[73,100].

They include inhibins, activins, relaxin, lectins, and the growth factors insulin-like growth factor-1 (IGF-1), Transforming growth factor (TGF)-α, and TGF-β, and cytokines.

2.5.3.1.2 Progesterone

Progesterone belongs to a group of steroid hormones called progestogens, and is the major progestogen in the body. It is a sex hormone produced in the ovaries by the corpus luteum which serve as an essential steroidogenic precursor of several gonadal, and non-gonadal hormones such as cortisol, testosterone, estradiol, and aldosterone. It is also produced by the placenta in pregnancy during which it plays a major role. Low level of progesterone is also present in the male body [101]. It plays important roles in ovulation, pregnancy, embryogenesis while progesterone-based drugs are used for important functions including contraception, immune response, treatment of dysfunctional uterine bleeding, and prevention of cancer[77]. Progesterone's most important functions are to cause the endometrium to secrete special proteins during the second phase of the menstrual cycle, preparing it to receive, and nourish an implanted fertilized egg[99]. During menstruation, the progesterone level is on peaks, and results into several symptoms that can be observed as a Pre-Menstruation Syndrome (PMS) or during menstruation such as mood swings, bloating, food cravings, breast tenderness, and acne on face. Progesterone level remains elevated throughout the period of pregnancy. Progesterone also encourages the growth of milk-producing glands in the breast during pregnancy. When progesterone is consumed as a hormonal contraceptive, its level increases in the body, and the regular usage can disturb the normal functioning of the body causing toxicity, and further health problems[101].

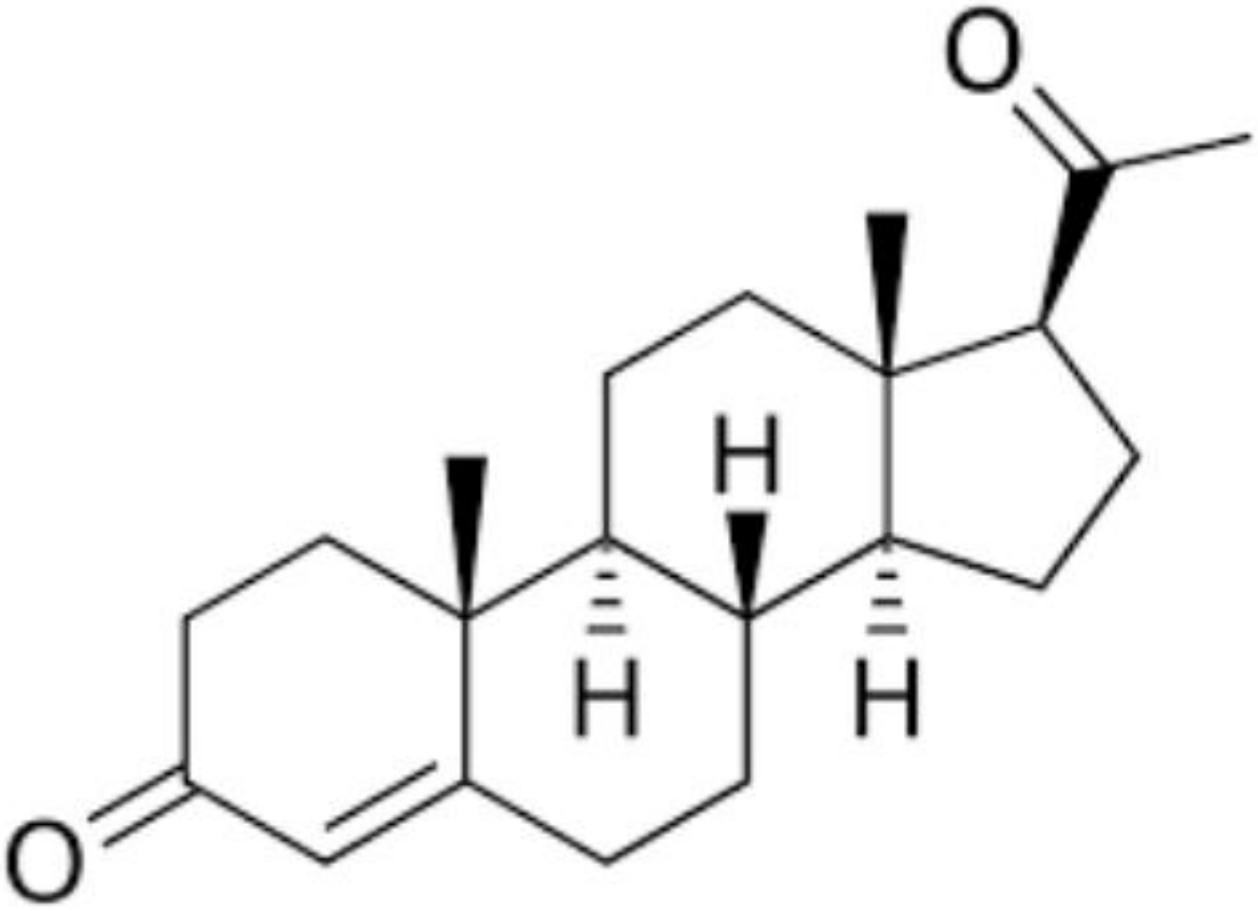

Figure 2.1: Chemical Structure of Progesterone

Source [101]

The other hormones secreted from the ovaries includes testosterone which is made in small amounts. In women, like in men, testosterone is important for bone health, and muscle mass, making new blood cells, and sex drive (or libido). In women, most of the testosterone made by the ovary is converted into estradiol (E2). The other one is Anti-Mullerian hormone (AMH) which is important during development of the ovarian follicles. Inhibin A, and Inhibin B are released from cells within the ovarian follicles. They play important roles in regulating follicle stimulating hormone (FSH) release from the pituitary gland at different stages of the ovarian cycle[100].

2.5.3.1.3 Effects of Hormonal Modulation on Implantation

Progesterone plays a pivotal role in implantation that allows the uterus to support the development of the embryo. The advent of advanced omics technologies provides unique insight of embryo implantation using targeted proteomics by identifying endometrial epithelial cellular, and secreted protein changes in response to ovarian steroid hormones[97].

The binding of progestin to PR leads to conformational changes in the receptors leading to dimerization, dissociation of chaperones, binding to progestin response elements in the promoter regions of target genes, and the recruitment of specific coactivators, and general transcription factors which results in the modulation of transcription of the genes[77].

The proliferative (follicular) phase is influenced by rising estrogen levels due to growing ovarian follicles, leading to proliferation of the epithelium, stroma, and vascular endothelium which causes regeneration of the endometrium. Increasing levels of estrogen are observed before the receptive phase, but it remains to be determined whether this mid-luteal phase estrogen is necessary for implantation in humans or non-human primates. Although estrogen can induce the

receptivity of the uterine, WOI remains open for an extended period at lower estrogen levels but rapidly closes at higher levels in the mouse model. Uterine receptivity is improved when estrogen levels are decreased during the preimplantation period in patients undergoing In Vitro Fertilization, and Embryo Transfer (IVF-ET)[98].

Progesterone induces the formation of pinopodes which are epithelial cells that lose their polarity, and microvilli through down regulation of cell-cell adhesion, and develop smooth protrusions along the apical surface. Pinopodes are most importantly noted for the removal of the cell surface glycoprotein mucin 1 (MUC1) which inhibits cell to cell adhesion during WOI. However, the validity of pinopodes as a marker of uterine receptivity is controversial as pinopodes are detected throughout the luteal phase of the human menstrual cycle, and pregnancy. The undeveloped embryo enters the uterine cavity as a morula, and becomes a 32 to 256-cell blastocyst before implantation. Implantation starts with the loss of the zona pellucida known as hatching, 1-3 days after the morula enters the uterine cavity in preparation for attachment. The active blastocyst goes through structural changes such that a more irregular surface with more microvilli is observed with accumulation of glycogen granules in the cytoplasm[77].

2.5.3.2 Cytokines

Cytokines are regulatory extracellular polypeptides or glycoproteins of low molecular weights synthesized mainly by the T cells, neutrophils, and macrophages. Unlike hormones, cytokines usually act as paracrine or autocrine signals in local tissue, and only occasionally, they have more distant effects as endocrine mediators. They are responsible for the promotion, and regulation of immune responses such as activities, differentiation, proliferation, and production of cells, and other cytokines. They also act on signaling molecules, and cells, stimulating them toward sites of infections, inflammation, and traumas, acting on primary lymphocyte growth

factors, and other biological functions. Cytokines are categorized based on where they act such the site where they are produced (autocrine action), in nearby cells (paracrine action) or in distant cells (endocrine action). This makes them important in the development, and regulation of immune system cells. There are different types of cytokines which include chemokines, interferons (IFN), interleukins (IL), lymphokines, and tumor necrosis factor (TNF)[103].

2.5.3.3 Leukemia Inhibitory Factor (LIF)

Leukemia-inhibitory factor (LIF), a member of the interleukin-6 family of cytokines, is a major mediator of estrogen action. In a study using mice, a knock-out of LIF gene results in infertility, characterized by a defect in implantation, and decidualization that can be rescued by administering recombinant LIF. The expression of LIF is higher around the time of implantation in fertile women as opposed to lower levels observed in infertile women. LIF mediates a shift from a proliferative state of luminal epithelium to a differentiated state through down-regulation of cell-cell junctional molecules acting as a barrier to embryo invasion. LIF also drives stromal proliferation through regulation of the epidermal growth factor (EGF) signaling pathway[103]. Mice with an inactivating mutation in the colony-stimulating factor-1 (CSF-1) gene are infertile due to lower rates of implantation, and fetal viability. Both the embryo, and endometrium express CSF-1 receptor mRNA, and it has been suggested that cross-talk of endometrial epithelial CSF-1 with trophectodermal CSF-1 receptor enhances attachment. Interleukin-1 (IL-1) is believed to be involved in implantation, and the pre-implantation development of the embryo. IL-1 stimulates the expression of vascular endothelial growth factor (VEGF), and regulates the activities of matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs)[102].

Interleukin-6 (IL-6) displayed the highest protein concentration in the endometrial epithelial cells. Assessment of IL-6 through ELISA showed viable blastocysts taking up IL-6 as compared with blastocysts that failed to result in a pregnancy, suggesting a potential role for IL-6 in blastocyst development, and implantation. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is another crucial molecule in the interactions between the uterus, and the embryo during the attachment to the endometrium. It was shown in a study using rodents that it is expressed exclusively in the luminal epithelium surrounding the blastocyst 6 - 7 hours before the attachment process. Its expression is very important in the receptive epithelium, and is concurrent with pinopodes. HB-EGF, as a transmembrane protein, is a synthesized protein that can be processed to release the soluble growth factor, and together they influence the blastocyst through the EGF family of receptors expressed on the blastocyst surface as a juxtacrine adhesion factor. HB-EGF in addition promotes blastocyst growth, and zona-hatching[104].

2.5.3.3.1 Effect of LNG on Uterine Leukemia Inhibitory Factor (LIF)

The cytokine, LIF, is essential for embryo attachment, and it is induced by estrogen. Its expression was found to be reduced in mice treated with 300 µg/ Kg/ day LNG, and the mice showed embryo failure on day 5 of pregnancy of treatment while all the control mice had normal pregnancy due to normal attachment sites. Treatment with LNG is associated with downregulated of LIF expression which may contribute to the contraceptive effect of LNG[105].

Treatment with P4 before implantation decreased decidual progesterone receptor (PGR) expression, and induced decidualization defect apart from LIF downregulation. These findings indicate that progestogens cause embryo attachment inhibition through downregulation of uterine LIF expression, and compromised decidualization through downregulation of PGR independently of LIF reduction [105]. Recent research has shown that LIF may play a role in the

development of endometriosis, highlighting its importance in reproductive health. Additionally, LIF has been implicated in polycystic ovary syndrome (PCOS), another common reproductive disorder. As such, LIF represents a potential target for treatment of these conditions. Looking to the future, further research into the role of LIF in fertility could yield new insights into the mechanisms underlying reproductive health, and lead to new clinical applications. Overall, it is clear that LIF plays a critical role in reproductive health, and warrants continued investigation into its potential therapeutic applications. Both the proliferative, and the secretory phases of the menstrual cycle has been reported to result in a dysregulation of LIF synthesis in the endometrium. In patients with multiple failures of implantation (MFI), the malfunction of cytokine production was reported to be more severe. This led to the suggestion that unknown infertility, and recurrent failures of implantation may be caused by the dysregulation of endometrial LIF secretion during the menstrual cycle[49].

2.5.3.4 Growth Factors

Growth factors are classified according to both structural, and evolutionary associations, ordering them into larger families of proteins. There are a great increase in the number of growth factors, and their families such as bone morphogenic protein (BMP), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], and neurotrophin [NT3]), colony stimulating factors (CSF), platelet-derived growth factor (PDG F), erythropoietin (EPO), thrombopoietin (TPO), myostatin (GDF-8), growth differentiation factor 9 (GDF-9), epidermal growth factors (EGFs), hepatocyte growth factor (HGF), and more in recent times[106].

The term growth factor is sometimes used interchangeably with the term cytokine. Growth factor implies a positive effect on cell division while cytokine is a neutral term with respect to

whether a molecule affects proliferation or not. In this sense, some cytokines can be growth factors, for example granulocyte colony-stimulating factor (GCSF), and granulocyte-macrophage colony-stimulating factor (GMCSF). However, some cytokines have an inhibitory effect on cell growth

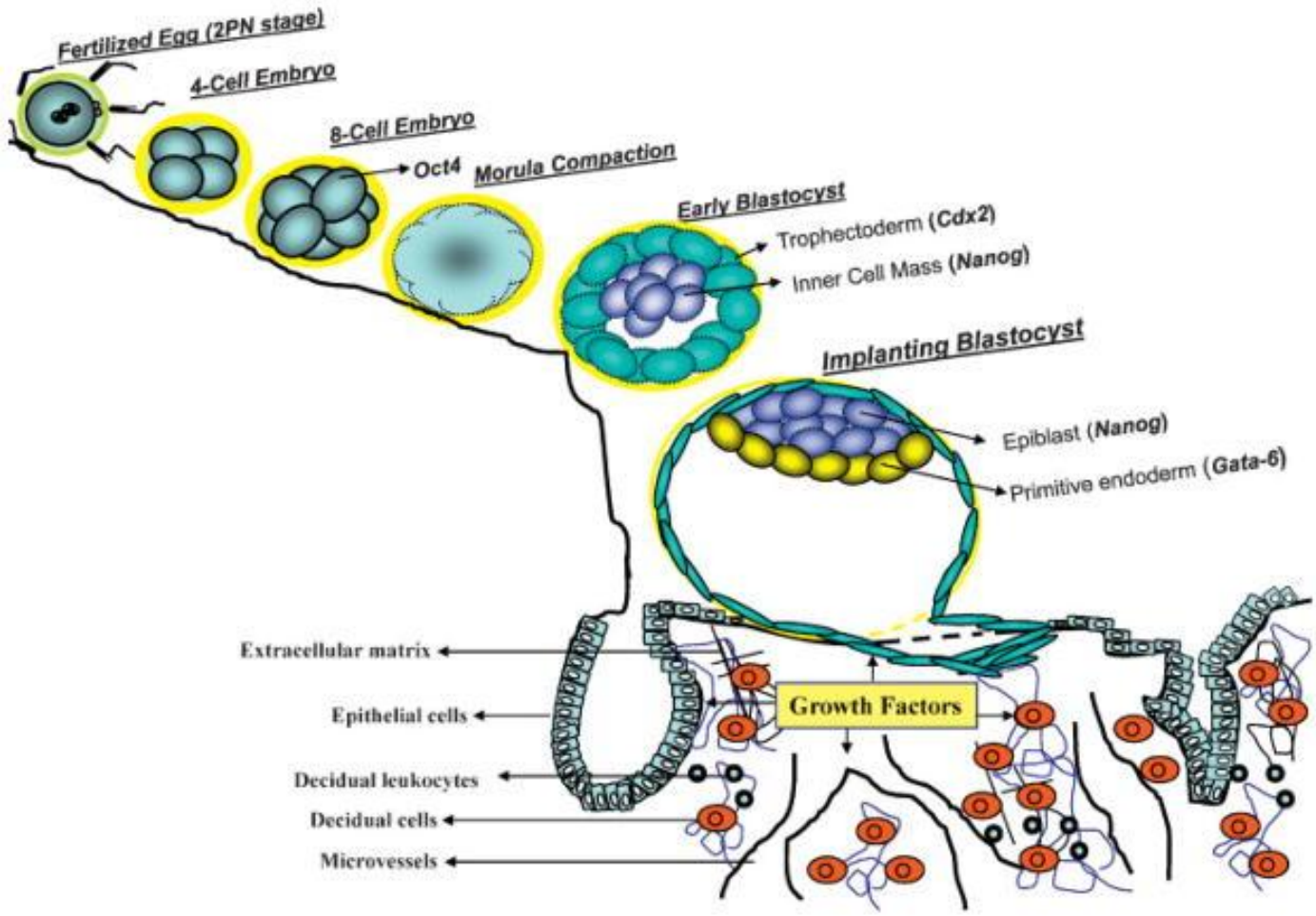

Figure 2.15: Roles of Growth Factors in Implantation

Source [107]

or proliferation, others, such as the Fas and lig, are used as "death" signals because they cause target cells to undergo programmed cell death namely apoptosis[105]. Their roles in implantation is illustrated in figure 2.20. Also, Glycodelin suppresses the monocyte chemotaxis, and facilitates its apoptosis, and reduces TNF-α secretion by macrophages, which further influences innate immunity. It also impairs the maturation of DCs, and inhibits their immunogenic T-cell stimulatory capacity. Glycodelin inhibits the proliferation, IgM secretion, and MHC class II expression of stimulated B cells to regulate humoral immunity, and also decreases the cytotoxicity of NK cells, and upregulates its secretion of IL-6, IL-13, and GM-CSF which results in attenuated lethal effect against target cells. The expression of glycodelin in females is related to some reproductive system diseases such as premature ovarian failure, recurrent spontaneous abortion, and unexplained infertility. Up till recent times, more studies have reported that glycodelin is expressed in various cancers from female-specific malignancies, such as endometrial cancer, ovarian cancer, and breast cancer, to non-gender specific cancers including lung cancer, and colon cancer[84,85].

2.6.1 Hormones of Importance in the Reproductive Tract

The function performed by the endocrine system is imperative in the management of the human reproductive cycle. LH along with the two other gonadotropin hormones, follicle-stimulating

hormone (FSH), and human chorionic gonadotropin (hCG), are very important in regulating sexual, and reproductive functioning[108].

2.6.1.1 Luteinizing Hormone (LH)

LH has an important evolutionary function as it acts on both male, and female gonads (testes or ovaries). Also known as lutrophin or lutropin, it plays key roles in biological processes such as sex steroid synthesis (for both sexes), and the critical reproductive mechanism of ovulation in females. LH is a member of a heterodimeric glycoprotein family along with thyroid-stimulating hormone (TSH), and the other gonadotropin hormones[109]. Members from this hormone family all share the same 92-amino acid a subunit, yet they each have their own respective hormone-specific b subunit that garners receptor specificity, and subsequent functional specificity. In humans, the gene sequence encoding for the LH b subunit is located among a cluster of gene sequences on chromosome 19q13.32. Transcription of the LH subunit is particularly important as it also happens to be the step-in which production rates of LH are modulated. The structure of the highly conserved b subunit amino acid sequence is what provides LH with its essential biochemical behavior, and function. Gonadotropin-releasing hormone (GnRH) mediate the production of LH from the hypothalamus[110]. In females, an acute rise of LH "also known as LH surge" triggers ovulation, and development of the corpus luteum (Figure 2.16). LH stimulates the thecal cell-mediated production of, androgens, which are ultimately converted into an estradiol by aromatase within the granulosa cells.

LH is also involved in progesterone production, and promotes the final stages of follicle maturation through direct interaction with the granulosa cells[111]. It acts synergistically with follicle-stimulating hormone (FSH). LH works to assist FSH in the stimulation of follicle, and it also stimulates ovulation, and release of the ovum. This is accomplished by a large LH surge

which occurs by positive feedback activity of rising estrogen on the pituitary gland, which triggers completion of meiosis I in the primary oocyte, rupture of the follicular wall, and subsequent ovulation 9 hours proceeding peak LH levels. In the postovulatory phase, LH subsequently forms/maintains the corpus luteum by promoting progesterone secretion. The corpus luteum is a small yellowish hormone secreting structure that is formed from the remains of the follicular sac that once held the developing ovum. Its main function is releasing large amounts of progesterone, and small amounts of estrogen, which are very important to implantation, and preparation for pregnancy[108].

In the ovaries, LH supports theca cells that provide, androgens, and hormonal precursors for estradiol production. During menstruation, FSH initiates follicular growth, which specifically affects the granulosa cells. With the rise in estrogens level, LH receptors are also expressed on the maturing follicle, which causes it to produce more estradiol. As the follicle fully matures, a spike in 17α-hydroxyprogesterone production by the follicle inhibits the production of estrogens, leading to a reduction in estrogen-mediated negative feedback of GnRH in the hypothalamus, which then stimulates the release of LH from the anterior pituitary. However, another theory of the LH peak is a positive feedback mechanism from estradiol. The levels keep rising through the follicular phase until they reach an unknown threshold which results in the peak of the LH[111].

2.6.1.2 Follicle Stimulating Hormone (FSH)

Follicle stimulating hormone (FSH) is produced by the anterior pituitary gland in response to gonadotropin releasing hormone (GnRH) from the hypothalamus into the pituitary via the hypophyseal portal system. FSH plays a role in sexual development, and reproduction in both males, and females. It is a glycoprotein dimer with alpha, and beta subunits. The beta subunit (FSHβ) of 111 amino acids is unique to FSH as it confers on it the specific biological action, and

also responsible for interaction with the follicle stimulating hormone receptor, while the alpha subunit is the same as found in Thyroid stimulating hormone (TSH), human Chorionic G, and LH[113]. The hypothalamus produces GnRH, and it is released into the hypophyseal portal circulation to act on G protein coupled receptors at gonadotropic cells of the anterior pituitary.

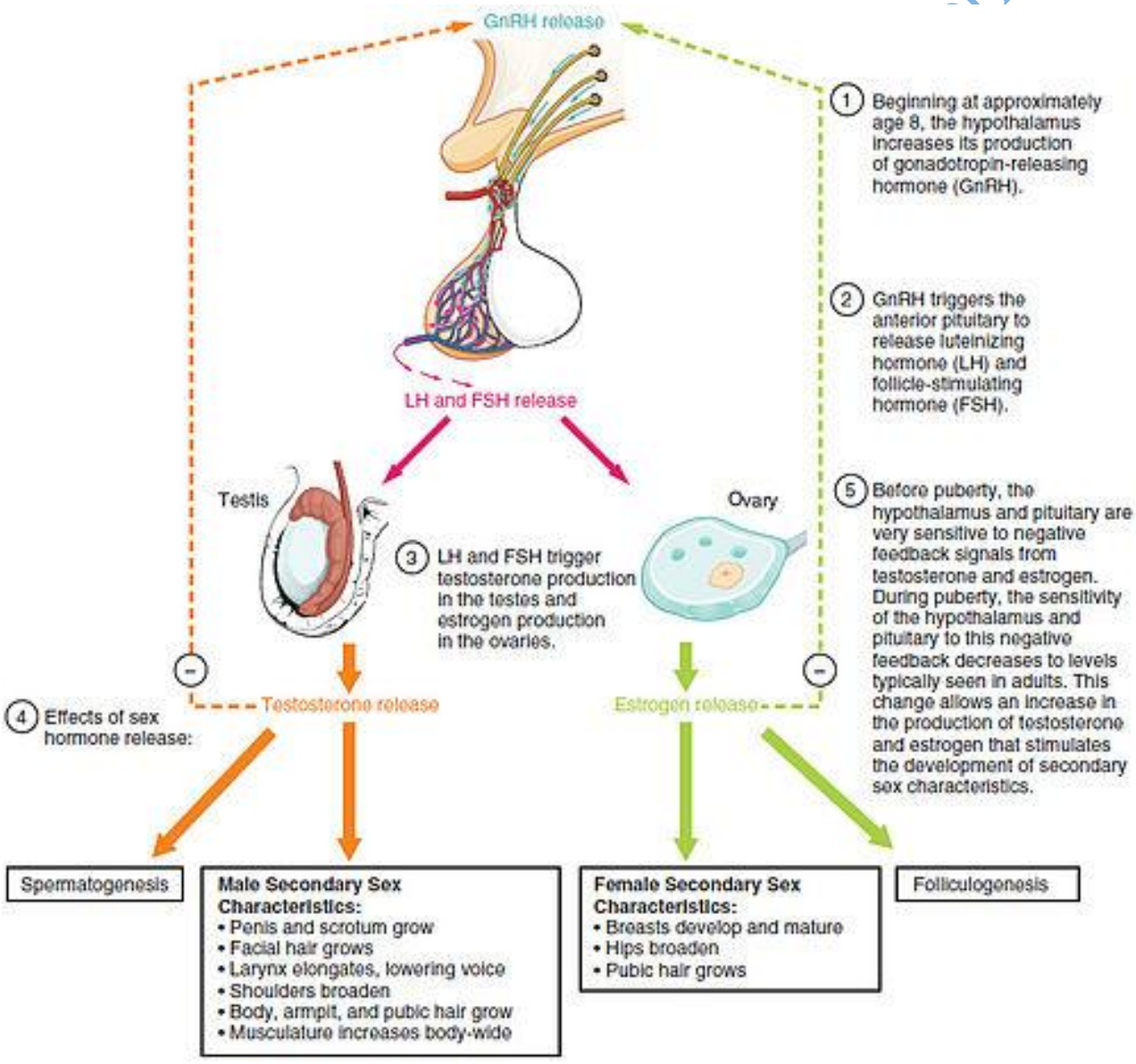

Figure 2.16: Effect of LH in the Body

Source [111]

Those gonadotropic cells produce FSH, and luteinizing hormone (LH), and release them into the peripheral circulation. GnRH release occurs in a pulsatile manner, with low pulse frequencies stimulating more FSH production, and high pulse frequencies stimulating more LH production[111]. The gene for the alpha subunit is located on chromosome 6q14.3 expressed only in two cell types most notably the basophils of the anterior pituitary. The gene for the synthesis of the beta subunit is located on chromosome 11p13, and expressed on gonadotropes of the pituitary cells which is controlled by GnRH, enhanced by activin, and inhibited by inhibin[113].

During the follicular phase of the menstrual cycle, FSH stimulates the maturation of ovarian follicles. As a dominant follicle takes over, and secretes estradiol, and inhibin, FSH secretion is suppressed. When the dominant follicle produces enough estradiol to maintain levels of 200 to 300 pg/mL for 48 hours, the hypothalamus responds with a surge of GnRH which stimulates the secretion of gonadotropic hormones instead inhibiting them[114]. FSH peaks at the same time as the LH surge that causes ovulation. FSH then remains low throughout the luteal phase, preventing the development of new follicles. Continuous use of medications which are GnRH agonist suppresses or even inhibits the release of FSH, and LH from the anterior pituitary as a result of negative feedback from estrogen, which inhibits ovulation, and estrogen production in women. Clinically, GnRH agonists like leuprolide work via this mechanism[115].

The efficiency of FSH is regulated by three main factors: one, the intrinsic bioactivity of the hormone, two, its serum concentration, and three, the efficacy of FSHR signal transduction in response to hormonal stimulation. The rate-limiting step in FSH production is the transcription of the FSH beta subunit coding FSHB gene. The other component in the biologically active FSH heterodimer, the common alpha subunit, is produced normally in excess, and is not known to limit the production of bioactive FSH. Activation of FSH receptor coded by the FSHR gene is necessary for the hormonal functioning of FSH. Medications that can influence the regulation of FSH in any of the three factors will affect its efficiency as suggested by[116]. The effect of LNG on the pituitary level, their clinical value in pharmacogenetic applications requires further studies[117].

2.6.3 Biochemical Profile and Synergistic Action of LH and FSH

LH along with FSH are both stored in the anterior segment of the pituitary gland–pea-sized endocrine gland sitting at the base of the brain. Their secretion is stimulated by gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus into the pituitary via the hypophyseal portal system[113]. They play complementary roles in follicle development, and ovulation via a complex interaction in the hypothalamus, anterior pituitary gland, reproductive organs, and oocytes. Impairment of the production or action of gonadotropins causes relative or absolute LH, and FSH deficiency that compromises gametogenesis, and gonadal steroid production, thereby reducing fertility. In women, LH, and FSH deficiency is a wide range of conditions with different functional or organic causes that are associated by low or normal gonadotropin levels, and low oestradiol levels[118]. While the causes, and effects of reduced LH, and FSH production are very well known, the notion of reduced action has received less attention by researchers. Diminished action combined with reduced endogenous gonadotropin production caused by

GnRH analogue protocols, may lead to resistance to gonadotropins, and, thus, to an unexpected low response to ovarian stimulation[112].

FSH stimulates the synthesis of estrogens, and the maturation of cells lining the spherical egg-containing structures known as Graafian follicles. In menstruating women, there is a preovulatory surge in serum FSH, and LH concentrations. The preovulatory surge of LH is essential for rupture of the Graafian follicle (ovulation), after which the egg enters the fallopian tube, and travels to the uterus. The empty Graafian follicle becomes filled with progesterone-producing cells, transforming it into a corpus luteum. Inhibin, a hormone secreted by the Graafian follicles of the ovaries, and by the Sertoli cells of the testes, inhibits the secretion of FSH from the pituitary gonadotrophs[110]. Medications such as endogenous hormones, that are GnRH agonists initially stimulate secretion of LH, and FSH, but when continuously taken, suppress the release of LH, and FSH. This results in ovarian suppression, and decreased estrogen levels. GnRH antagonists can acutely suppress LH, and FSH secretion. Although it has been shown that both GnRH agonists, and antagonists are useful in the treatment of certain breast, and prostate cancers, endometriosis, and uterine leiomyomas[111,114]. Impairment in the production, and/or action of gonadotropins causes absolute or relative FSH, and LH deficiency that affects the gametogenesis, and gonadal steroid production, invariably reducing fertility[113].

2.6.4 Hormonal Modulation and Infertility

Infertility, the inability to conceive by natural means, is a worldwide problem affecting 8-12 % couple during their reproductive lives with high prevalence around the world. It is a disease of the reproductive system characterized by inability to achieve pregnancy after 12 or more months of regular unprotected sexual intercourse. It is estimated that female factor accounts for 40-50 % of infertility among couples[119]. Infertility can be hormonal, related to age, exercise, obesity or

infectious disease; it can be immunological, psychological, result from surgery or blockage, or be associated with defined abnormalities in the gametes. Many factors are implicated in the etiology of infertility, it may be male or female associated[115].

A variety of factors, including ovulation defects, spermatogenic failure, parental age, obesity, and infections have been linked with infertility, in addition to specific karyotypes, and genotypes. The study of genes associated with infertility in rodent models has expanded the field of translational genetics in identifying the underlying cause of human infertility problems[117]. The issues of infertility are underappreciated in developing countries. Childlessness has negative psychological consequences, and leads to social stigma. Nearly 70 million couples are infertile worldwide, and assisted reproductive technologies are expensive, and particularly unaffordable for couples in developing countries such as Nigeria[120]. In ovarian development, the number of primordial follicles is finite. Thus, there is a subsequent loss of follicles during female development, and puberty, and eventual follicle exhaustion leads to menopause. Moreover, reproduction physiology involves several paracrine, autocrine, and endocrine processes. All of these processes are tightly regulated by a plethora of genes, and discrepancies in any of these pathways can lead to infertility[121].

Infertility is a complex disease, and hence an approach to identify the causative, and contributing factors is imperative. The cause of infertility can be hormonal, immunological, infectious, or psychological. The effects of varying concentrations of estrogen, and progesterone throughout the period of the menstrual cycle have characteristic effects on the endometrium[115]. In the luteal phase progesterone reduces the biologic activity of estradiol on the endometrium by decreasing the concentration of estradiol receptor, increasing the enzymatic activities of 17-

hydroxylsteroid dehydrogenase type II, the enzyme responsible for the conversion of estradiol to estrone, and by increasing the activity of estrone sulfotransferase[122].

2.7 The Rats' Estrous Cycle

The estrus cycle of rats is the reproductive cycle identical to the human menstrual cycle. The cycle lasts for 4 - 5 days and it comprises of four phases which are proestrus, estrus, metestrus and diestrus. The proestrus phase last for 14 days, the estrus phase lasts for about 24 – 48 hours, the metestrus phase lasts for 6 – 8 hours while the diestrus phase lasts for 48 – 72 hours. These phases are associated with different morphological, behavioural, histological and cytological changes. The onset and length of the cycle varies among different rodents[123]. The vaginal opening is associated with an increase in estradiol concentration and the first ovulation usually around post natal day 30 (PND 30).

2.7.1 The Proestrus Phase

The proestrus, the preparatory stage for an animal coming into heat, is associated visually with a full, swollen, and moist vaginal opening[124]. The tissues are pink, with striations in both the dorsal and ventral lips of the vulva. Cytologically, the proestrus phase is characterized by the predominance of nucleated epithelial cells. At late proestrus phase there can be few anucleated cells that are either cornified or not. This phase is associated with a significant surge in the concentration of E2 which in turn cause a peak in the level of LH and FSH as well as P4[125].

2.7.2 The Estrus Phase

The appearance of the vagina looks similar to that in the proestrus, but less pink, less swollen, and less moist, with more prominent striations the estrous phase shows a smear dominated by anucleated cornified cells. This phase sees all hormones return to baseline except for E2 which

has a peak at the evening of the estrus phase. The proestrus and the estrus together makes the ovulation phase in human's menstrual cycle [125].

2.7.3 The Metestrus Phase

This is a brief period characterized with decline of corpus luteum functions in the absence of conception when the activities of reproductive organs gradually subside. The vaginal opening appears pale and dry with a slew of white cellular debris. The metestrus phase is characterized by the same proportion among leukocytes, cornified, and nucleated epithelial cells. Progesterone and estradiol circulate at relatively low concentrations. This is synonymous to the luteal phase in humans[125].

2.7.4 The Diestrus Phase

The features of diestrus include a very wet vaginal opening, which is ometimes too small, and closure in some cases with no tissue swelling. This phase is characterized by a predominance of leukocyte cells is the period of short rest during the breeding season, and anestrus which a non-breeding period when reproductive organs are quiescent[123]. Here, P4 concentration increases sharply and then returns to baseline at the end of the day. This is late luteal period[125].

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Chapter Three

Methodology

3.1. Study Design

This is an experimental animal study.

3.2 Administration of Questionnaires

500 copies of questionnaire (Appendix I) were administered among Lead City University students from various departments to evaluate their awareness, perception, and knowledge of contraceptives. This was done between June and August, 2022.

3.2.1 Analysis of Questionnaires

The data from the questionnaires was analyzed Microsoft Excel Package. The most commonly used emergency contraceptive pill, Postinor-2 (levonorgestrel only), reported from the survey was administered to Wistar rats in this study.

3.3 Study Area

Acclimatization, treatment (drug administration), and observation of the experimental animals were carried out at the institutional animal house under suitable environmental conditions. Biochemical analyses of the tissues of interest post-periodical administration were performed at the biochemistry, physiology, and pharmaco-chemistry laboratories at Lead City University as well as other pertinent research facilities outside the university.

3.4 Drug Procurement

Postinor-2, which is one of the brand names of levonorgestrel, was purchased from Kunle-Ara Pharmacy, a major distributor of drugs, government-approved pharmacy and highly patronized shop, in Ibadan, Oyo State.

3.5 Drug Dosage Determination and Drug Dilution

The normal dose of Postinor-2, comprising two tablets each containing 0.75 mg of the active compound (taken at a 12-hour interval), was used for the study. Each table was weighed and estimated to be 0.11 g (110 mg). The female average body weight (BW) was assumed to be 60 kg. Therefore, 60 kg body-weight animals required 110 mg of Postinor 2 tablet. This was used to obtain the Postinor 2 dosage used in this study, as shown in Appendix II.

3.6 Experimental Animals

Sexually mature female rats of the Wistar strain weighing between 110 and 120 g were used for the study. They were purchased from the Physiology Department of the University of Ibadan,

Nigeria. The animals were acclimatized in the departmental animal house under suitable environmental conditions in plastic cages with wire nets for 28 days. They were given standard rat pellets (Top Feeds Limited, Challenge, Ibadan) and water *ad libitum*. The animals were handled in compliance with the National Institutes of Health guidelines for the care and use of animals in research. The body weight of the animals was measured before and after treatments, prior to each batch of sacrifices.

3.7 Experimental Design

A total of sixty (60) rats were used for the study. The rats were randomly assigned into three (3) groups of twenty animals per group (n = 20). All the animals were allowed equal access to food, and water *ad libitum.* The treatments of the animals lasted for a period of ninety (90) days and were carried out as outlined below.

Group A (Once weekly): The animals in this group were orally administered Postinor-2 at a dose of 1.83 mg per kilogram of body weight (BW) one day in a week at a 12-hour interval (morning at 7 a.m. and evening at 6 p.m.). They were given normal saline on the other day of treatment for group B.

Group B (Twice weekly): The animals in this group were orally administered Postinor-2 at a dose of 1.83 mg per kilogram of body weight (BW) two days in a week, Tuesdays and Thursdays at 8 a.m. in the morning and 5 p.m. in the evening each day.

Group C: The control group was orally administered normal saline, the same volume used as the vehicle for the drug in the treated groups, at the same time as the treated groups.

After One (1) month of treatment, five (5) animals from each group were sacrificed by cervical decapitation; the tissues of interest, namely the blood, liver, kidney, ovaries, and uterus, were

harvested and processed for biochemical analysis and histopathological examination (excluding the blood). This procedure was repeated at the end of the second and third months, respectively. The remaining five animals in each group were left for a 30-day post-treatment recovery period. Thereafter, they were sacrificed, organs weighed and analyses (histological and biochemical) were carried out on the harvested and processed tissues. The summary of the design is shown in table 3.1

Table 3.1: Experimental Design

	Group A (n=20)	**Group B** (n=20)	**Control** (n=20)
Treatment	Food + water + drug (once a week)	Food + water + drug (twice a week)	Food, and water only

At the end of the first month, five rats were sacrificed from each group.	n = 15	n = 15	n = 15
At the end of the second month, five additional rats were sacrificed from each group.	n = 10	n = 10	n = 10
At the end of the third month, five rats were also sacrificed from each group.	n = 5	n = 5	n = 5

Recovery period: 1 month after termination of treatment, the remaining 5 rats in each group were sacrificed.

3.8 Determination of Body Weight of Rats

The body weights of both control and experimental rats were determined and recorded before the treatments started and before each batch of sacrifice using an electrical weighing balance.

3.9 Physical Observation of the Experimental Model (Wistar rats) before, during and after Treatments

The animals were monitored closely before and after each administration of Postinor 2 for any physical or behavioral changes. Considering any changes such as hyperactivity, change in

motion style, loss of hair, redness of the eye, excessive body weight gain or fat accumulation, change in food consumption, prolonged sleeping time, or change in excreta (urine and feces).

3.10 Identification of the Effect of Postinor 2 Treatment on the Rats Estrous Cycle

3.10.1 Visual Assessment of the Estrous Cycle to observe Effect of Postinor 2 Treatment

Visual assessment, one of the most widely accepted methods for evaluating and identifying the estrous cycle, was done according to the method of Macondes and Colleagues[1] and updated by Ajayi and Akhigbe[2]. Each cage was placed on a table in a well-illuminated area of the animal house. Each rat was then picked up by the tail and allowed to stand on the edge of the cage using its forepaws. The tail was raised to expose the vaginal area, and the vulva was then examined. A digital image for documentation was taken, and the observed features were documented. The examinations were done carefully to prevent misinterpretations. The appearance of the vagina at various phases of the estrous cycle is different. In the proestrus phase, the vaginal opening should appear full, swollen, and moist. The tissues are pink, with striations in both the dorsal and ventral lips of the vulva. In the estrus phase, the vagina should appear similar to that in the proestrus, but less pink, less swollen, and less moist, with more prominent striations. In metestrus, the vaginal opening should be pale and dry with a slew of white cellular debris. The features of diestrus include a very wet vaginal opening, which is sometimes too small, and closure in some cases with no tissue swelling.

3.6.2 Determination of the Effect of Postinor 2 on the Estrus Cycle of Rats through Cytological Evaluation

The reproductive status of the rats was monitored for possible effects of Postinor 2 on their estrous cycle and hormonal dysregulation by monitoring two successive cycles for 10 days. The

vaginal smears were collected every day through a non-invasive method described by Macondes *et al*[3]

The procedure was carried out on the animals after 8 weeks of continuous treatment. Every morning between 8:00, and 9:00 a.m. Each animal was held appropriately to allow easy collection of vaginal fluids. This was done by firmly grabbing the back fur from the neck to the back of the head and turning the rat over to expose the chest area and the vagina. Vaginal secretion was collected with a Pasteur pipette filled with 10 µL of normal saline (NaCl 0.9 %) by inserting the tip into the rat vagina but not deeply. The fluid is gently released inside, and withdrawn by gently releasing the bulb of the pipette. This is repeated 2 to 3 times to ensure adequate uptake of the cells. The fluid was gently released onto a labeled, frosted glass slide. Different Pasteur pipettes and glass slides were used for each animal. An adequate quantity of a few drops was collected with a clean tip from each rat. Unstained material was first observed under a light microscope (model) without the use of the condenser lens at 10 X objective lenses. The fluid on a glass slide was thereafter air-dried, stained accordingly with 0.1 % crystal violet stain (0.1 g of crystal violet powder in 100 mL of double distilled water), and viewed again under a light microscope immediately at 10 X. Pictures were taken for proper documentation.

Three types of cells that constitute the estrous cycle were observed: the epithelial cells are round and nucleated; the cornified cells are irregular in shape and without nuclei; and the leucocytes are little round ones. The proportion among them was used for the determination of the estrous cycle phases. The proestrus phase is characterized by the predominance of nucleated epithelial cells; the estrous phase shows a smear dominated by anucleated cornified cells; the metestrus phase is characterized by the same proportion among leukocytes, cornified, and nucleated epithelial cells; and the diestrus phase primarily consists of a predominance of leukocyte cells.

3.11 Sacrifice of Experimental Animals and Harvest of Organs

The animals were weighed, fasted overnight after the last administration for 12 hours, and sacrificed via cervical dislocation[4] and blood was collected into non-heparinized bottles and left to stand for about 30 minutes to facilitate clotting as described by Donovan and Brown[5]. The blood samples were then centrifuged at 3000 g for 15 minutes using a table centrifuge. The serum obtained was stored at -5 ^{o} C, and used for biochemical analyses. The organs of interest, particularly, the uterus (endometrium), ovary, liver, and kidney, were harvested with dissecting scissors and forceps, and rinsed in ice-cold 1.15 % KCl. The organs were examined for gross morphological changes and weighed. The organ weight and body weights were used to determine the relative organ weight of each rat.

3.12 Homogenization and Centrifugation

The organs of interest harvested were rinsed in ice-cold 1.15 % KCl solution, blotted with filter paper, and weighed. 0.2 g of each organ was taken and homogenized with a 1.8 ml phosphate buffer using a Teflon homogenizer to make 2 % homogenate. The resulting homogenate was centrifuged at 10,000 g for 10 minutes in a cold centrifuge (4 ^{0}C) to obtain the post-mitochondrial fraction. The supernatant was then collected, and used for biochemical assays.

3.13 Histopathological Examination of Tissues

Sections of about 3 to 5 µm of the organs of interest, namely, uterus, ovary, liver, and kidney from all groups, were fixed in 10 % phosphate-buffered formalin immediately after excision[6].

3.13.1 Protocol for the Processing of Tissues

The formalin preserved tissues were thereafter processed by dehydration in ascending grades of alcohol, cleaned in xylene, and impregnated in molten paraffin wax using an automatic tissue processor. The tissues were then embedded in paraffin wax by using an Embedding System (Leica EG 1160), followed by sectioning with microtome at 4 microns. The sections were thereafter floated on water using water bath at 45 ^{0}C, and picked unto an appropriately labeled frosted end slide. The slides were fixed on a hot plate for about 30 minutes, and stained with Heamatoxylin, and Eosin method

3.13.2 Heamatoxylin and Eosin Staining

i. All sections were brought into distilled water, and properly rinsed.
ii. The nucleus was stained with Erlichs heamatoxylin for 5 minutes.
iii. It was then rinsed in running tap water for a few minutes.
iv. It was differentiated with 0.5 % acid alcohol for 1- 2 seconds., and rinsed again in running tap water, and Scotts tap water substitute.
v. It was rinsed again in tap water, and stained with eosin for 2 minutes.
vi. Lastly, it was dehydrated, cleared, and mounted in DPX.

3.13.3 Microscopic Examination

The slides were then observed under the microscope at different magnifications on a Brunel light microscope, 20 mega pixels (Brunel SP35 Digital Trinocular). This was done using standard procedures as described by Alam *et al*[6].

3.14 Assessment of Sex Hormones

3.14.1 Quantitative Determination of Progesterone (P4)

Progesterone showed a diversity of end-organ effects, and its primary role was displayed by the reproductive organs. The progesterone level was analyzed by an Enzyme linked Immunosorbent

assay (ELISA) kit designed for the quantitative measurement of total P4 in tissues. The kit used was purchased from Perkin Elmer Health Sciences, Incorporated, Hayward California.

3.14.1.1 Principle of P4 Determination

The principle of progesterone determination by EIA was based on the competitive binding of progesterone in the sample to the progesterone – HRP conjugate for a specific amount of rabbit antiprogesterone. An incubation process allowed the goat anti-rabbit IgG-coated wells to combine with the progesterone in the standard, samples, and controls as well as the rabbit anti-progesterone reagent. During the incubation process, a specific amount of labeled progesterone contends with the endogenous progesterone in the standards, samples, and control for a limited number of binding sites on the given progesterone antibody. Hence, the quantity of P4 peroxidase conjugates immunologically bound to the well progressively decreases as the concentration of progesterone decreased in the sample. Unbound P4 peroxidase conjugate was removed by washing. Thereafter, a TMB solution was added, and incubated at room temperature for 20 minutes which gave rise to colour development. The colour development was stopped with the addition of a 100 µL Stop solution. The intensity of the colour was measured at a wavelength of 450 nm spectrophotometrically. The colour intensity was directly proportional to the amount of enzyme existing in the samples, and inversely proportional to the quantity of the unlabeled progesterone. The absorbance of the standards, and the concentration were used to plot a standard curve, the concentration of P4 in the samples, and controls were calculated from the standard plot.

3.14.1.2 Reagents for P4 Determination

Provided with the test kits were the following materials

i. Goat anti-Rabbit IgG-coated microtiter 96 wells

ii. 0.5 mL each Reference Standards of progesterone ranging from 0, 0.5, 3.0, 10, 25, and 50 ng/mL

iii. 7 mL Rabbit Anti-Progesterone Reagent

iv. 1.3 mL Progesterone – HRP Conjugate concentrate

v. 13 mL Progesterone – HRP Conjugate diluent

vi. 15 mL Wash Buffer Concentrate (50 X)

vii. 12 mL TMB substrate

viii. 12 mL Stop solution

3.14.1.3 Procedure for P4 Determination

The number of coated wells to be used was appropriately labeled against the reference standards, samples, and controls in an Elisa sheet. 25 µL of each was orderly dispensed into the correct wells. Using a different micropipette tip, 50 µL of rabbit anti-progesterone reagent into each well. Then, 100 µl of working Progesterone – HRP Conjugate Reagent was dispensed into each well. This was rigorously mixed for 30 seconds as it was an important step to ensure adequate interaction. Incubation was done at room temperature by placing the microplate wells in the aluminum bag provided for 90 minutes. It was then washed 5 times in an automatic washer containing the washing buffer previously diluted. 100 µL of TMB Substrate was pipetted into the wells, and carefully mixed for 10 seconds. Incubation was done again at room temperature for 20 minutes after which the reaction was stopped by the addition of 100 µl Stop solution into the wells. This was gently mixed for another 30 minutes to ensure a complete change in colour from blue to yellow. The microplate was placed in a microtiter plate reader within 5 minutes, and the absorbance read at 450 nm.

3.14.1.4 Calculation of P4 Assay Results

The mean absorbance value for each set of reference standards, controls, and samples was calculated. The result was used to construct a standard curve of mean absorbance on the vertical Y axis, and the concentration in ng/mL on the horizontal X axis. The absorbance of each sample was thereafter used to determine the corresponding concentrations on the standard curve in ng/mL.

3.14.2 Quantitative Determination of Estrogen (E_2)

The quantitative determination of estradiol was done using a microplate enzyme-linked immunoassay (ELISA) method. The measurement of E2 in serum or plasma is considered to be the most reliable way to assess its rate of production in the body. The kit used was purchased from Monobind Incorporated, USA. The kit used a specific anti-estradiol antibody which does not require prior sample extraction of serum or plasma.

3.14.2.1 Principle of E_2 Assay

The principle of the assay was based on the interaction between a biotinylated antibody, enzyme-antigen, and a native antigen. When the biotinylated antibody was mixed with the antigen in a sample, a reaction occurred as illustrated below:

$Ag + Ab_{Btn} \quad \leftrightarrow \quad AgAb_{Btn}$

Where;

Ab_{Btn} = Biotinylated Antibody

Ag = Antigen

$AgAb_{Btn}$ = Immune Complex formed between Antigen, and antibody

The enzyme conjugate added after a short incubation, allows a competitive reaction between the enzyme analogue, and the antigen in the sample for a limited number of antibody binding sites.

The short incubation period was to allow an increased in sensitivity in cases of low concentration samples.

$^{Enz}Ag + Ag + rAb_{Btn}$ $AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$

^{Enz}Ag = Enzyme – antigen Conjugate (Constant Quantity)

$^{Enz}AgAb_{Btn}$ = Enzyme- antigen Conjugate – Antibody Complex

rAb_{Btn} = Biotinylated antibody not reacted in the first incubation

Ka = Rate of Association Constant

K.a = Rate of Disassociation Constant

K = Ka/ K.a = Equilibrium Constant

Thereafter a reaction between the biotin attached to the antibody, and the streptavidin immobilized on the microwell caused the separation of the bound fraction of antibody after aspiration or decantation.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + Streptavidin_{cw} \rightarrow$ Immobilized complex

Where;

$Streptavidin_{cw}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface

The activity enzyme in the antibody bound fraction was inversely proportional to the native antigen concentration. By utilizing several serum references of known antigen concentration, a dose-response curve can be generated from which the unknown antigen concentration can be extrapolated.

3.14.2.2 Reagents for E_2 Assay

i. Estradiol Enzyme Reagent: 6 mL/vial: A single vial of E2 containing horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with red dye

ii. Estradiol Biotin Reagent: One bottle of this reagent was made up of anti-estradiol biotinylated purified rabbit IgG conjugate in a buffer, green dye, and preservative

iii. Estradiol Calibrators 1 mL/vial labelled A-G: Seven bottles of estradiol calibrators containing serum reference for E2 at concentrations of A - 0; B – 20; C – 100; D – 250; E - 500; F – 1500, and G – 3000 pg/mL.

iv. Streptavidin Coated Plate of 96 wells: One microplate of 96 wells is also provided in an aluminum bag containing a drying agent which has been precoated with 1.0 µg/mL streptavidin

v. Wash Solution Concentrate – 20 mL This contains a surfactant in buffered saline with an added preservative

vi. Substrate Reagent – 12 mL/vial: This contains a buffer made up of tetramethylbenzidine (TMB), and Hydrogen peroxide (H_2O_2)

vii. Stop Solution: One bottle of 8 mL strong acid (0.5 M H_2SO_4)

3.14.2.3 Procedure for E_2 Assay

At room temperature, the microplate wells were highlighted in an Elisa sheet against the calibrators, control (in duplicates), and samples. 25 µL of the calibrators, control or sample was pipetted into appropriate wells. 50 µL of E_2 Biotin Reagent was added to the wells, the plate was swirled gently for 30 seconds to mix thoroughly. This was placed in the aluminum bag to incubate for 30 minutes at room temperature. Thereafter, 50 µL E_2 Enzyme Reagent was dispensed directly on the reagents in the wells, and swirled again for 30 seconds to properly mix. The plate was placed back in the aluminum bag for 90 minutes for another round of incubation at room temperature. The content of the plate was then discarded by decantation, and excess liquid

was blotted on an absorbent paper. An automatic plate washer filled with the prepared wash buffer was used to wash the plate 3 times, and the excess liquid was blotted on an absorbent paper repeatedly. 100 µL of substrate solution was pipetted to all wells in the same order, and incubated at room temperature for 20 minutes. 50 µL of stop solution was then added, and gently mixed for 20 seconds. The absorbance was read at 450 nm using a microtiter plate reader within 5 minutes

3.14.2.4 Calculation of E_2 Assay Results

A dose-response curve was used to ascertain the concentration of E2 in an unknown sample. The absorbance of the standard in duplicates is plotted against the known concentration in pg/mL on a linear graph paper. The points were connected with a best-fit curve, and the intersecting points on the curve is used to determine the concentration of E_2 in the unknown samples.

3.14.3 Quantitative Determination of Luteinizing Hormone (LH)

Quantitative determination of luteinizing hormone (LH) concentration in the serum, ovaries, and uterus was performed using an Enzyme Immunoassay test kit purchased from Perkin Elmer Health Sciences, Incorporated, Hayward, California. The kit was prepared according to the assay procedure of Beastall *et al*.[6]

3.14.3.1 Principle of LH Assay

The principle of the analysis was based on a solid-phase enzyme-linked immunosorbent assay (ELISA). The assay system employs an anti-LH antibody for solid phase immobilization which is the microtiter plate wells, and another mouse monoclonal anti-LH antibody in the antibody-enzyme horseradish peroxidase (HRP) conjugate solution. The test sample was allowed to react in a simultaneous manner with the antibodies resulting in the LH molecules being sandwiched between the solid phase, and enzyme-linked antibodies. A chromogen solution of 3,3',5,5'-

tetramethylbenzidine (TMB) was added, and incubated for 20 minutes resulting in the development of a blue colour. The colour change was stopped with the addition of 2N HCl, and the colour changed to yellow, and measured spectrophotometrically at a wavelength of 450 nm. The concentration of LH was directly proportional to the intensity of the test sample.

3.14.3.2 Reagents for LH Determination

i. Antibody-coated microtiter plate with 96 wells

ii. Sets of standards containing 0, 5, 20, 50, 100, and 200 mIU/mL

iii. 12 mL enzyme conjugate reagent

iv. 12 mL 3,3',5,5'- tetramethylbenzidine (TMB) substrate

v. 12 mL stop solution and 15 mL Wash buffer concentrate (50X)

3.14.3.3 Assay Procedure for LH

i. At room temperature, the lyophilized standards provided in the kit were reconstituted with 0.5 mL distilled water and allowed to stand for 20 minutes before starting the assay

ii. 15 mL (1volume) of the wash buffer (50X) was diluted with 735 mL (49 volumes) of distilled water to make 750 mL (50 volume) of washing solution.

iii. The data sheet was properly labeled according to the samples, standards, and controls added to the coated wells

iv. 50 µL of the samples, standard, and controls into appropriate wells in the microtiter plate

v. 100 µL of Enzyme conjugate substrates were added to each well. This was carefully mixed for 30 seconds to allow complete mixing of the reaction, and incubated for 1 hour at room temperature

vi. The contents of the plate were thereafter decanted into a sink, and the plate was set in a washer to wash 5 times with the washing solution

vii. The residual solution in the plate was emptied on an absorbent paper, after which 100 µL of TMB solution was pipetted into each well, and gently mixed for 5 seconds

viii. Incubation was done again for 20 minutes at room temperature

ix. The reaction was ended by adding 100 µL of Stop solution to the wells, gently mixed for half a minute as the blue colour changed completely to yellow

x. The microtiter plate was placed in the microtiter plate reader, and absorbance was read at 450 nm within 5 minutes.

3.14.3.4 Calculation of LH Results

The mean absorbance value (OD 450) for the standards, and controls was calculated, and used to construct a curve by plotting the mean absorbance of the standards on the vertical Y axis, and the concentration (mIU/mL) on the horizontal X axis. The graph was then used to determine the concentration of the samples in mIU/mL.

3.14.4 Determination of Follicle Stimulating Hormone (FSH) Activity

Quantitative determination of FSH in the serum, uterus, and ovaries was done using an Enzyme immunoassay kit purchased from Diasino Laboratories Co., Limited, Zhengzhou China.

3.14.4. Principle of FSH Analysis

The kit employed the enzyme-linked immunosorbent assay-double antibody sandwich method according to Beastall *et al*[6]. The sample was allowed to react with Anti-FSH coated microwells strip plate, and enzyme-labeled Anti-FSH combined. This coated strip plate was provided in the kit alongside other reagents. FSH molecules from the sample are allowed to react simultaneously with the two antibodies during incubation, sandwiching them between the solid phase, and enzyme-linked antibodies. Following washing, an immunological reaction creates a complex between the solid phase, the samples FSH, and enzyme-linked antibodies. The subsequent

addition of the substrate solution was catalyzed by this complex, producing a chromogenic reaction from blue to yellow. A microplate reader was used to measure the chromogenic reaction at a wavelength of 450 nm. The absorbance read correlated with the concentration of FSH in the sample.

3.14.4.2 Reagents for FSH Assay

i. Standards: S0, S1, S2, S3, S4, and S5 were 0, 5, 10, 25, 50, and 100 mIU/L respectively

ii. FSH Coated Microplate: 8 x 12 strips, 96 wells, pre-coated with mouse monoclonal Anti-FSH.

iii. FSH Calibrators: 6 vials of 1 mL each, prepared, and ready to use at concentrations: 0 (A), 5 (B), 20 (C), 50 (D), 100 (E), and 200 (F) mIU/mL.

iv. FSH Enzyme Conjugate: 1 vial, 11 mL of HRP (horseradish peroxidase) labeled mouse monoclonal Anti-FSH in Tris-NaCl buffer containing bovine serum albumin (BSA). This contains 0.1 % ProClin300 preservative.

v. Substrate - 1 vial of 1 mL Tetramethylbenzidine (TMB), ready to use

vi. Stop Solution - 1 vial, 6.0 mL of 1 mol/L sulfuric acid.

vii. Wash Solution Concentrate: 1 vial, 25 mL PBS-Tween wash solution (40 X concentrated)

viii. A piece of Plate Lid

3.14.4.3 Preparation of Wash Solution

Wash solution was diluted with distilled or water at a ratio of 1:20. 50 mL of Wash Solution Concentrate with 975 mL of distilled water to a final volume of 1950 mL, and poured into the microplate strip washer (EMP Model W206).

3.14.4.4 FSH Assay Procedure

At room temperature, all reagents provided were gently mixed by inversion before use. 25 µL of each labeled sample was pipetted into the microplate wells. 100 µL of enzyme conjugate was then added to each well. Therefore, the microplate was gently shaken for about 30 seconds to allow proper mixing. The plate was then covered with the plate lid for 60 minutes at room temperature to allow incubation. The contents of the plate were discarded by decantation, and blotted with absorbent paper. The microplate was placed carefully in the plate washer, and set to wash 5 times after which the plate was inverted, and tapped out on an absorbent paper to ensure the removal of any residual wash solution. The substrate (25 mL of Wash Solution Concentrate with 975 mL of deionized water to a final volume of 1000 mL) was then added to each well, and covered to be incubated at ambient temperature in a dark drawer for 20 minutes. Caution was taken to avoid shaking the microplate after the addition of the substrate. 25 mL of Wash Solution Concentrate was diluted with 975 mL of deionized water to a final volume of 1000 mL. At room temperature, the stop solution was pipetted into each well, and properly shaken for about 20 seconds to allow mixing of the liquid in the wells. The blue colour changed completely to yellow, and the microplate was placed in a microplate reader (EMP Model 201), and the absorbance read at 450 nm. The result was printed out within 5 minutes.

3.14.4.5 Calculation of FSH Assay Results

The standard curve was generated by plotting the average O.D. (450 nm) obtained for each of the six standards on the vertical (Y) axis versus the concentration of FSH concentration on the horizontal (X) axis. The standard curve was used to determine the unknown FSH activity in the sample in mIU/mL.

3.15 Determination of Endometrial Proteins Level

3.15.1 Progesterone Associated Endometrial Protein (PAEP) in the Serum

This Quantitative Sandwich ELISA kit for research use only was purchased from MyBioSource, USA. This kit is intended to be used to determine the level of PAEP analyte in undiluted Rat serum, plasma, or tissue homogenates samples.

3.15.1.1 Principle of PEAP Immunoassay

Sandwich enzyme immunoassay was the testing method used in this kit. The Progesterone Associated Endometrial Protein (PAEP)-specific antibody has been pre-coated on the microtiter plate included in this kit. A biotin-conjugated antibody directed against the PAEP was then added to the appropriate microtiter plate wells after the necessary standards or samples have been added. Each microplate well was then filled with, and incubated with Avidin conjugated to Horseradish Peroxidase (HRP). Only the wells containing Progestagen Associated Endometrial Protein (PAEP), biotin-conjugated antibody, and enzyme-conjugated Avidin will show a change in color after the TMB substrate solution was applied. The addition of sulphuric acid solution stopped the enzyme-substrate reaction, and the color change was detected spectrophotometrically at a wavelength of 450 nm. Comparing the OD of the samples to the standard curve allowed the determination of the concentration of PAEP in the samples.

3.15.1.2 Reagents for PAEP Assay

i. 1 48 well Micro Elisa Strip plate
ii. 6 vials of 0.5 mL each Standard solution: S1- (Red); S2- (Pink); S3- (Blue); S4- (Green); S5- (Yellow), and S6- (White)
iii. 1 vial of 3.0 mL Sample Diluent (Blue)
iv. 1 vial of 5.0 mL HRP-Conjugate Reagent (Red)
v. 1 bottle of 15 mL 20 × Wash Solution (White)
vi. 1 bottle of 3.0 mL Stop Solution (Yellow)

vii. 1 bottle of 3.0 mL Chromogen Solution A (Purple)

viii. 1 bottle of 3.0 mL Chromogen Solution B (Black)

ix. 2 pieces Closure Plate Membrane

3.15.1.3 Procedure for PAEP Assay

i. Each bottle of reagents was properly checked, and identified

ii. At room temperature, the microplate was removed from the foil pouch.

iii. The wells were carefully marked, and labeled for Blank; Standard, and samples

iv. 50 µL of each Standard solution (S1, S2, S3, S4, S5, S6), and samples were pipetted into corresponding Standard wells, and Sample wells. The blank wells (2) were left empty as instructed in the manual.

v. 100 µL HRP-Conjugate Reagent was then added to all wells except the blank

vi. The Plate was placed in the provided Closure Plate Membrane, and incubated for 60 minutes at 37 °C.

vii. Washing was done for all wells (including all Blank wells) using the 30X washing solution 4 times using an automatic washer. The excess liquid was blotted against an absorbent paper

viii. 50 µL of Chromogen Solution A was thereafter added to every well followed by 50 µL Chromogen Solution B.

ix. The solution was gently mixed, and incubated for 15 minutes at 37 °C.

x. 50 µL Stop Solution was added to every well.

xi. The Optical Density (O.D.) was read at a wavelength of 450 nm using an ELISA reader within 5 minutes after adding Stop Solution.

3.11.1.4 Calculation of Results of PAEP

The mean values of the duplicate readings for each standard, and sample to subtract average optical density of the Blank (OD 0).

Table 3.2: Standard Curve for PAEP

Concentration:	Blank	S1	S2	S3	S4	S5	S6
Mean O.D. (450 nm):	OD0	OD1	OD2	OD3	OD4	OD5	OD6

Professional curve fitting software (GraphPad Prism 9) was used to make the standard curve, and used to calculate the level of the PAEP in the sample.

3.15.2 In-vitro Quantitative Measurement of Leukemia Inhibitory Factor (LIF)

The in-vitro quantitative measurement of LIF in serum was done with an ELISA kit purchased from MyBioSource Biotechnology Company, USA. The kit employs a sandwich enzyme immunoassay for LIF determination.

3.15.2.1 Principle of LIF Assay

The ELISA kit employed the Sandwiched-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat LIF. Standards, and samples were added to the micro-ELISA plate wells, and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat LIF, and Avidin-Horseradish Peroxidase (HRP) conjugate was added successively to each microplate well, and incubated. Free components were washed away. The substrate solution was then added to each well. The wells that contain Rat LIF, biotinylated detection antibody, and Avidin-HRP conjugate appear blue in color. The enzyme-substrate reaction was terminated by the addition of Stop solution, and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The

OD value was proportional to the concentration of Rat LIF. The concentration of Rat LIF in the samples was calculated by comparing the OD of the samples to the standard curve.

3.15.2.2 Reagents for LIF Determination

i. 1 96-wells (8 wells ×12 strips) Micro ELISA Plate
ii. 2 vials of Reference Standard
iii. 1 vial of 120 µL Concentrated Biotinylated Detection Ab (100X)
iv. 1 vial of 120 µL Concentrated HRP Conjugate (100X)
v. 1 vial of 20 mL Reference Standard and Sample Diluent
vi. 1 vial of 14 mL Biotinylated Detection Ab Diluent
vii. 1 vial of 14 mL HRP Conjugate Diluent
viii. 1 vial of 30 mL Concentrated Wash Buffer (25X)
ix. 1 vial of 10 mL Substrate Reagent and 1 vial of 10 mL Stop Solution
x. Plate Sealer 5 pieces

3.15.2.2.1 Preparation of Reagents for LIF Assay

i. Wash Buffer: The wash buffer was diluted by mixing 30 mL of Concentrated Wash Buffer with 720 mL of distilled water to prepare 750 mL of Wash Buffer.

ii. Standard Working Solution: The standard solution was centrifuged at 10,000 g for 1 minute. Add 1.0 mL of Reference Standard, and Sample Diluent were also added, allowed to stand for 10 minutes, and inverted gently several times. After it has fully dissolved, mixed thoroughly with a pipette. This reconstitution produces a working solution of 1000 pg/mL. Then make serial dilutions as needed.

The recommended dilution gradient is as follows:

1000 500 250 125 62.5 31.25 15.63 0 pg/mL.

Dilution method: 8 plain tubes were labeled, and 500 µL of Reference Standard, as well as Sample Diluent, was added to each tube. 500 µL of the 1000 pg/mL working solution was pipetted to the first tube, and mixed to produce a 500 pg/mL working solution. 500 µL of the solution from the former tube was pipetted into the latter one according to this step as shown below. The last tube is regarded as a blank of 0 pg/mL.

Table 3.3: Dilution Procedure for Standard and Sample Diluent in LIF Assay

Tube	1	2	3	4	5	6	7	8
Stock Standard (µL)	500	500	500	500	500	500	500	------
Concentration (pg/mL)	1000	500	250	125	62.5	31.25	15.63	0

iii. Biotinylated Detection Ab Working Solution: The required amount was calculated before the experiment (100 µL/well), and prepared slightly more than calculated was prepared. The stock tube was centrifuged before use, and the 100X Concentrated Biotinylated Detection Ab was diluted to 1X working solution with Biotinylated Detection Ab Diluent.

iv. Concentrated HRP Conjugate Working Solution: The required amount was calculated before the experiment 100 µL/well, and prepared slightly more than calculated was prepared. 100X Concentrated HRP Conjugate was diluted to 1 X working solution with Concentrated HRP Conjugate Diluent.

3.15.2.3 Procedure for LIF Assay

i. The Standard working solution was added to the first two columns. Each concentration of the solution was added in duplicates into different wells, side by side (100 µL for each well). Serum samples were also added to the other wells. 100 µL for each standard, and sample. The plate was

then covered with the sealer provided in the kit, and allowed to incubate for 90 minutes at 37 °C. Solutions were added to the bottom of the micro-ELISA plate well to avoid touching the inside wall, and causing foaming as much as possible.

ii. The solution was poured out of each well without washing, and 100 µL of Biotinylated Detection Ab working solution was promptly added to each well, covered with the Plate sealer, gently mixed by tapping, and incubated for an hour at 37 °C.

iii. The solution was properly decanted from each well, and washed in a microplate washer 5 times using the diluted washing buffer. After this, the plate was patted against a clean absorbent paper.

iv. 100 µL of HRP Conjugate working solution was added next to each well, covered with the Plate sealer, and incubated for another 30 minutes at 37 °C.

v. The solution was decanted again from each well, and washed as previously done in step 3 five different times in an automatic washer. The plate was damped against an absorbent paper to remove excess liquid.

vi. 90 µL of Substrate Reagent was subsequently pipetted into each well, covered with a new plate sealer, and incubated at room temperature for about 20 minutes. The wrapped plate was placed in a dark wardrobe to protect it from light.

vii. Lastly, the addition of 50 µL of Stop Solution was done into each well in the same order as the substrate solution.

viii. The absorbance (the optical density/ OD value) was read in a microplate reader at 450 nm

3.11.2.4 Calculation of LIF Results

The duplicate readings for each standard, and sample were averaged, then subtracted from the average zero standard optical density. A graph was plotted with the standard concentration on the

x-axis, and OD values on the y-axis from which the concentration of the samples was determined in pg/mL.

3.15.3 Quantitative Determination of Prolactin

The in-vitro quantification assay for prolactin was done in serum, uterus, and ovaries homogenates through an enzyme-linked immunosorbent assay as described by Smith and Norman[7]. The ELISA kit employed for this analysis was purchased from DiaSino Laboratories Company Limited, China.

3.15.3.1 Principle of Prolactin ELISA Assay

The assay was based on a sandwich principle that uses Anti-Prolactin coated microwells, and enzyme-labeled Anti- prolactin mixed. An incubation process allowed prolactin in the sample to combine simultaneously with the antibodies which then resulted in the prolactin in the sample being sandwiched between the solid phase (Anti-prolactin), and the enzyme-linked antibodies. Subsequently, a washing process generated a complex via immunologic reaction between the anti-prolactin, the prolactin in the sample, and enzyme-linked antibodies. An addition of a substrate solution catalyzed the complex resulting in a color development which was measured by absorbance. The absorbance was directly proportional to the quantity of Prolactin in the sample.

3.15.3.2 Reagents for Prolactin Assay

i. Prolactin coated Microplate: 8 x 12 strips of 96 wells precoated with mouse monoclonal Anti – Prolactin

ii. Prolactin Calibrators: 6 vials of 1 mL each labeled A – F with the following concentrations: A = 0, B = 5, C = 10, D = 20, E = 50, F = 150 ng/mL

iii. Prolactin Enzyme conjugate: 1 vial containing 11 mL Horseradish peroxidase labeled mouse monoclonal Anti-prolactin in Tris-NaCl buffer containing Bovine serum albumin (BSA). A preservative, 0.1 % ProClin 300 was also added

iv. Substrate solution: 1 vial containing 11 mL tetramethylbenzidine (TMB)

v. Stop Solution: I vial of 6.0 mL containing 1 mol/L sulfuric acid

vi. Wash Buffer Concentrate (40X): 1 vial of 25 mL phosphate buffered saline (PBS) – Tween wash solution.

vii. Plate lid

3.15.3.3 Procedure for Prolactin Assay

The manual was carefully read before the analysis, and reagents preparation were done accordingly. At an ambient temperature, the 25 µL of calibrators, and samples were added to each well previously labelled against the calibrators, and sample on an Elisa test sheet. 100 µL of enzyme conjugate was also added to each well. This was mixed by gently shaking the microplate for 30 seconds. Incubation was then done by covering the microplate with a plate lid at room temperature for 60 minutes. This was done to permit a complete reaction between the solid phase anti-prolactin, prolactin in the sample, and the labelled enzyme anti-prolactin in the Enzyme conjugate. The content of the plate was then discarded by decantation, and the plate was blotted dry on an absorbent paper. An automatic washer, previously filled with the diluted wash buffer solution was used to wash the microplate at a total time of 5. Excess liquid was blotted on an adsorbent paper repeatedly to ensure complete removal of residual wash solution. 100 µL Substrate was pipetted into the wells, covered, and incubated at ambient temperature in a dark

drawer for 20 minutes. Care was taken not to shake the plate after adding the Substrate solution. Thereafter, 50 µL Stop Solution was added to each well, shaken for 20 seconds, and the blue colour was allowed to completely change to yellow. The absorbance was read using a microtiter plate reader at 450 nm within 10 minutes.

3.15.3.4 Calculation of LIF Results

The absorbance printed from the microplate reader was recorded against the calibrators, and samples. The mean absorbance of the calibrators was used to plot a graph against the concentrations in ng/mL. The concentration of prolactin in the samples was then extrapolated from the graph

3.15.4 Quantitative Determination of Sex Hormone Binding Globulin (SHBG)

The in-vitro quantitative measurement of SHBG in serum, and other biological fluids was done with an ELISA kit purchased from MyBioSource Biotechnology Company, USA. The kit employed a sandwiched enzyme immunoassay technique for SHBG determination.

3.15.4.1 Principle of SHBG Determination

The microtiter plate provided in the kit has been pre-coated with an antibody specific to SHBG. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific to SHBG. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well, and incubated. After TMB substrate solution was added, only those wells that contained SHBG, biotin-conjugated antibody, and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of SHBG in the samples was then determined by comparing the O.D. of the samples to the standard curve.

3.15.4.2 Reagents for the Determination of SHBG

i. 1 pre-coated, ready to use 96-well strip plate

ii. 2 pieces of Plate sealers

iii. 2 Standard solutions and 1 vial of 45 mL Diluent Buffer

iv. 1 vial of 120 µL Detection Reagent A and 1 vial of 120 µL Detection Reagent B

v. 1 vial of 9 mL TMB Substrate and 1 vial of 20 mL Wash Buffer (30X Concentrate)

vi. 1 vial of 6 mL Stop Solution

Other materials required but not supplied include: Microplate reader, Precision single, and multi-channel pipettes, and disposable tips, Tubes for diluting samples, Deionized or distilled water, Absorbent paper for blotting the microtiter plate, Container for Wash Solution

3.15.4.2.1 Preparation of Reagents for SHBG Assay

i. All kit components, and samples were opened at ambient temperature.

ii. The standard was reconstituted within 15 minutes of use with 1 mL of Diluent Buffer, and put aside for 10 minutes at room temperature. It was then shaken gently to prevent foaming. The concentration of the standard provided in the stock solution is 2000 pg/mL. 7 plain tubes were prepared with 0.5 mL Diluent Buffer, and the diluted standard were used to produce a double dilution series according to the table below. For instance: tube 1 = 2000 pg/mL, tube 2 = 1000 pg/mL, tube 3 = 500 pg/mL, tube 4 = 250 pg/mL, tube 5 = 125 pg/mL, tube 6 = 62.5 pg/mL, tube 7 = 31.2 pg/mL, and the 8th tube with Diluent Buffer only is the blank with a concentration of 0 pg/mL

Table 3.4. Preparation of Reagents for SHBG Assay

Tube	1	2	3	4	5	6	7	8
Stock Standard (µL)	500	500	500	500	500	500	500	------

Concentration (pg/mL)	2000	1000	500	250	125	62.5	31.2	0

iii. Detection Reagent A, and Detection Reagent B were centrifuged briefly, and diluted with the Diluent buffer to a working concentration (1:100), for instance, 100 µL reagent in 1900 µL diluent buffer. Detection Reagents A, and B were slowly pipetted to reduce volume errors because they are sticky.

iv. Wash Solution – 20 mL of Wash Solution concentrate (30X) was diluted with 580 mL of distilled water to prepare 600 mL of Wash Solution (1X).

3.15.4.3 Procedure for SHBG Assay

i. Wells was determined for diluted standards, blank, and samples using an appropriate ELISA assay sheet. 7 wells for the standards, 1 well for blank. 100 µL each of dilutions of standard, blank, and samples were pipetted into the appropriate wells. The microplate was covered with the Plate sealer, and incubated for 120 minutes at 37 ^{0}C.

ii. The liquid was decanted from each well, and damped on an absorbent paper but not washed

iii. 100 µL of Detection Reagent A working solution was added to each well, and incubated for 60 minutes at 37 ^{0}C after covering it with the Plate sealer.

iv. The solution was washed with 300 µL of 1X Wash Solution added to each well using a squirt bottle, multi-channel pipette allowed to sit for 2 minutes. The excess liquid from all wells was removed completely by tapping the plate onto absorbent paper. Then washed thoroughly 3 additional times. After the last wash, the remaining Wash Buffer was removed by decantation, and blotted against absorbent paper.

v. 100 µL of Detection Reagent B working solution was thereafter added to each well, and incubated for 60 minutes at 37 ^{0}C after covering it with the Plate sealer.

vi. The washing process was repeated for a total of 5 times as conducted in step 4.

vii. 90 µL of Substrate Solution was pipetted into each well, covered with a new Plate sealer, and incubated 25 minutes at 37 ^{0}C away from light. The liquid in the wells turned blue with the addition of the Substrate Solution.

The addition of 50 µL of Stop Solution to each well turned the liquid from blue to yellow. The liquid was mixed by tapping the side of the plate against the palm gently to ensure thorough mixing.

viii. Excess drops of water were removed from the plate, and placed in a microplate reader, and readings were taken at 450 nm immediately.

3.15.4.4 Calculation of SHBG Results

The average optical density of the zero standard was subtracted before averaging the duplicate readings for each standard, and control. A standard curve by was then constructed with the mean absorbance, and concentration for each standard by placing the concentration of SHBG on the y-axis, and the absorbance on the x-axis then tracing a line of best fit between the points on the graph in pg/mL.

3.16 Assessment of Inflammatory Markers

3.16.1 Nuclear Factor Kappa B (NF-κB)

The quantitative determination of TNF-α in serum, uterus, and ovaries were assayed using a Microplate Immunoenzymometric assay (ELISA) whose kits was supplied by Mornmed Medical Equipment Company Limited, United Kingdom.

3.16.1.1 Principle for the Determination of NF-κB

A set of calibration standards supplied with the kit is used to determine the concentration of NF-κB in the samples. The calibration standards are assayed at the same time, and conditions as the samples whose optical density is determined at a wavelength of 450 nm by measuring the intensity of the colour change brought about by the addition of a Stop solution which terminates the reaction of a set of chromogenic solutions previously introduced. The chromogenic solution is added after, HRP- conjugate has been pipetted into each well containing the standards, and samples in duplicates, and thoroughly washed.

3.16.1.2 Reagents for NF-κB Assay

i. 1 Micro Elisa strip plate
ii. 6 tubes of 0.3 mL standards: S0, S1, S2, S3, S4, S5 at concentrations of 0, 75, 150, 300, 600, and 1200 pg/mL
iii. 6.0 mL Sample Diluent and 10.0 mL HRP- Conjugate reagent
iv. 25 mL 20X wash buffer; reconstituted with distilled water at ratio 1:20
v. 6.0 mL chromogen A solution and 6.0 mL Chromogen B solution
vi. 6.0 mL Stop solution
vii. 2 pieces of closure plate membrane

3.16.1.3 Procedure for the Determination of NF-κB

i. All standards were labelled in duplicates in the microplate well, and 50 µL of each standard.
ii. 10 µL of each sample were pipetted into appropriate well as labeled on the Elisa sheet, and 40 µL of Sample diluent was added.
iii. 100 µL of HRP – conjugate reagent to all well, after which the plate was covered in with the adhesive strip, and incubated for 60 minutes at 37 ^{0}C.

iv. The plate was washed using the automatic plate washer set at 5 times with 400 µL Wash solution. Excess solution was blotted out by damping on an absorbent paper

v. 50 µL of chromogen A, and Chromogen B were added successively into each well, mixed gently, and incubated for 15 minutes at room temperature.

vi. The chromogenic reaction was stopped by adding 50 µL Stop solution, and tap gently to mix which changed the colour from blue to yellow

vii. The absorbance was read at 450 nm in a microplate reader

3.16.1.4 Calculation of NF-κB Elisa Results

The OD of the standard was first averaged, and subtracted from the value of the zero standard before result interpretation. This was used to construct a standard curve with the OD values on the Y axis, and the corresponding concentration on the X axis. This was used to extrapolate the concentration of the analyte in the samples

3.16.2 Determination of Tumor Necrosis Factor-α (TNF-α)

The quantitative determination of TNF-α in serum, uterus, and ovaries were assayed using a Microplate Immunoenzymometric assay (ELISA) whose kits were supplied by Mornmed Medical Equipment Company Limited, United Kingdom.

3.16.2.1 Principle for the Quantitative Determination TNF-α

The concentration of NF-B in the samples was calculated using a set of calibration standards included with the kit. The optical density of the samples was determined at a wavelength of 450 nm by measuring the intensity of the color change caused by the addition of a Stop solution, which stopped the reaction of a group of chromogenic solutions that were previously introduced. The calibration standards were assayed at the same time, and under the same conditions as the

samples. After the HRP- conjugate has been pipetted into each well containing the standards, and samples in duplicate, and thoroughly cleaned, the chromogenic solution was added.

3.16.2.2 Reagents for TNF-α Elisa Assay

i. 1 Micro Elisa strip plate

ii. 6 tubes of 0.3 mL standards: S0, S1, S2, S3, S4, S5 at concentrations of 0, 20, 40, 80, 160, and 320 pg/mL

iii. 6.0 mL Sample Diluent and 10.0 mL HRP- Conjugate reagent

iv. 25 mL 20X wash buffer; reconstituted with distilled water at ratio 1:20

v. 6.0 mL chromogen A solution and 6.0 mL Chromogen B solution

vi. 6.0 mL Stop solution

vii. 2 pieces of closure plate membrane

3.16.2.3 Procedure for TNF-α Elisa Assay

i. All standards were labelled in duplicates in the microplate well, and 50 µL of each standard.

ii. 10 µL of each sample were pipetted into appropriate well as labeled on the Elisa sheet, and 40 µL of Sample diluent was added.

iii. 100 µL of HRP – conjugate reagent to all well, after which the plate was covered in with the adhesive strip, and incubated for 60 minutes at 37 ^{0}C

iv. The plate was washed using the automatic plate washer set at 5 times with 400 µL Wash solution. Excess solution was blotted out by damping on an absorbent paper

v. 50 µL of chromogen A, and Chromogen B was added successively into each well, mixed gently, and incubated for 15 minutes at room temperature.

vi. The chromogenic reaction was stopped by adding 50 µL Stop solution, and tap gently to mix which changed the colour from blue to yellow

vii. The absorbance was read at 450 nm in a microplate reader

3.16.2.4 Calculation of TNF-α Elisa Results

The optical density (OD) of the standard was first averaged, and subtracted from the value of the zero standard before result interpretation. This was used to construct a standard curve with the OD values on the Y axis, and the corresponding concentration on the X axis. The concentration of the TNF-α in the samples was determined by extrapolation on the standard curve.

3.17 Liver and Kidney Function Indices

3.17.1 Estimation of Aspartate Transaminases (AST)

Aspartate transaminases was assayed Spectro photochemically using Randox kits purchased from Randox Laboratories Limited, United Kingdom. The principle is based on that developed by Reitman *et al*[8], which measures the concentration of AST by monitoring oxaloacetate hydrazone formed with 2,4 dinitrophenylhydrazine in serum, and liver homogenates.

L-aspartate + α-oxoglutarate $\xrightarrow{\textbf{SGOT}}$ L-glutamate + Oxaloacetate

Where SGOT is serum glutamic oxaloacetic transaminase

3.17.1.1 Composition of Reagent used for AST Analysis

The reagents used for the assay are:

i. Buffer (R1): This comprises of 100 mmol/L of phosphate buffer (pH 7.4), 100 mmol/L L-aspartate, and 2 mmol/L α-oxoglutarate.

ii. 2 mmol/L 2,4-dinitrophenylhydrazine (R2)

iii. 0.4 mol/L Sodium Hydroxide solution: This was prepared by dissolving 1.6 g of NaOH in 100mL distilled water

3.17.1.2 Procedure for AST Assay

The reagent blank, and sample were prepared into different test tubes by following the procedure in table 3.5 below

Table 3.5: Procedure for AST Assay

	Reagent Blank	**Sample**
Sample	___	0.1 mL
R1	0.5 mL	0.5 mL
Distilled water	0.1 mL	____
The mixture was thoroughly mixed, and incubated at 37^0C for 30 minutes		
R2	0.5 mL	0.5 mL
The mixture was thoroughly mixed again, and allowed to stand at 25^0C for 20 minutes		
Sodium Hydroxide	5.0 mL	5.0 mL

Thereafter, it was mixed again, and allowed to stand for 5 minutes before pouring in a cuvette, and the absorbance of each sample was read at 546 nm against the reagent blank.

3.17.1.3 Calculation of AST Activity

The activity of AST in the tissues were extrapolated from the standard calibration curve which was plotted from the values provided in the kit manual

3.17.2 Estimation of Alanine Aminotransferase (ALT)

Alanine aminotransferase (ALT) was determined quantitatively in the serum, and liver homogenate using a Randox kit purchased from Randox Laboratories Limited, United Kingdom. The kit employed the spectrophotometric methodology by Schmidt and Schmidt[9], which allows for a rapid measurement of ALT activities in serum by double-beam spectrophotometry in a short-interval enzyme-activity analyzer.

3.17.2.1 Principle of ALT Estimation

The amount of pyruvate hydrazone produced by 2,4-dinitrophenylhydrazine was monitored to determine the amount of ALT

L – alanine + α - oxoglutarate $\xrightarrow{\text{SGPT}}$ L – glutamate + pyruvate

where SGPT is serum glutamic pyruvic transaminase

3.17.2.2 Procedure for ALT Assay

The reagent blank was first prepared by pipetting 0.5 mL of solution R1 into a test tube, and then adding 0.1 mL of distilled water. This was then thoroughly mixed, and incubated for exactly 30 minutes at 37 ^{0}C. 0.5 mL of solution R2 was then added, mixed, and allowed to stand for exactly 20 minutes at 25 ^{0}C. Sodium Hydroxide (0.5 mL, 0.4 mol/L) was finally added before mixing again, and using it to blank at 546 nm. The samples were added first into the test tubes before following the same steps for the addition of reagents in the blank solution.

3.17.2.3 Standard Calibration Curve for ALT Activity

The standard calibration curve for ALT activity was obtained from the values provided in the kit manual as shown below (table 3.6), and the ALT activity in the tissues were estimated by extrapolation using the calibration curve.

Table 3.6: Procedure for ALT Standards

Absorbance	U/I	Absorbance	U/I
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.5000	94

Source: Randox kit manual, Randox Laboratories Limited, United Kingdom.

3.17.3 Calculation of AST/ALT Ratio

AST/ALT ratio alternatively known as De Ritis ratio was calculated by dividing the value of AST with that of ALT in the serum, and liver[10].

3.17.4 Estimation of Alkaline Phosphatase (ALP)

The ALP activity was estimated in serum, and liver samples using a Randox kit (AP) The kit employed the method according to Deutsche Gesellschaft Fur Klinische Chemie (DGKC)[11].

3.17.4.1 Principle of ALP Determination

p-nitrophenylphosphate + H2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol

This was done by directly mixing with 2-amino, 2-methyl, 1-propanol (AMP) buffer at pH 10.5 followed by estimation of absorbance of the resultant yellow color solution at 405 nm

3.17.4.2 Composition and Preparation of the Reagents

Provided in the kits were the following reagents

i. Buffer (R1a) was made up of 1 mol/L Diethanolamine (pH 9.8), and 0.5 mmol/L Magnesium chloride ($MgCl_2$).

ii. Substrate (R1b)

This contains 10 mmol/L p-nitrophenylphosphate. This was reconstituted by diluting one vial of R1b with 5000 µL of R1a.

3.17.4 Procedure for the ALP Assay

Into a micro cuvette, the following were pipetted at room temperature: Sample 100 µL, Reagent 500 µL. The cuvette was mixed by inversion, and the initial absorbance was read, and timer was started simultaneously. Readings were taken again at 60, 120, and 180 seconds at a wavelength of 405 nm by blanking with distilled water.

3.17.4.3 Calculation of ALP Activity

To calculate the activity of ALP, the formula below was used

U/l = 2760 x ΔA 405 nm/min

3.17.5 Estimation of Total Protein in Serum and Liver Homogenate

With certain changes, such as the inclusion of potassium iodide to stop the precipitation of $Cu2^{+}$ ions as cuprous oxide, the biuret method was used to measure the protein concentration of the various homogenates[12].

3.17.5.1 Principle of Total Protein Assay

Protein amino groups convert cupric ions to cuprous ions in alkaline solutions, which then combine with the proteins to form a blue complex that absorbs most strongly at 540 nm. To stabilize the compound, sodium potassium tartrate was added.

3.17.5.2 Reagents for Total Protein Estimation

The reagents used in total protein estimation include the following:

i. Sodium Hydroxide (NaOH) 0.2 M: Distilled water was used to dissolve 1.6 g NaOH, and the solution was made up to 200 mL

ii. Biuret reagent: Biuret reagent was prepared by dissolving 0.6 g of copper sulphate ($CuSO_4.5H_2O$), and 1.8 g of sodium potassium tartrate in 150 mL of 0.2 M NaOH. 1g of Potassium iodide was then added, and the solution made up to 200 mL with 0.2 M NaOH.

iii. Stock bovine serum albumin (BSA) solution: 4 mL of distilled water was used to dissolve 20 mg of BSA to give a stock solution of 5 mg/ mL.

3.17.5.3 Standard Protein (BSA) Curve

The stock solution, which contained 2 to 10 mg/mL of protein, was diluted several times. One milliliter of each protein standard solution was mixed with three milliliters of the biuret reagent in a test tube. After the mixture was allowed to stand at room temperature for 30 minutes, it was compared to a blank made out of 1 mL of distilled water, and 3 mL of biuret reagent. The optical densities of the resultant solutions were then measured at 540 nm in a spectrophotometer. The protocol for the preparation of the standard curve is shown in table 3.7.

Table 3.7 Protocol for Protein Standard Curve Preparation

Test tube No	**1**	**2**	**3**	**4**	**5**
Stock BSA (mL)	0.2	0.4	0.6	0.8	1.0
Distilled water (mL)	0.8	0.6	0.4	0.2	0
Biuret reagent (mL)	3	3	3	3	3
BSA concentration (mg/mL)	0.25	0.50	0.75	1.00	1.25

Source [12]

3.17.5.4 Procedure for Total Protein Estimation in Samples

To create a 1 in 4 dilution of the sample, 0.2 mL of the sample was combined with 0.8 mL of distilled water. 3 mL of the biuret reagent was then added to the diluted sample. The combination was incubated at room temperature for 30 minutes, and then the reagent blank was used to read the absorbance at 540 nm. The actual protein concentration in each sample was determined by extrapolation from the standard curve, and multiplied by 5.

3.17.6 Gamma Glutamyl Transferase (γGT) Determination

Gamma-glutamyl transferase is usually most significantly elevated by obstructive disease and has good specificity for the liver. γGT can also help to differentiate between mechanical, and viral cholestasis, and drug induced cholestasis. The highest concentration of γGT is found in the luminal membrane of the proximal tubules of the kidney. The method employed by Spectrum kits produced by the Egyptian Company for Biotechnology, Cairo, Egypt, was the kinetic colorimetric method developed by Szasz, and Persijn,[13]. The principle of the assay was based on the determination of **γ-**GT by following the reaction below:

L- γ-Glutamyl-3-carboxyl-4-nitroanilide + Glycylglycine

↓ γ-GT

L- γ-Glutamyl-glycylglycine + 5-amino-2-nitrobenzoate

A yellow-coloured 5-amino-2-nitrobenzoate liberated was proportional to the activities of **γ-**GT in the tissue whose quantity was equivalent to the rate of increase in absorbance read at 405nm.

3.17.6.1 Reagents used for γGT Assay

i. Buffer (Reagent 1): R1 buffer is made up of 120 mmol/L Tris buffer (pH 8.2), Glycylglycine 300 mmol/L, and 12 mmol/L sodium azide.

ii. R2 starter (Reagent 2): This consist of modified L- γ- Glutamyl-3-carboxyl-4-nitroanilide 1.0 mmol/L, and sodium azide, 8 mmol/L. All reagents were prepared according to instructions, and precautions taken on storage, and stability.

3.13.6.2 Procedure for the Assay: In a test tube, 0.5 mL R1, 0.5 mL R2, and 100 µL of sample was carefully pipetted, mixed, and the initial absorbance read at 60 seconds, 1 minute, 2 minutes, and 3 minutes by using a stopwatch which was started immediately after the initial reading at 60 seconds. The mean absorbance was calculated as change per minute (ΔA/minute)

3.17.6.3 Calculation of γ-GT Activity

The formula used for the determination of γ-GT activity is

U/L = 1450 x ΔA 405 nm/minute; where 1450 is the conversion factor

3.18 Determination of Kidney Function Indices

3.18.1 Quantitative Determination of Cystatin C

The quantitative determination of cystatin C in serum was done using the Roche/Hitachi CYSC2 reagents (Tina -quants Cystatin C Gen. 2) and Cobasc 501 analyzers by the method of Kyhse-Andersen *et al*[14].

3.18.1.1 Principle of Cystatin C Assay

The procedure employs the particle enhanced immunoturbidimetric assay where the cystatin c present in the serum agglutinates with latex particles coated with anti-cystatin c antibodies. The aggregate is determined turbidimetrically at 546 nm

3.18.1.2 Reagents Preparation for Cystatin C Determination

i. R1 is a solution of polymers in MOPS-buffered saline containing stabilizers and preservatives

ii. R2 contains latex particles in glycine buffer coated with anti-cystatin C antibodies, also containing stabilizers and preservatives

3.18.1.3 Procedure for Cystatin C Assay

i. The reagent containers were carefully inverted multiple times prior to use to facilitate proper mixing of the reagent components

ii. Properly labelled and centrifuged samples were

iii. the sample and regent blank were prepared as follows:

Table 3.8: Procedure for the Determination of Cystatin C

	Sample	Blank
R1	154 µL	-
R2	34 µL	20 µL
Sample	2 µL	-

The mixture was placed in an analyzer and determined against the blank at a wavelength of 546 nm.

3.18.1.4 Calculation of Cystatin C Results

The results obtained were analyzed from the standard curve with the formula y = 1.031x – 0.153 mg/L where r is 0.988

3.18.2 Creatinine Determination in Tissues

The body typically produces creatinine at a fairly consistent pace since it is a breakdown product of creatine phosphate in muscles. Chemically speaking, creatinine is a cyclic derivative of creatine that naturally forms. Every day, the body converts about 2 % of its creatine into creatinine. The kidney receives creatinine through the circulation. The kidney tubules do little to no tubular re-absorption of creatinine. Therefore, the creatinine clearance (CrCl), which represents the glomerular filtration rate (GFR), may be calculated using the levels of creatinine in the blood, and urine. Because it measures renal function, the GFR is significant. The blood creatinine level will most likely increase if there is a disorder that affects the kidneys' ability to operate. Higher levels of creatinine are seen in persons with high blood pressure, diabetes mellitus, and medication use. The principle of creatinine determination is based on its reaction

with picric acid in alkaline medium forming a yellow orange color complex which is measured at 492 nm [15]:

Creatinine + Picric Acid ⟶ Creatinine Picrate

The reaction was carried out up to fixed time in order to minimize interference of other substances reacting with Picric acid.

3.18.2.1 Reagent Composition for Creatinine Determination

i. 9 mmo/L picric acid

ii. 400 mmol/L Sodium Hydroxide

iii. 2 mg/dl creatinine standard

3.18.2.2 Procedure for Creatinine Analysis

Pre-warm working reagent at 37 ^{0}C for 2 minutes prior to addition of sample.

For working standard, 1000 µL of working reagent, and 100 µL of creatinine standard into a test tube. For the sample determination, 1000 µL of working reagent, and 100 µL of each sample were pipetted into the spectrophotometer cuvette, and mixed properly. The initial absorbance A1 was read at 30 seconds of mixing, and the final absorbance A2 was read after 90 seconds at 37 ^{0}C.

3.18.2.3 Calculation of Creatinine Concentration in Sample

Creatinine concentration in the sample was calculated using the following formula:

$$\Delta A = A2 - A1$$

where, ΔA= change in absorbance, Δ Absorbance of Sample = change in the absorbance of sample

Δ Absorbance of Standard = change in the absorbance of standard

3.18.3 Determination of Urea Concentration

Urea is the major end product of protein nitrogen metabolism. it is synthesized by the urea cycle in the liver, and excreted through the kidneys. The circulating levels of urea depend upon protein intake, protein catabolism, and kidney function. Urea concentration was determined in the serum, and tissue homogenates using a Spectrum kit. This kit employed the urease Berthelot colorimetric method developed by Batton, and Crouch[16]. The method measured the amount of ammonia produced after reaction with phenol in the presence of hypochlorite. The principle of the assay is as shown below:

Urea + water $\xrightarrow{\text{Urease}}$ $2NH_3$ + CO_2

The amount of urea present was related to the green color of the complex formed by the free ammonia in an alkaline pH, and the presence of an indicator.

3.18.3.1 Reagents for the Determination of Urea

i. 8.33 mmol/L (50 mg/dL) of aqueous urea standard

ii. Reagent 1 (R1 buffer) which was made up of 100 mmol/L of phosphate buffer with pH of 8.0, 80 mmol/l sodium salicylate, 6.0 mmol/L sodium nitroprusside, and 30.0 mmol/L Ethylenediamine tetra acetic acid (EDTA)

iii. Reagent 2 (R2 Enzyme) which is made up of about 6000 U/L and Reagent 3 (R3 Alkaline Reagent) made up of 400 mmol/L sodium hydroxide and 20.0 mmol/L sodium hypochlorite

3.18.3.2 Procedure for the Assay of Urea

Blank, standard, and sample preparation were carried out as described in the manual following table 3.9

Table 3.9: Procedure for the Assay of Urea

	Blank	Standard	Sample
R1 (Buffer)	1000 µL	1000 µL	1000 µL

R2 (Enzyme)	50 µL	50 µL	50 µL
Standard Sample	--------	---------	---------
The mixture was mixed properly, and incubated for 3 to 5 minutes at 37 ^{0}C.			
R3 (Alkaline Reagent)	200 µL	200 µL	200 µL

These were properly mixed, and incubated again for 5 minutes at 37 ^{0}C. Thereafter, the absorbance of each sample, and standard was read at 578 nm against the reagent blank. The absorbance was read at 578 nm against the prepared blank.

3.18.3.3 Calculation for Urea Concentration

$$\text{Urea concentration (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times n$$

Where n is dilution factor 50.0 mg/dL (8.33 mmol)

3.18.4 Urea BUN/ Creatinine Ratio

Blood urea nitrogen (BUN)/creatinine ratios were calculated by dividing the BUN, and creatinine values. BUN is calculated by dividing blood urea by 2.14 or multiplying it by 0.467[17].

Blood urea = 2.14 x BUN

Urea BUN/ creatinine ratio = BUN/creatinine

3.19 Assessment of Oxidative Stress Biomarkers

3.19.1 Catalase Activity (CAT) Determination

Catalase activity was determined in all tissue homogenates according to the method of Claiborne,[18]. The principle of the method was based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. The absorbance correlates with concentration to allow its use in quantitative assay and an extinction coefficient of 0.0436 $mM^{-1}cm^{-1}$ was used.

3.19.1.1 Preparation of Reagents for CAT Assay

i. Phosphate Buffer (0.05 M, pH 7.4): In 90 mL of distilled water, dipotassium hydrogen phosphate trihydrate (0.696 g), and potassium dihydrogen phosphate (0.265 g) were dissolved. The pH was then adjusted to 7.4, and the volume was increased to 100 mL with distilled water.

ii. Hydrogen peroxide (19 mM): 50 mL of 0.05 M phosphate buffer, pH 7.4, and 194 µL of 30 % H_2O_2 were combined to make 100 mL of the same solution.

3.19.1.2 Assay Procedure for CAT Assay

50 µL of the sample were pipetted into a 1 cm quartz cuvette together with 2.95 mL of hydrogen peroxide (19 mM solution). The liquid was added quickly inverted to mix before placing it into a spectrophotometer. The absorbance change was measured at 240 nm every minute for 5 minutes

3.19.1.3 Calculation of CAT Activity

$$\text{Catalase activity} = \frac{\Delta A_{240}/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{0.0436 \times \text{sample volume} \times \text{mg protein/mL}}$$

$$= \mu\text{mole } H_2O_2/\text{min/mg protein}$$

3.19.2 Determination of Reduced Glutathione (GSH) Level

Reduced glutathione level was estimated in all tissue homogenates using the method of Ellman, [19]. The principle was based on the development of a relatively stable yellow colour when 5',5'-dithios-(2-nitrobenzoic acid) also known as Ellman's reagent is added to sulfydryl compounds. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced GSH, 2-nitro,5-thiobenzoic acid, possesses a molar absorption at 412 nm. Reduced GSH is proportional to the absorbance at 412 nm.

3.19.2.1 Reagents Preparation for GSH Analysis

i. **Phosphate Buffer (0.1 M, pH 7.4):** 2.098 g Dipotassium hydrogen phosphate trihydrate, and 0.791 g potassium dihydrogen phosphate were dissolved in 120 mL distilled water, the pH adjusted to 7.4, and the volume made up to 150 mL with distilled water.

ii. **GSH Stock Solution:** 80 mL of the prepared 0.1 M phosphate buffer (pH 7.4) was used to dissolve 40 mg of GSH, and made up to 100 mL with the same.

iii. **Ellman's Reagent:** 120 mL of the same 0.1 M phosphate buffer was also used to dissolve 60 mg of Ellman's reagent, and made up to 150 mL with the same.

iv. **Sulphosalicylic Acid (4 % solution):** 20 mL distilled water was used to prepare 0.8 g of sulphosalicylic acid.

3.19.2.2 GSH Standard Curve Preparation

The GSH stock solution was diluted serially, as indicated in the table 3.10 below. Within 30 minutes, at 412 nm, the absorbance of the yellow color that Ellman's reagent produced was measured in comparison to a blank consisting of 1.5 mL of Ellman's reagent, and 0.5 mL of phosphate buffer. The concentration of reduced GSH was then plotted versus absorbance

Table 3.10: GSH Standard Curve Protocol

GSH Stock (mL)	Phosphate Buffer (mL)	Ellman's Reagent (mL)	GSH Concentration (µg/mL)
0.01	0.49	1.5	1
0.03	0.47	1.5	3
0.05	0.45	1.5	5
0.10	0.40	1.5	10

0.15	0.35	1.5	15
0.20	0.30	1.5	20

Source[18]

3.19.2.3 Procedure for the Determination of GSH in Samples

A precipitating solution, and a sample volume of 0.4 mL each were combined, vortexed, then centrifuged at 4000 rpm for five minutes. After that, 1.5 mL of Ellman's reagent was mixed with 0.5 mL of the supernatant. At 412 nm, the reaction mixture's absorbance was measured against a blank for the reagent.

3.19.3 Estimation of Glutathione Peroxidase (GPx) Activity

The activity of Glutathione peroxidase (GPx) was measured according to the method of Rotruck *et al*[20], which was based on the reaction between glutathione remaining after the action of GPx. GPx is known to most efficiently scavenge hydrogen peroxide (H_2O_2) by using GSH as an electron donor.

3.19.3.1 Reagents Preparation for GPx Activity Assay

All reagents were prepared carefully as described in different beakers, and properly labelled

i. Dipotassium Hydrogen Orthophosphate ($K_2HPO_4.3H_2O$) (0.3 M): 8.22 g of $K_2HPO_4.3H_2O$ was dissolved in distilled water, and the volume made up to 120 mL with the same.

ii. Phosphate Buffer (0.1 M, pH 7.4): Dipotassium hydrogen phosphate trihydrate (2.798 g), and potassium dihydrogen phosphate (1.054 g) was dissolved in 180 mL of distilled water, the pH adjusted to 7.4, and the volume made up to 200 mL with distilled water.

iii. Sodium Azide (NaN_3) (10 mM): 100 mL of distilled water was used to dissolve 6.5 mg of sodium azide.

iv. Reduced Glutathione (4 mM): 24.6 mg of GSH was dissolved in 20 mL of 0.1 M phosphate buffer, pH 7.4.

v.Hydrogen Peroxide (2.5 mM): 4 µL of hydrogen peroxide (30 %) was added to distilled water, and the volume made up to 50 mL with the same

vi. Trichloroacetic Acid (10 %): 4 g of TCA was liquidized in distilled water, and the volume made up to 40 mL with the same

vii. Ellman's Reagent (Dinitrothiocyanobenzene: DTNB): 39.6 mg of DTNB was dissolved in 100 mL of 0.1 M phosphate buffer, pH 7.4.

3.19.3.2 Procedure for GPx Analysis

In a test tube containing 0.5 mL of phosphate buffer, 0.1 mL of NaN_3, 0.2 mL of GSH, 0.1 mL of H_2O_2, and 0.5 mL of sample was added last. After the reaction mixture had been incubated at 37 °C for three minutes, 0.5 mL of TCA was added, and the finished product was centrifuged at 3000 rpm for five minutes. 2 mL of K_2HPO_4, and 1 mL of DTNB were added to 1 mL of the supernatants, and the absorbance was measured at 412 nm (Bosch Model 752N) in comparison to a reagent blank made up of 1 mL of distilled water, 2 mL of K_2HPO_4, and 1 mL of DTNB.

3.19.3.3 Calculation of GPx Activity

GSH consumed = initial GSH amount (129.39 µg) – GSH remaining (µg/mL × 4 mL)

GPX activity = GSH consumed/mg protein

= µg GSH/mg protein

3.19.4 Estimation of Glutathione-S–Transferase (GST) Activity

Glutathione -s- transferase activity was determined according to the method of Habig *et al*[21]. The principle of the assay was based on the fact that all known isotypes of glutathione-S-Transferase

demonstrate a relatively high activity with 1–Chloro-2,4- dinitrobenzene (CDNB) as the second substrate. When CDNB was conjugated to the reduced form of glutathione, its absorption maximum shifts to a longer wavelength, and the absorption increase at the new wavelength of 340 nm provided a direct measurement of the enzymatic reaction.

3.19.4.1Reagents Preparation for GST Assay

i. Phosphate buffer (0.1 M, pH 6.5): 0.381 g of Dipotassium hydrogen phosphate trihydrate, and 1.134 g of potassium dihydrogen phosphate were dissolved in 90 mL of distilled water, the pH was adjusted to 6.5

ii. (0.1 M) phosphate buffer: 30.73 mg GSH was dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.5)

iii. 1-Chloro-2,4-dinitrobenzene (CNDB) (20 mM): 16.85 mg of CDNB was dissolved in 5 mL absolute ethanol

iv. Reduced Glutathione (GSH) every 60 seconds against the blank at a wavelength of 340 nm.

3.19.4.2 Procedure for GST Analysis

As shown in the table 3.11 below, the reagents were added as shown. The reaction was allowed to run for 3 minutes, readings were taken 6.5, and the volume made up to 100 mL with distilled water

Table 3.11: Glutathione S-Transferase Assay Medium

Reagent	Blank	Test
CDNB (20 mM)	150 µl	150 µl
Reduced glutathione (0.1 M)	30 µl	30 µl

0.1 M Phosphate buffer, pH 6.5	2.82 mL	2.79 mL
Sample	-	30 µl

Source [21]

3.19.4.3 Calculations for the Determination of GST Activity

$$\text{GST activity} = \frac{\Delta A_{340}/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{9.6 \times \text{sample volume} \times \text{mg protein/mL}}$$

$$= \text{µmole/min/mg protein}$$

9.6 ($mM^{-1}cm^{-1}$) = The extinction coefficient of CDNB at 340 nm

3.19.5 Determination of Superoxide Dismutase (SOD) Activity

The activity of SOD was determined in all tissue homogenates by the method of Misra, and Fridovich[19]. The principle was based on the ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at a pH of 10.2. This makes the reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\cdot-}$) radical, and hence inhabitable by superoxide dismutase

3.19.5.1 Reagents Preparation for SOD Assay

i. **0.05 M Carbonate buffer (pH 10.2)**

1.573 g of Sodium carbonate ($Na_2CO_3.10H_2O$), and 0.588 g of Sodium bicarbonate or bicarbonate of soda ($NaHCO_3$) were dissolved in 200 mL of distilled water. The pH was adjusted to 10.2, and then made up to 250 mL.

ii. **0.3M Epinephrine**

This was prepared by dissolving 0.05 g of epinephrine in 200 mL of distilled water containing 0.5 mL of concentrated HCl (37 %).

3.19.5.2 Protocol for Sample Preparation

50 µL of each sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2), and 0.3 mL of epinephrine in a cuvette, mixed by inversion, and change in absorbance monitored every 30 seconds for 2.5 minutes at 480 nm. The reference cuvette was the same as for the samples with water replacing the samples.

3.19.5.3 Calculation of SOD Reaction

$$\% \text{ inhibition} = 100 - \left(100 \times \frac{\text{Increase in absorbance per min for sample}}{\text{Increase in absorbance per min for blank}}\right)$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50 % inhibition of the auto-oxidation of epinephrine.

3.19.6 Lipid Peroxidation (LPO) Assay

The generation of thiobarbituric acid reactive substances (TBARS) contained in the test sample was measured in order to quantify the level of lipid peroxidation in all tissue homogenates using the method of Varshney and Kale [23]. The fundamental interaction between 2-thiobarbituric acid (TBA), and malondialdehyde (MDA), a byproduct of lipid peroxide during peroxidation, is the basis of the technique. A pink complex that can be extracted into organic solvents like butanol was produced after heating in an acidic pH environment. This complex absorbs most light at 532 nm. The results are reported as the amount of free MDA produced because MDA is frequently used to calibrate this test.

3.19.6.1 Reagents Preparation for LPO Assay

i. 30 % Trichloroacetic acid (TCA): Distilled water was used to dissolved 13.5 g of TCA (CCl_3COOH), and made up to 45 mL with the same.

ii. 0.1M Hydrochloric acid (HCl): Distilled water was used to dissolve 13 µl of concentrated HCl (36.5-38 %), and the volume made up to 15 mL with the same.

iii. 0.75 % Thiobarbituric acid (TBA): 0.1M HCl was used to dissolve 0.3375 g of TBA, and made up to 45 mL with the same. Stirring in a hot water bath (50 ^{0}C) helped with the dissolution.

iv. Tris-KCl buffer (0.15 M, pH 7.4): In 45 mL of distilled water, 0.559 g of KCl, and 0.909 g of Tris base were dissolved. The pH was then raised to 7.4 with HCl, and the volume was increased to 50 mL with the same

3.19.6.2 Procedure of LPO Assay

0.4 milliliters of the test sample were combined with 1.6 milliliters of Tris-KCl buffer, and 0.5 milliliters of 30 % TCA. After that, 0.5 mL of 0.75 % TBA was added, and the mixture was submerged in water for 45 minutes at 80 ^{0}C. After being brought to room temperature with ice, this was centrifuged at 3000 rpm for 10 minutes. At 532 nm, the absorbance of the clear supernatant was measured in comparison to a reference blank of distilled water.

3.19.6.3 Calculation of MDA

An extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$ was used to calculate the concentration of MDA

$$\text{Lipid peroxidation (nmole MDA/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample} \times \text{mg protein/mL}}$$

3.20 Statistical Analysis

Data obtained was expressed as mean ± standard deviation (SD) using Graph Pad Prism version 9. The questionnaire was analyzed with Microsoft excel package. Treated and control

groups were compared using row statistics and 2- way ANOVA (multiple comparison TUKEY test). Statistical significance was set at 95 % confidence level ($p < 0.05$).

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Chapter Four

Results and Discussion of Findings

4.1 Analysis of Cross-Sectional Study using Questionnaire

The questionnaire administered randomly to students from different departments of Lead City University, Ibadan has the following data distributions.

4.1.1 Demographics Characteristics of Respondents

The demographic characteristics of respondents are represented in Figure 4.1. There were 483 respondents in all, 73 (15.11 %) were under the age of 18 years, 312 (64.59 %) were between the ages of 18 and 25 years, 85 (17.59 %) were between 26 and 32 years old, and 9 (1.85 %) above the age of 32 years.

Figure 4.2 shows the marital status of participants. 422 (87.7%) are single while 61 (12.63%) are married.

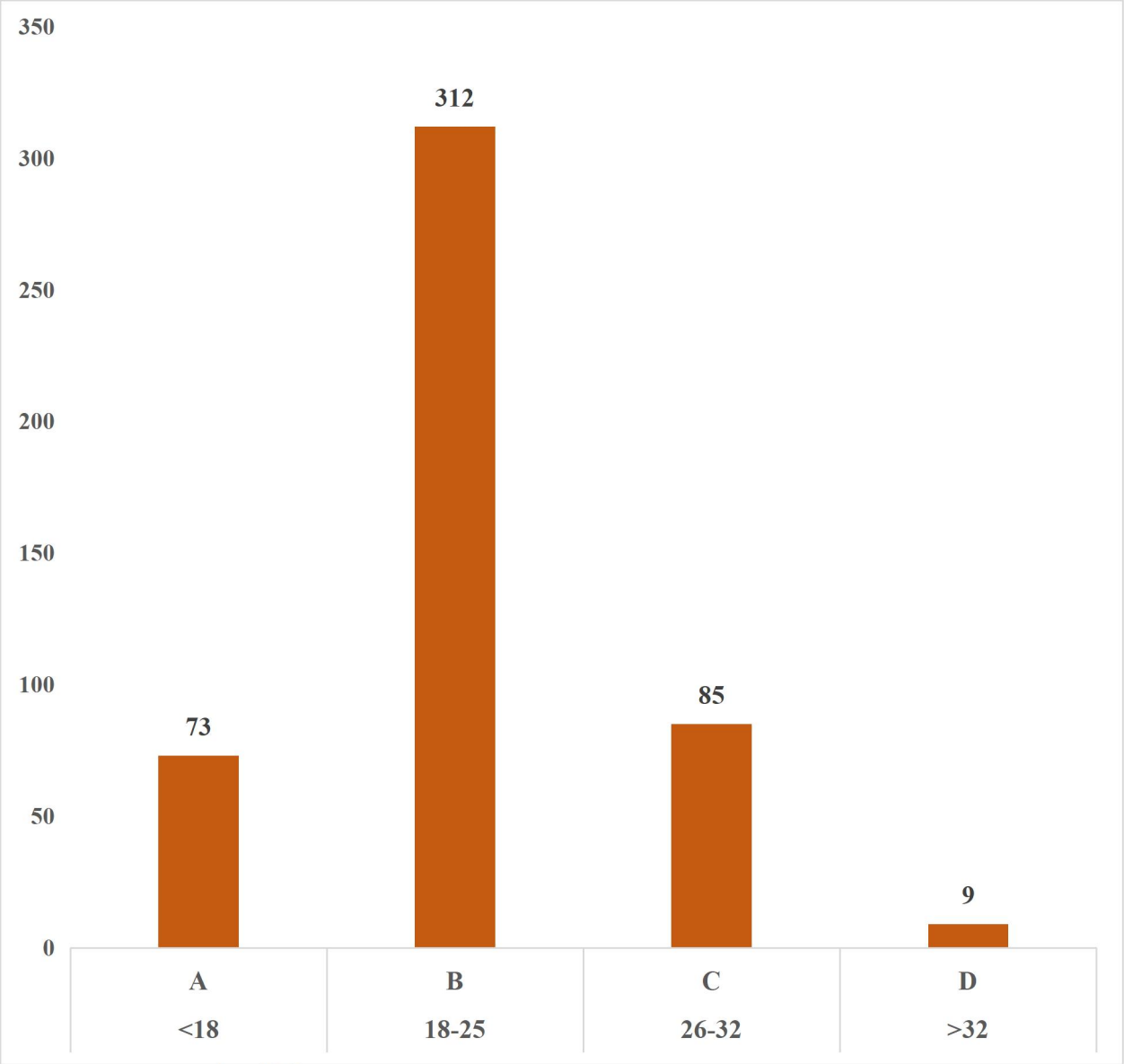


Figure 4.1: Age Distribution of Respondents

Source: Authors' Data Analysis

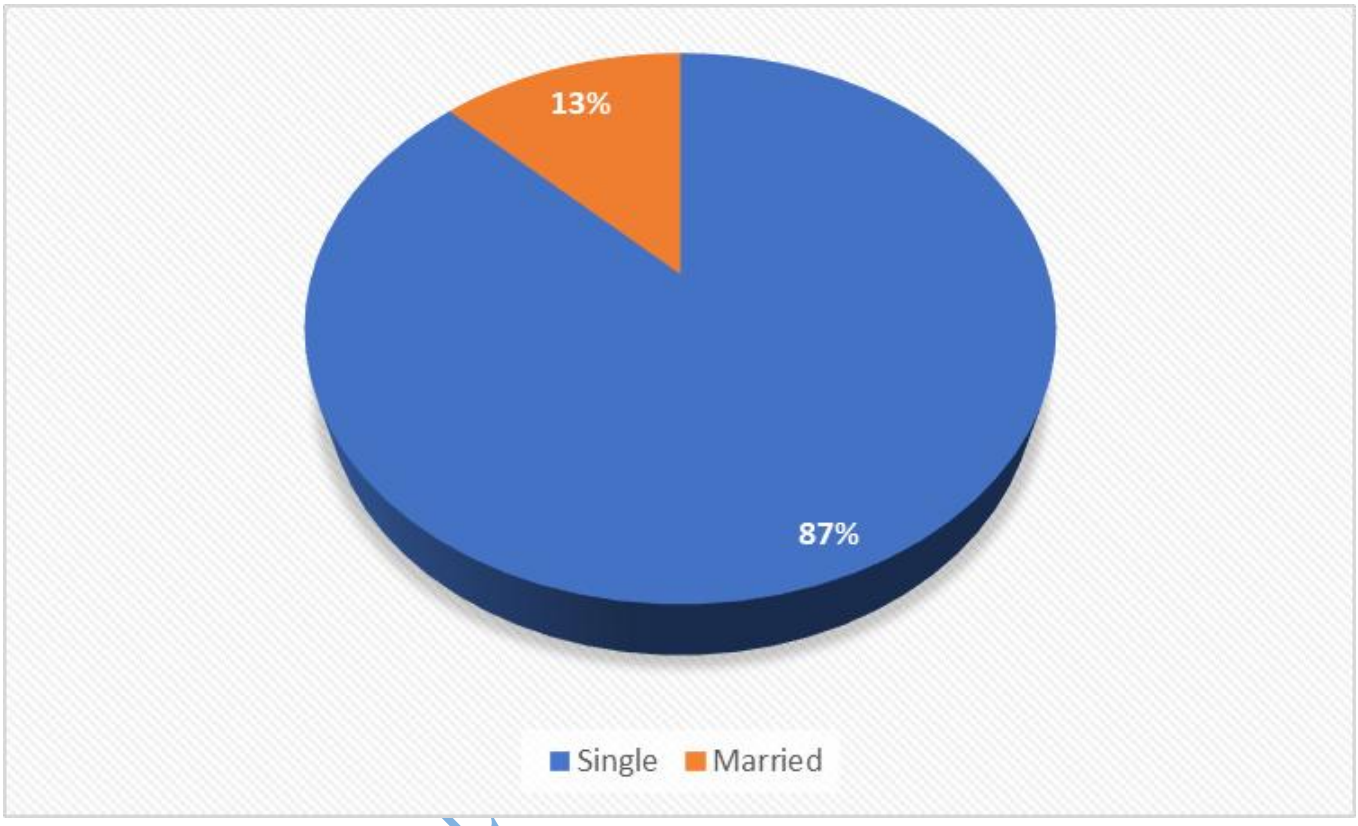


Figure 4.2: Marital Status of Respondents

Source: Author's Data Analysis

4.1.2 Respondents' Knowledge and Awareness of Contraceptive Methods and Use

The awareness and knowledge of contraception methods were enquired from participants, and the contraceptive methods used was reported as follows: 183 (37.97%) did not use any form of contraception because they have never had sex or by preference while 299 (62.03%) used one or more methods of contraception as shown in Figure 4.3.

Figure 4.4 shows the distribution of contraceptive use among the different age groups. 26 (35.62 %) out of the 73 under the age of 18 use contraception while 47 (64.38 %) did not. 208 (66.67 %) out of 312 participants between 18 and 25 use contraception while 104 (33.33 %) did not. 31 (36.47 %) out of 85 participants between the ages of 26 and 32 (11.18%) used no contraceptive while 54 (63.53 %) used. 8 (88.89 %) participants above 32 years used contraception while 1 participant (11.11 %) did not.

Figure 4.5 shows the response to the question on the method of contraception by those who used it. 140 (28.99 %) used condom alone, 82 (16.98 %) used a combination of condom and pill, 196 (40.58 %) used pills only, 24 (4.97 %) used injectible, 4 (0.83 %) used intrauterine devices, 11 (2.28 %) used herbs, concoction or substances such as ampicillin, salt and water mixture, alcohol or local herbs, 1 (0.21 %) used patches, and 2 (0.41 %) used implant.

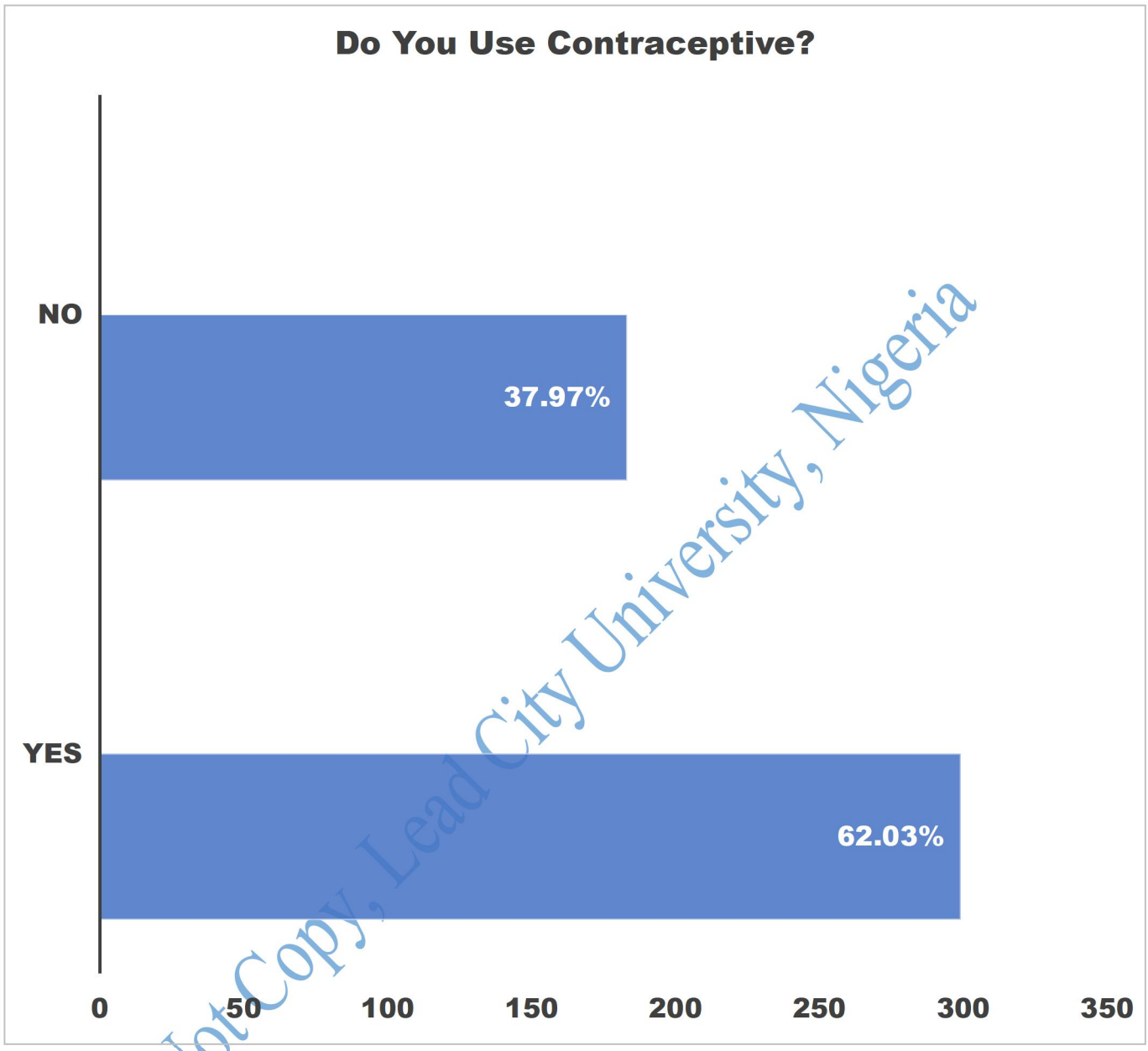


Figure 4.3: Response of Participants to Question on the Use of Contraception

Source: Authors' Data Analysis

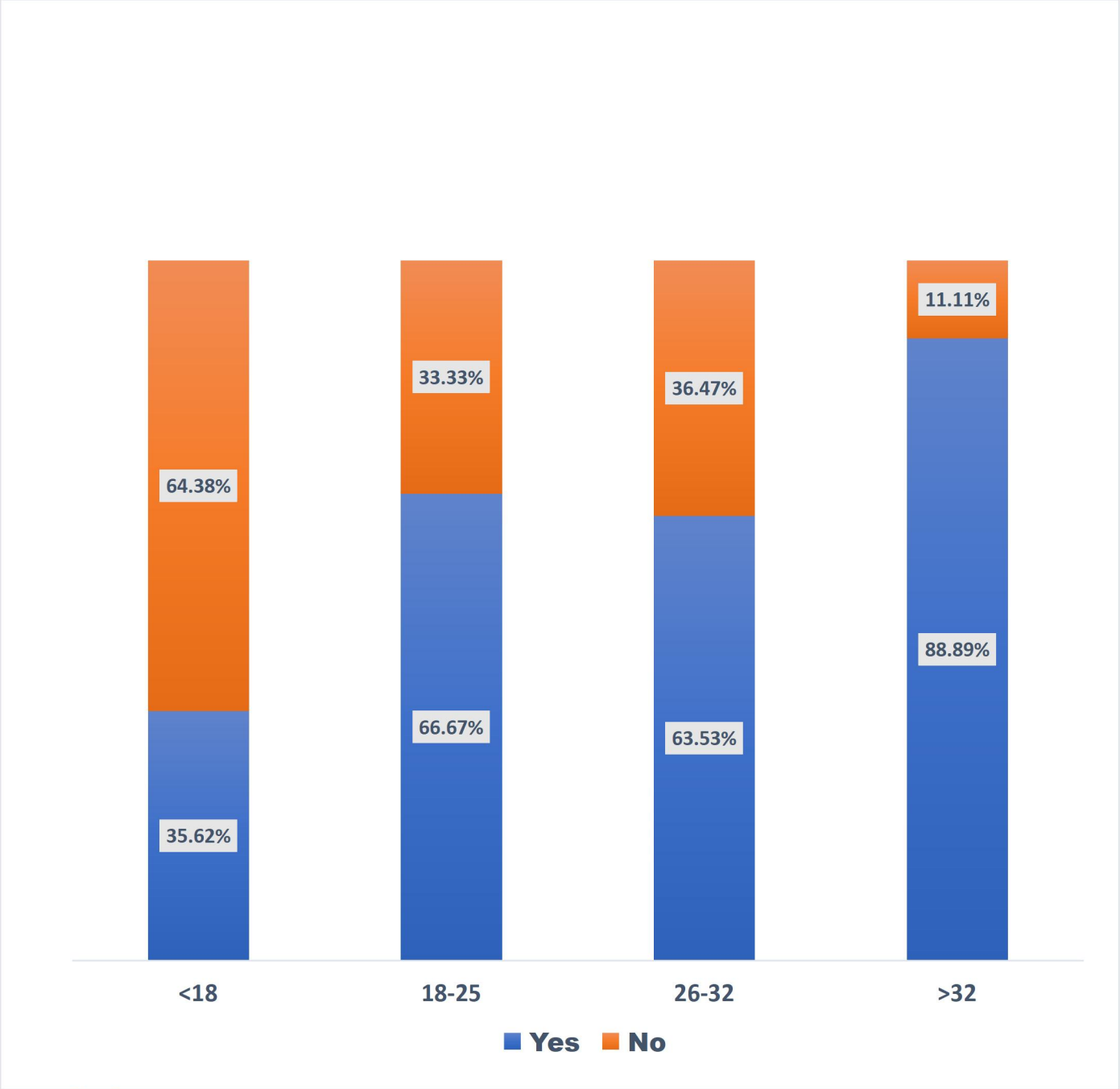


Figure 4.4: Distribution of Contraceptive Use among the Different Age Groups

Source: Authors' Data Analysis

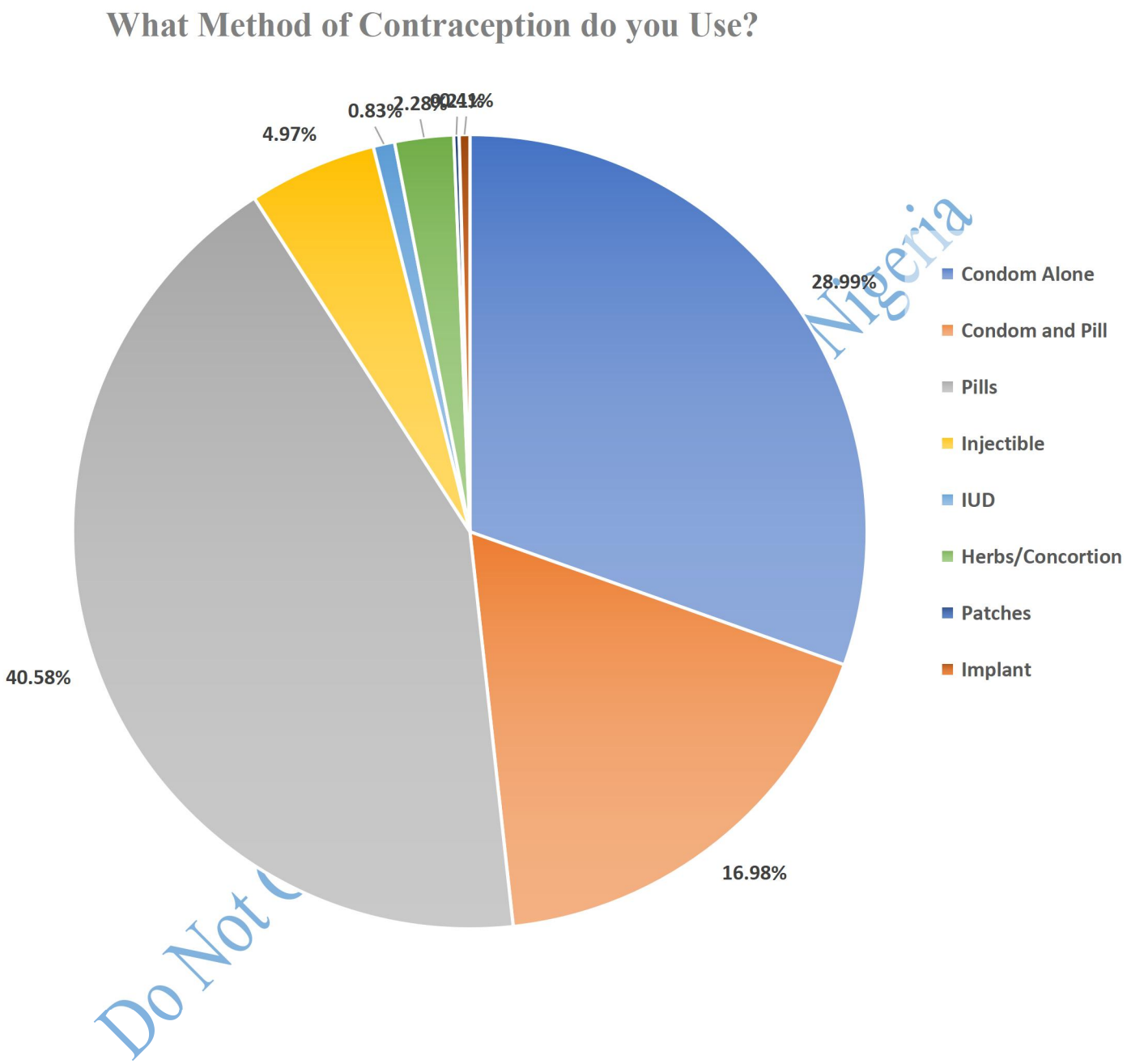
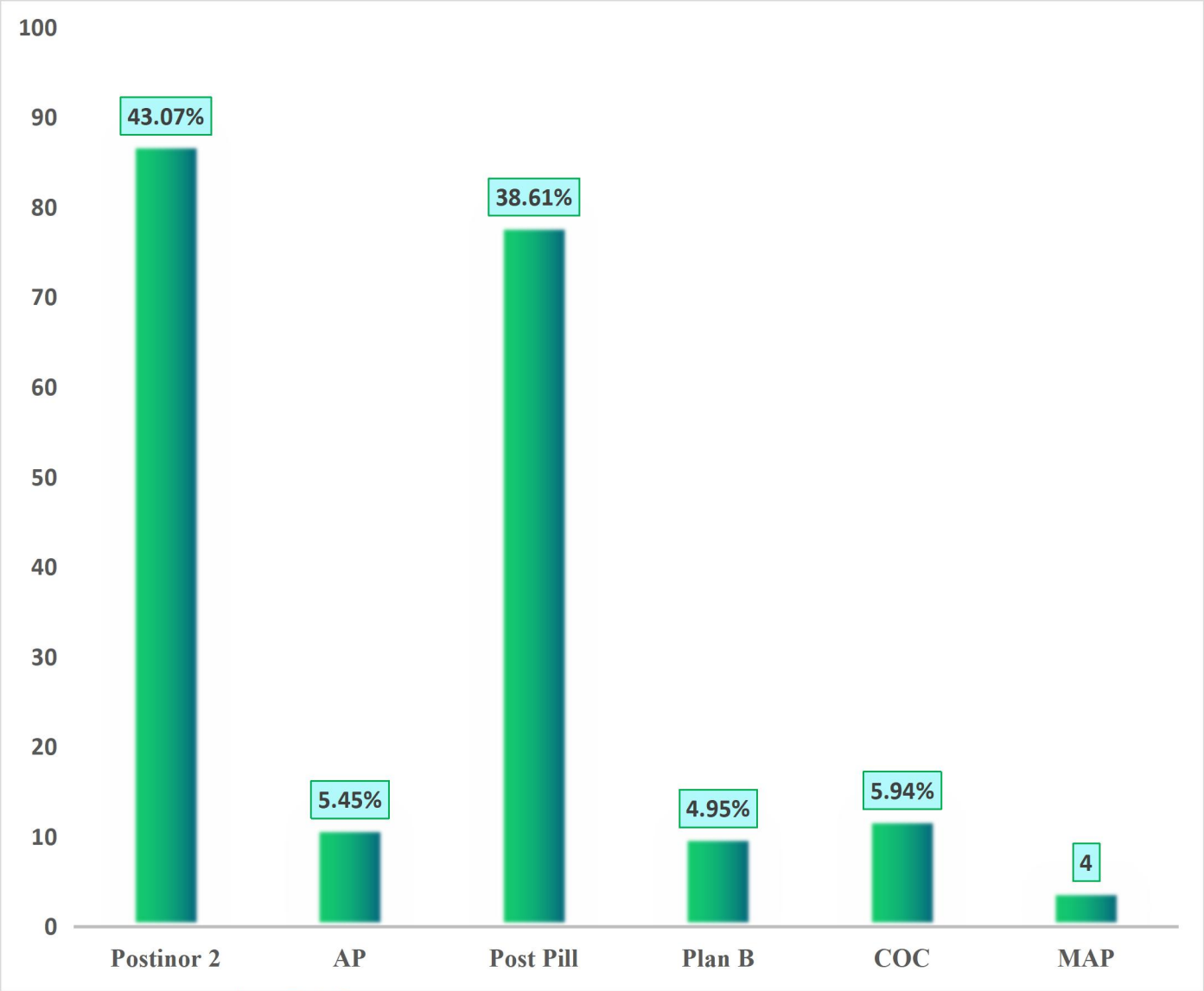


Figure 4.5: Response to Question on the Specific Method of Contraception used

Source: Authors' Data Analysis

The specific type of pill among the participants who chose pills as their preferred method of contraception is displayed in Figure 4.6. 87 (43.07 %) used Postinor 2, 11 (5.44 %) used Afterpill, 78 (38.61 %) used Postpill, 10 (4.95 %) used Plan B, 12 (5.94 %) used combined oral contraceptives while 4 (1.94 %) used Morning After Pill.

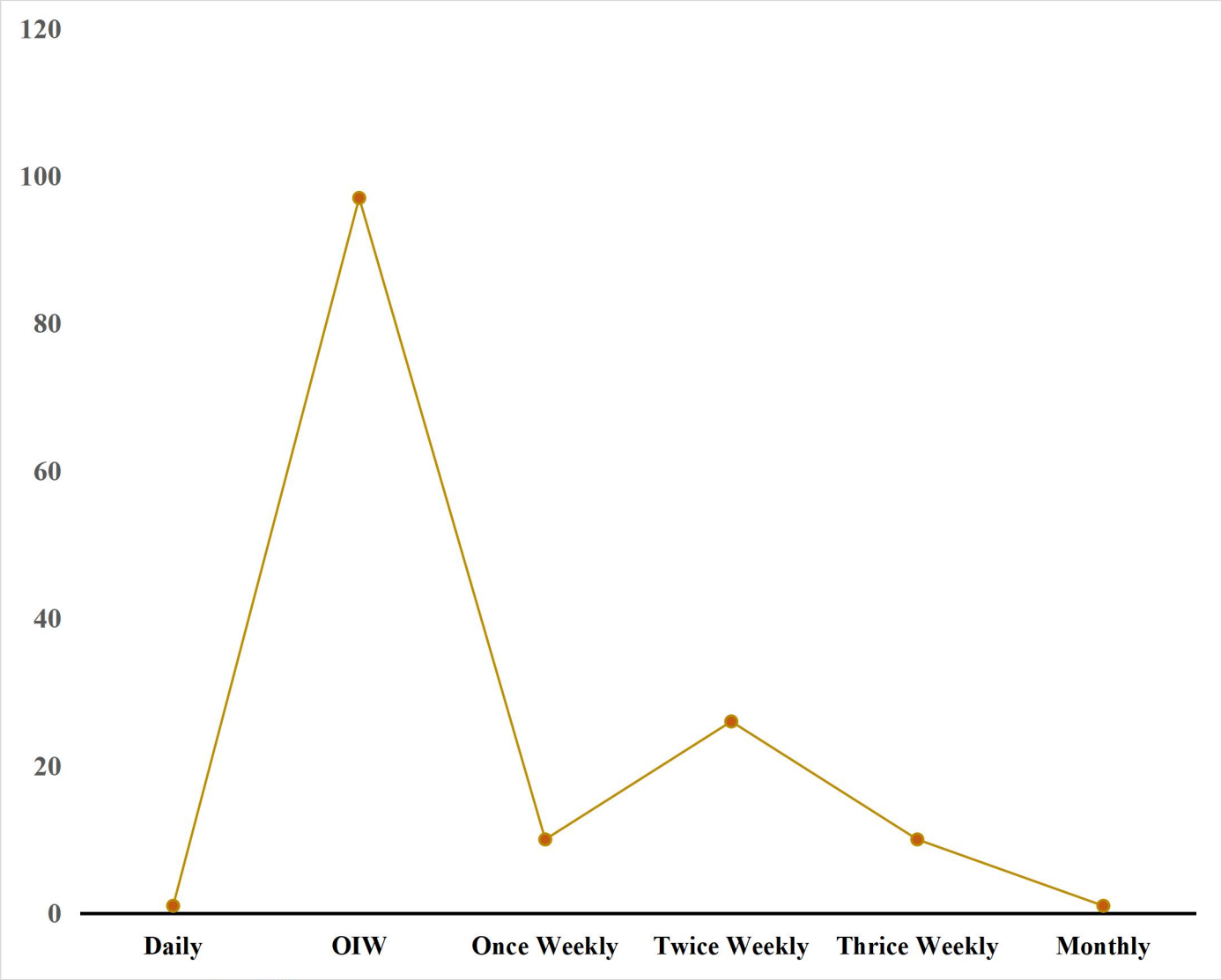
Figure 4.7 shows the frequency of pills taken by participants, 1 (0.69 %) used it daily, 97 (66.90 %) used it once in a while, 10 (6.90 %) used it once weekly, 26 (17.93 %) used it twice weekly, 10 (6.90 %) used it thrice weekly, while 1 (0.69 %) used it monthly.



AP – After Pill; COC – Combined Oral Contraceptives; MAP- Morning After Pill

Figure 4.6: Specific Type of Pills used by Respondents

Source: Authors' Data Analysis



OIW – Once in a While

Figure 4.7: Frequency of Pills used by Participants

Source: Authors' Data Analysis

4.1.2 Prevalence of Side Effects from Various Contraceptive Methods

Out of the 299 participants who used contraceptives, 49 (16.39 %) claimed they observed no side effects while 250 (83.61 %) said they did (Figure 4.8.)

Figure 4.9 shows the specific side effects observed by respondents from the use of contraceptive ranged from headache (8 %) to painful menstrual cycle (5 %), irregular menstruation (39 %), spotting (15 %), heavy menstrual flow (10 %), weight gain (20 %) and weight loss (3 %).

The distribution of the side effects from different types of contraception methods is shown in Table 4.1 with most effects reported from the use of pills.

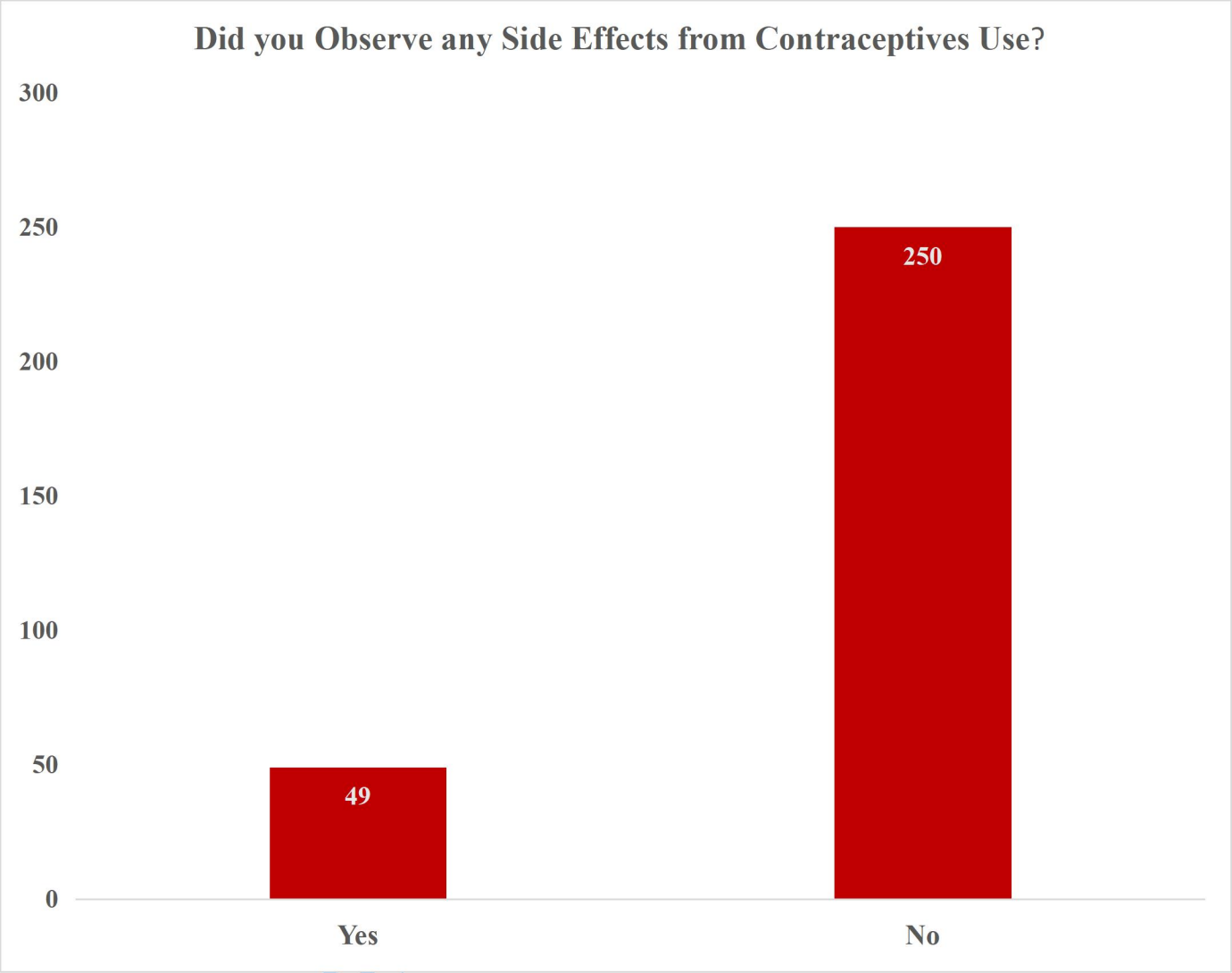


Figure 4.8: Side Effects Observed by Respondents from the use of Contraceptives

Source: Authors' Data Analysis

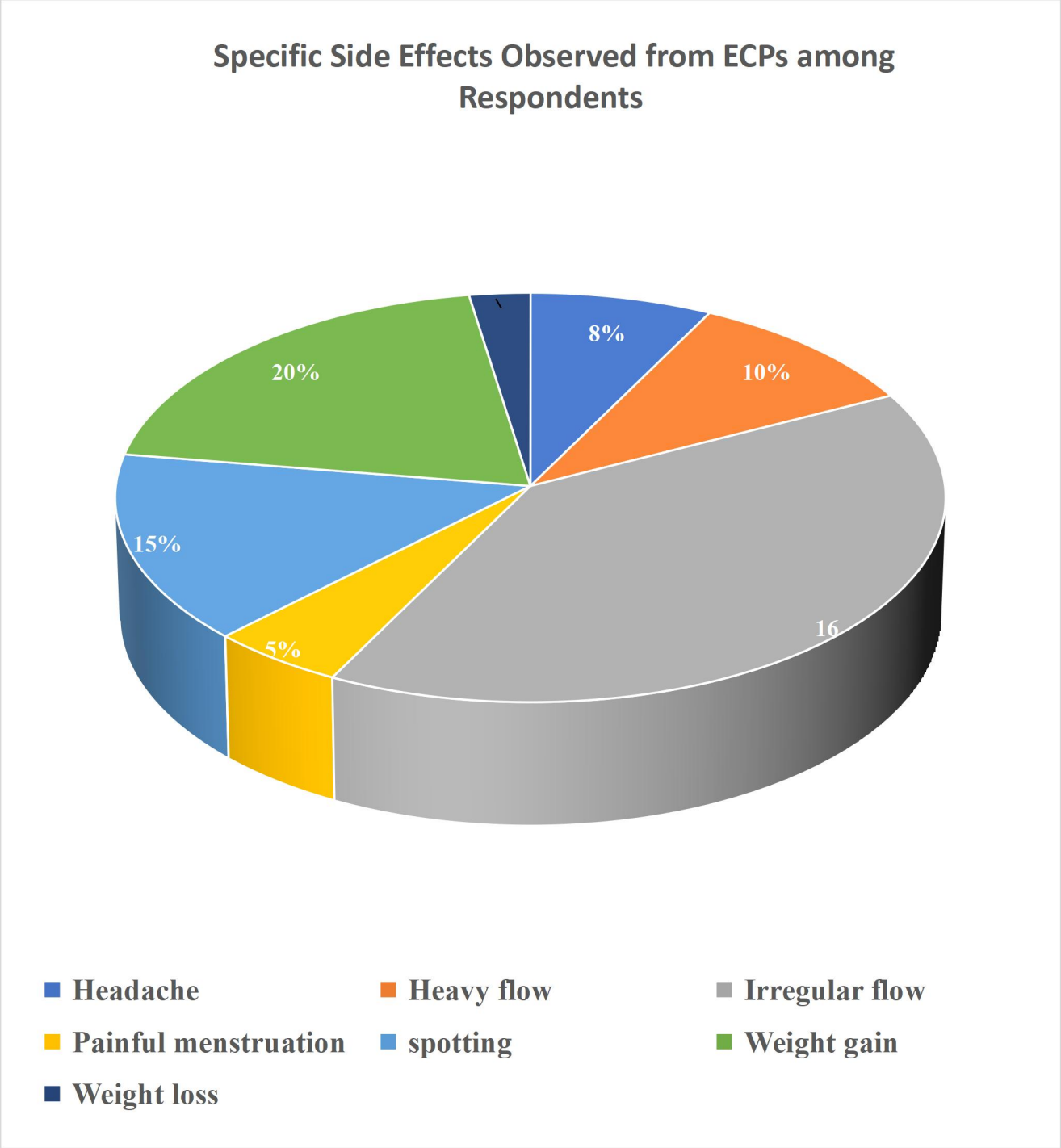


Figure 4.9: Side Effects Observed by Respondents from the Use of Contraceptives

Source: Author's Data Analysis

Table 4.1: Distribution of Side Effects Observed from the Use of Various Contraceptives

Side effects	Pills	Injectables	Intra Uterine Devices	Herb
Headache	3 (6 %)			
Heavy flow	4 (8 %)	3 (6 %)	3 (6 %)	
Irregular flow	16 (32%)	1 (2 %)		1 (2 %)
Painful Menstruation	2 (4 %)			
Spotting	6 (12 %)		1 (2 %)	
Weight gain	1 (2 %)	1 (2 %)		
Weight loss	8 (16 %)			

Source: Author's Data Analysis

4.2: Physical Observation of the Experimental Model (Wistar rats) before, during and after Treatments

The animals used for the experiment were closely monitored for any physical and behavioral differences across the three groups. The behavioral characteristics observed were shown in Table 4.2. the control group looked healthier fresh and active throughout the experiment. The treated groups are usually physically inactive immediately after treatment and took a while to resume normal physical activities. The control group had more appetite than treated groups on the days of treatment but more appetite in the treated groups than in control on other days. No mortality was observed in the control group throughout the experiment while 13 % mortality was observed in the treated groups (2 in once weekly and 6 in twice weekly).

Table 4.2: Observation of Wistar Rats before, during and after Treatments

Behavioral Patterns Observed	Control	Once Weekly	Twice Weekly
Food Consumption	Moderate	Fair	Low on treatment days, fair on other days
Appearance	Fresh and chunky	A little shrinky, not as fresh as control	Shabby due to hair loss
Body Weight	Moderate	Small	Smaller
Redness of Eyes	Nil	Nil	Observed in a few
Hair Loss	Low	Moderate	High
Physical Activities	Very Active	Slow after treatment	Slow and inactive for awhile after treatments
Mortality	Nil	2	6
Excreta (Feces and Urine)	Firm and whole	A little softer than in control	Slimy Feces after treatment and sometimes blood stained

Source: Author's Data Analysis

4.3 Comparative Evaluation of the Effect of Postinor 2 on the Body Weights of Wistar Rats Before and After Treatments

Figure 4.3 shows the comparative evaluation of average body weights of animals in the control, once-weekly, and twice-weekly treated groups before and after treatments. After 30 days, Postinor 2 caused a decrease in the body weights that was significant between twice weekly and control ($p < 0.05$). By 60 days into treatment, there was an upsurge in the body weights of the treated group that was very close to the control ($p > 0.05$).

At the end of 90 days, the end of treatment, the average weight of the treated groups especially the twice weekly group was again significantly lower than control group ($p < 0.05$).

Recovery was observed with treatment withdrawal as the weights of the animals returned to similar range as it was initially before treatment started ($p > 0.05$).

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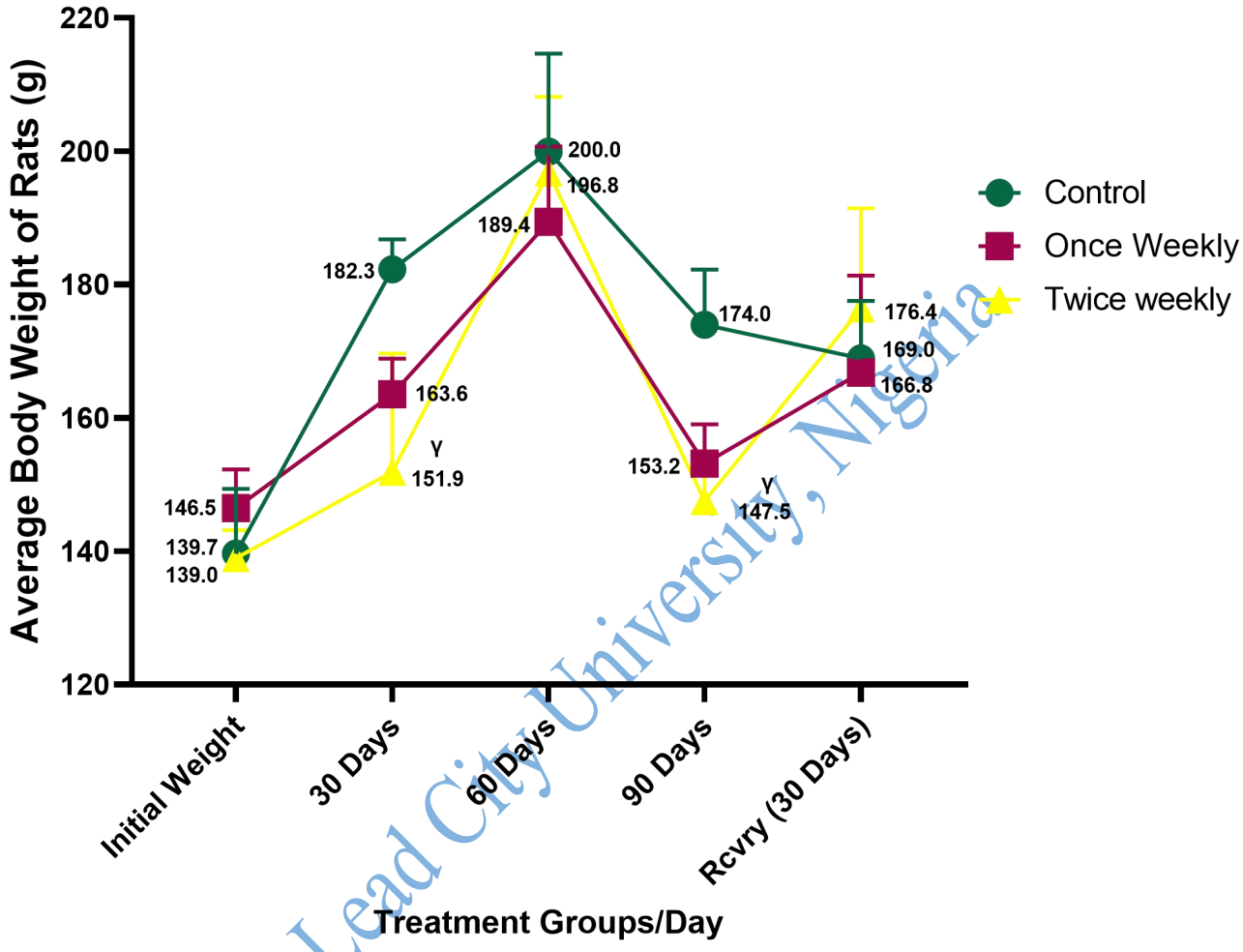


Figure 4.10: Effect of Postinor 2 on the Body Weights of Animals

Values were expressed as mean ± standard deviation (SD), n = 5. **y**' showed significance when twice weekly was compared with control ($p < 0.05$).

4.4 Effect of Postinor 2 Treatment on Rats' Estrous Cycle

4.4.1 Visual Assessment of the Effect of Postinor 2 on Rats' Estrous Cycle

While all the phases were randomly observed per time in the control group and those treated once a week, the group treated twice weekly were mostly in the metestrus phase with the whitish sloughs. Additionally, the proestrus phase was first observed in the group treated twice weekly long before it was seen in the other groups (Appendix 3)

4.4.2 Cytological Assessment of the Effects of Postinor 2 on the Estrous Cycle

Appendix 4 shows the phases of the estrus cycle observed cytologically under the microscope in the animals. Treatment with Postinor 2 prolonged the metestrus phase which was more evident in the ones treated twice weekly while the control proceeded accordingly (Table 4. 3).

Table 4.3: Cytological Assessment of the Estrus Cycle in Animals Treated with Postinor 2 Compared with Control

	Control	Once Weekly	Twice Weekly
Proestrus	14 – 15 Hours	10 – 12 Hours	8 – 12 Hours
Estrus	24 – 48 Hours	24 – 40 Hours	24 – 30 Hours
Metestrus	7 – 8 Hours	12 – 18 Hours	20 – 24 Hours
Diestrus	47 – 73 Hours	40 – 72 Hours	30 – 60 Hours

Source: Authors' Data Analysis

4.5 Effect of Postinor 2 Treatment on Relative Organ Weights

4.5.1 Effect on Relative Uterus Weight

The relative uterus weight compared between the various groups, the control group (0.14 ± 0.003) at the end of the first month of treatment, was significantly lower than twice weekly but higher than once weekly (0.12 ± 0.005 and 0.16 ± 0.002 respectively) as shown in Figure 4.11a.

At the end of the second month of treatment, there was no discernible difference between once-weekly and twice-weekly treated groups ($p > 0.05$) as they have both increased in value and significantly higher than control ($p < 0.05$).

After 90 days of continuous treatment, twice weekly (0.21 ± 0.002) had become significantly higher than both once weekly and control ($p < 0.05$). No significance difference was observed between the once-weekly and control groups ($p > 0.05$).

30 days recovery period and post-treatment brought about a reduction in the uterus weight of both treated groups which was significantly lower than the control ($p < 0.05$). The reduction in twice weekly was about 20 % while it was about 9 % in once weekly group.

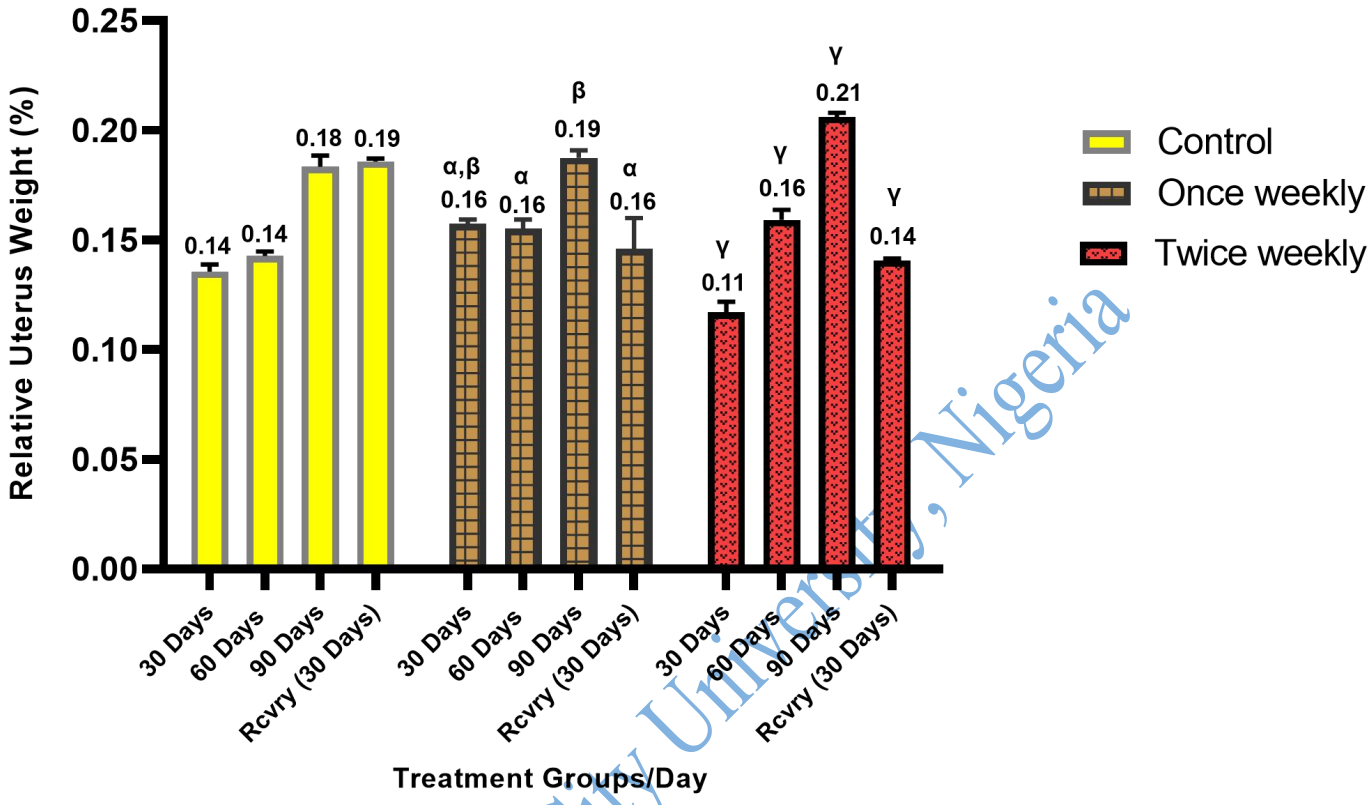


Figure 4.11a: Effect of Postinor 2 on the Relative Uterus Weight of Treated Wistar Rats compared with Control

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control. **β** indicated a statistical significance when once weekly was compared with those treated twice weekly. **γ**'showed significance when twice weekly was compared with control ($p < 0.05$).

4.5.2 Effect of Postinor 2 Treatment on Relative Ovary Weight

Figure 4.11b displays the relative ovarian weight of the respective groups. When the control group was compared with the treated groups at the end of the first 30 days of treatment, control was significantly lower ($p < 0.05$). The relative ovary weights evaluated at 60 days, the twice weekly group (0.048 ± 0.001) and once weekly group (0.042 ± 0.004) were significantly higher than the control group (0.037 ± 0.002) ($p < 0.05$). Additionally, twice weekly was significantly higher ($p = 0.0003$) than once weekly.

At 90 days of treatment, the relative ovary weight of once weekly had dropped to the same level as control and both were significantly higher than twice weekly ($p < 0.05$).

When the treatment was stopped for 30 days, there was recovery in both treated groups with the values very close to the control as in relative uterus weight.

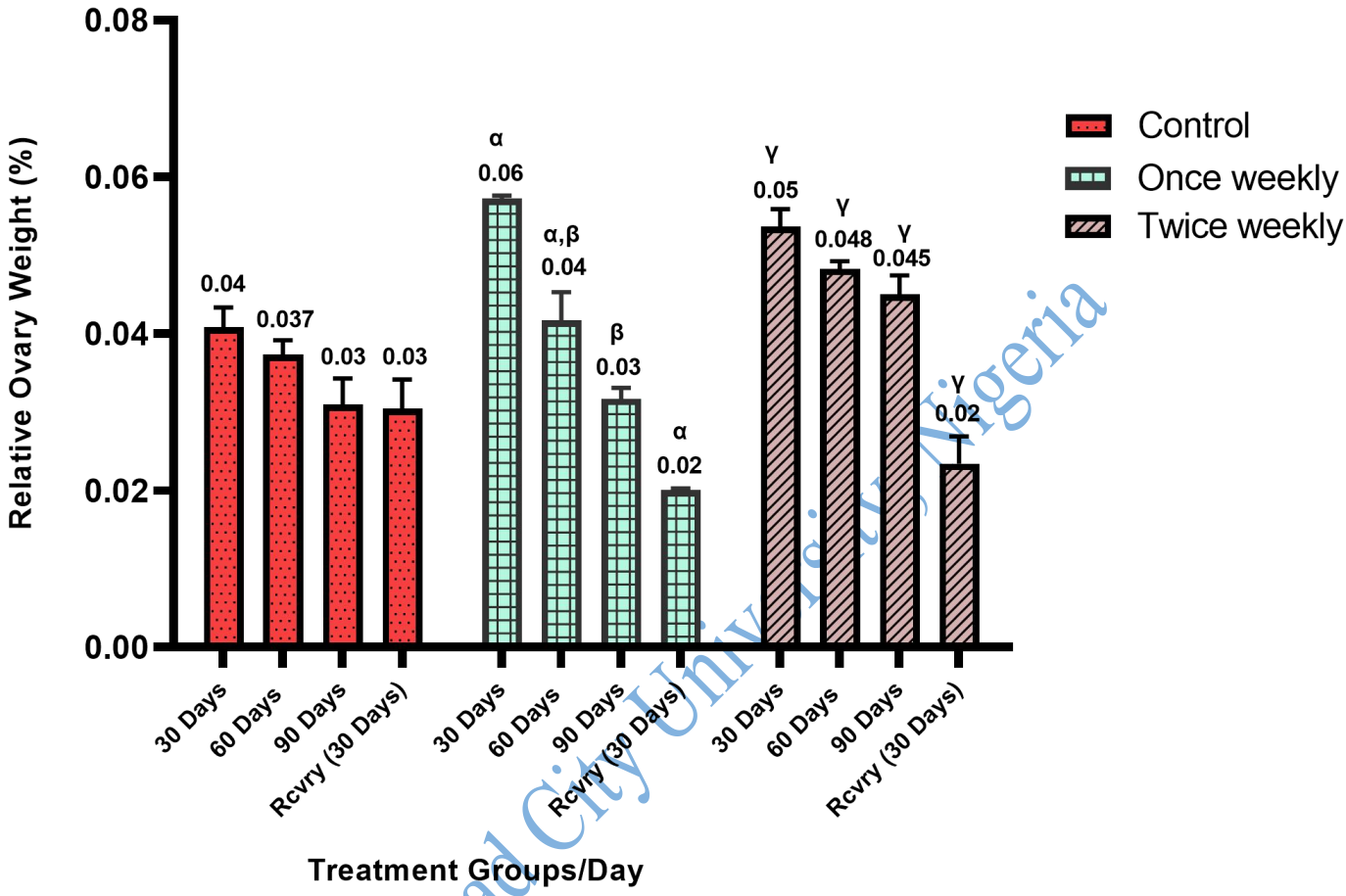


Figure 4.11b: The Relative Ovary Weights of Control and Treated Groups

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control. **β** indicated a statistical significance when once weekly was compared with those treated twice weekly. **γ**'showed significance when twice weekly was compared with control ($p < 0.05$).

4.5.3 Effect of Postinor 2 Treatment on Relative Liver Weight

As shown in Figure 4.11c, the relative liver weight of the treated groups was also compared with the control group. At the end of the first month of treatment, the relative liver weight was in the order once weekly < control < twice weekly and the differences were all significant ($p < 0.05$).

This trend continued throughout the treatment days showing once weekly to be lower than control while twice weekly was higher than the control groups until the recovery period when the order changed to control < once weekly < twice weekly ($p < 0.05$).

The recovery period brought about an increase in Relative liver weight that the values became significantly higher than in control ($p < 0.05$).

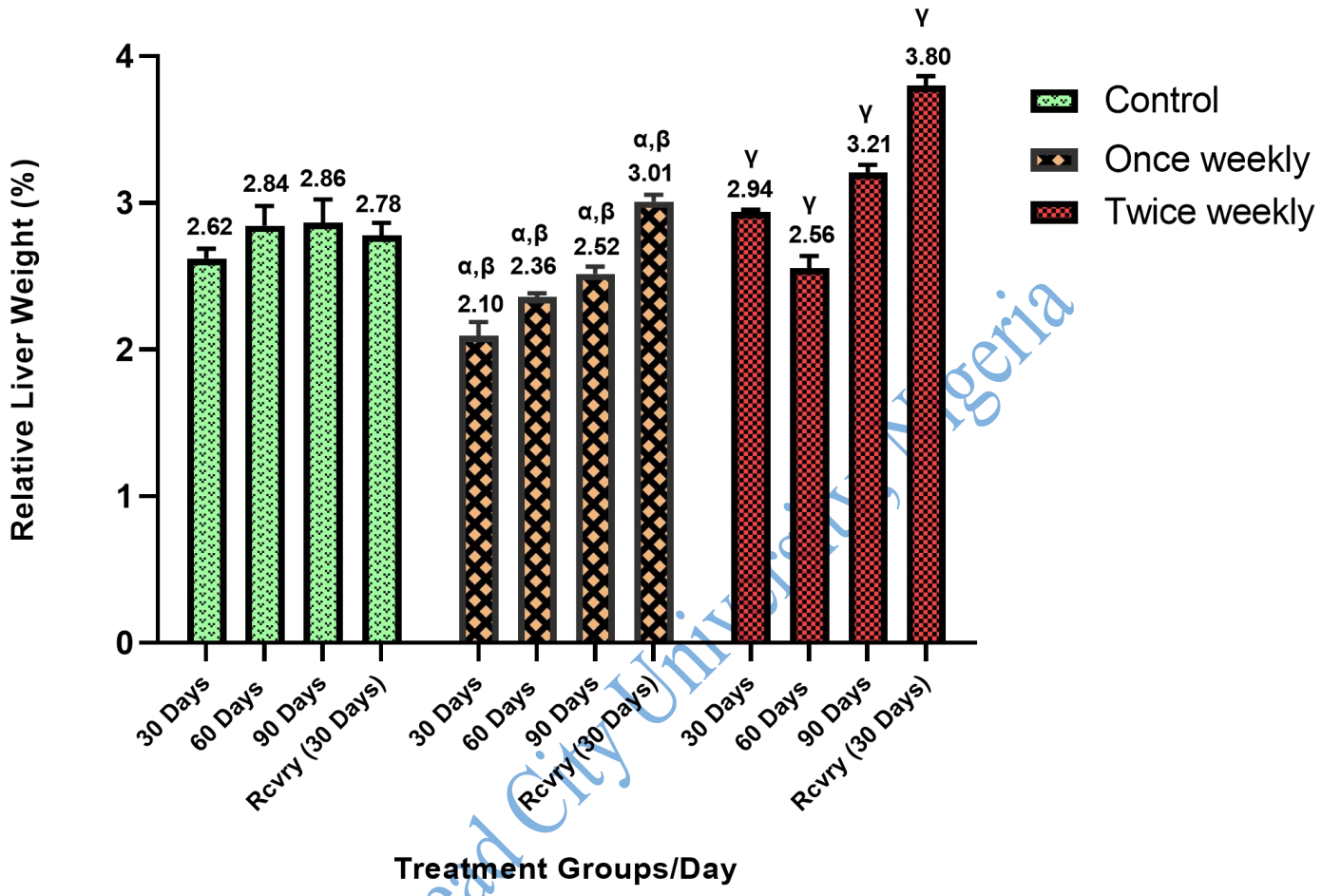


Figure 4.11c: Effect on Postinor 2 on the Relative Liver Weights

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control. **β** indicated a statistical significance when once weekly was compared with those treated twice weekly. **γ**'showed significance when twice weekly was compared with control ($p < 0.05$).

4.5.4. Effect of Postinor 2 on Relative Kidney Weight

When the relative kidney weight was compared among the different groups, at the end of the first month of treatment, control group (2.62 ± 0.067) was significantly higher than those treated once weekly (2.14 ± 0.093) ($p < 0.05$), and significantly lower than the group treated twice weekly (2.94 ± 0.016) ($p < 0.05$). There also existed a significant difference between once weekly and twice weekly ($p < 0.05$) as shown in Figure 4.11d.

A significant difference was also observed between once weekly and twice weekly treated groups ($p = 0.05$) with a mean ± SD values of 2.36 ± 0.026, and 2.56 ± 0.081 respectively at the end of the second month of treatment. Meanwhile, the relative kidney weights in the control (2.84 ± 0.134) had become significantly higher than both once weekly and twice weekly groups ($p < 0.05$).

At the end of 90 days, the group treated twice weekly (3.21 ± 0.051) was significantly higher than both control group (2.86 ± 0.159) and the group treated once weekly (2.52 ± 0.048) ($p < 0.05$). There was also a significant difference between control and once weekly ($p < 0.05$).

30 days post treatment showed an increase iin kidney weight across all groups which was significantly higher in twice weekly than in once weekly and both appeared significantly higher than the control group ($p < 0.05$).

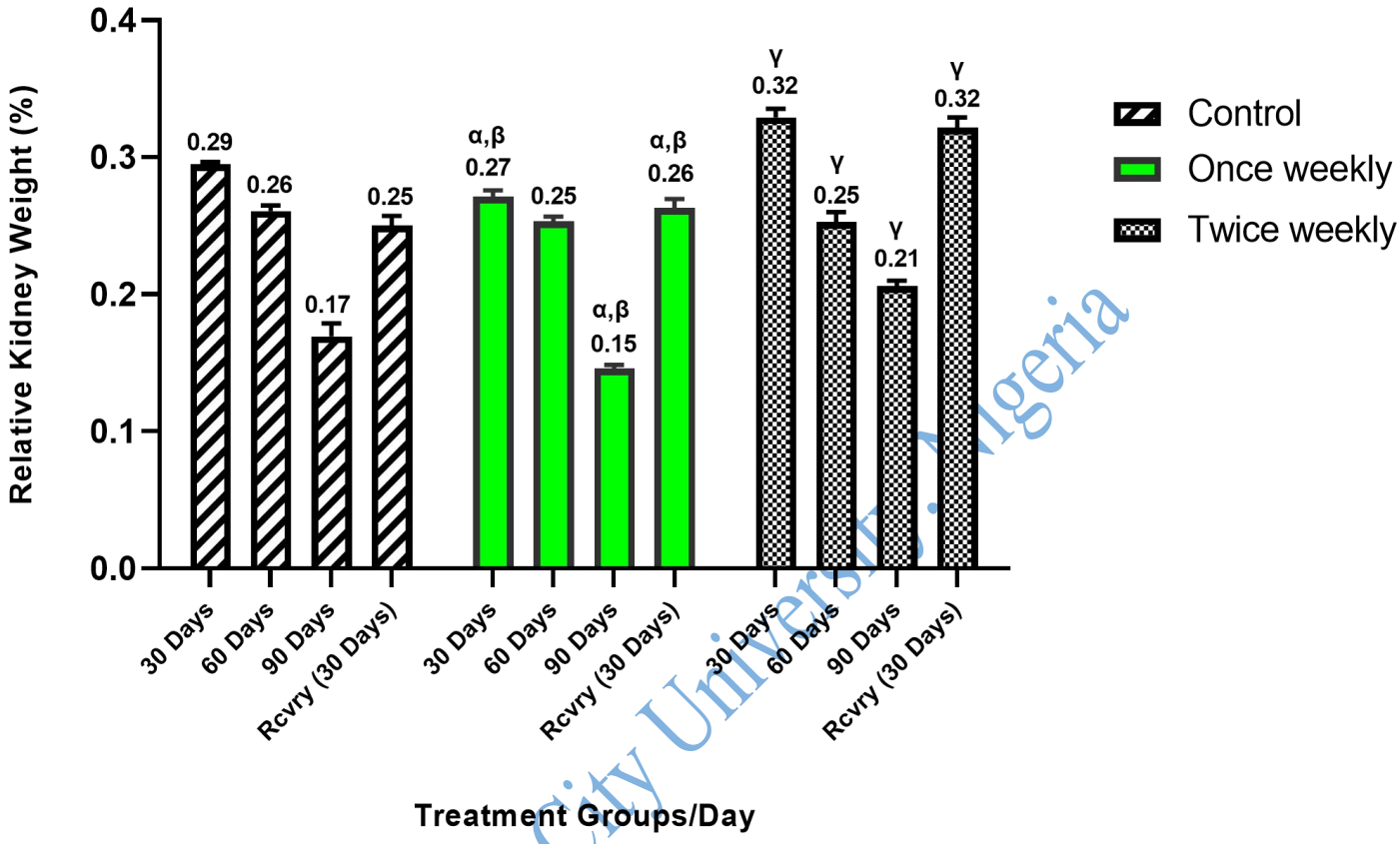


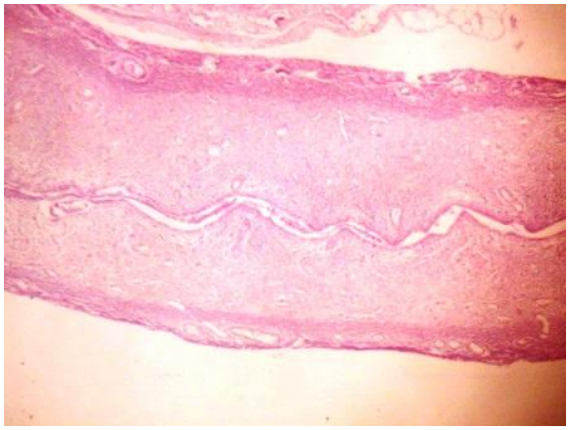
Figure 4.11d: The Effect of Postinor 2 on Relative Kidney Weights

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control. **β** indicated a statistical significance when once weekly was compared with those treated twice weekly. **γ**'showed significance when twice weekly was compared with control ($p < 0.05$).

4.6 Histopathological Examination of the Harvested Tissues for Observation of any Effect of Postinor 2 Intake

4.6.1 Histopathological Examination of the Uterus

The photomicrograph of the uterus harvested from the treated groups was compared with that from the control groups. The architecture of the control groups appeared normal no visible lesion as (plates 4.1 and 4.2). Conversely, the photomicrographs from tissues harvested from rats treated with Postinor 2 once weekly showed an infiltration of the mucosa as well as degenerated and hyperplastic uterine glands (plates 4.3 and 4.4). Whereas severe endometrial ulcerations were observed in animals treated with Postinor 2 twice weekly (plates 4.5).



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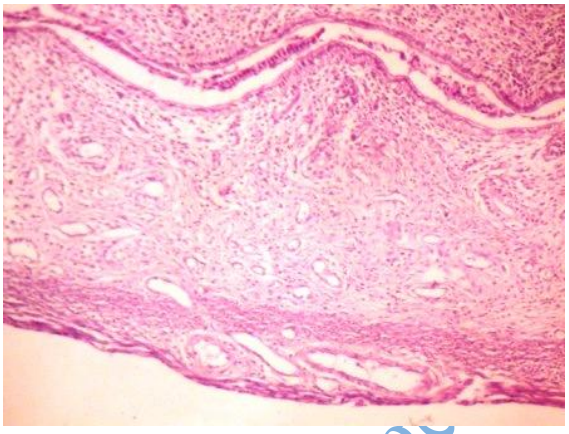

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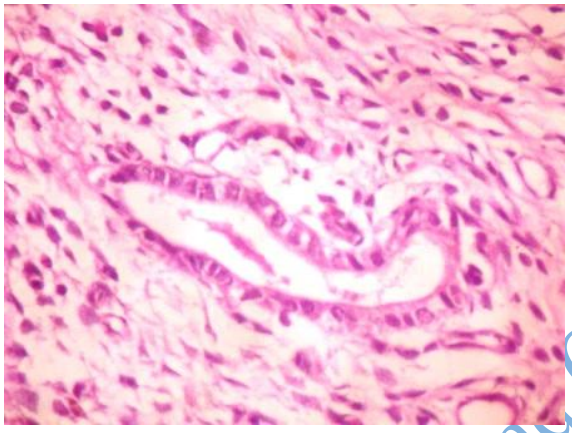

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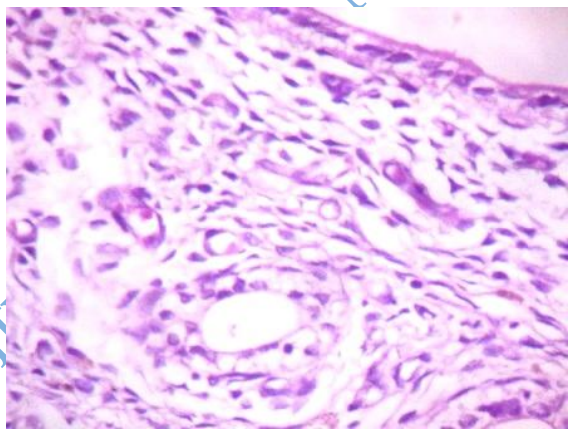

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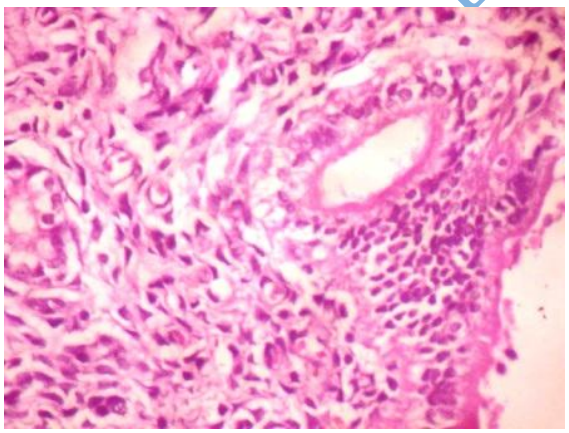

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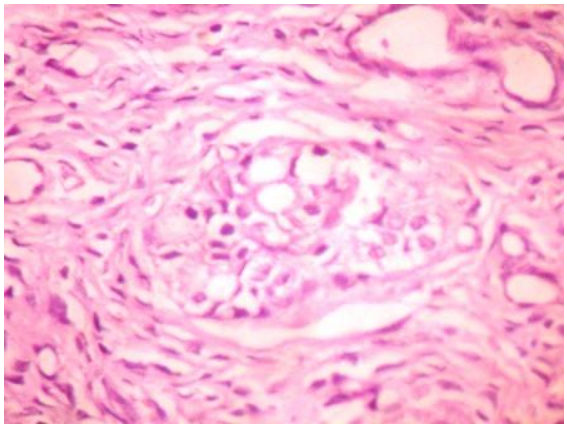

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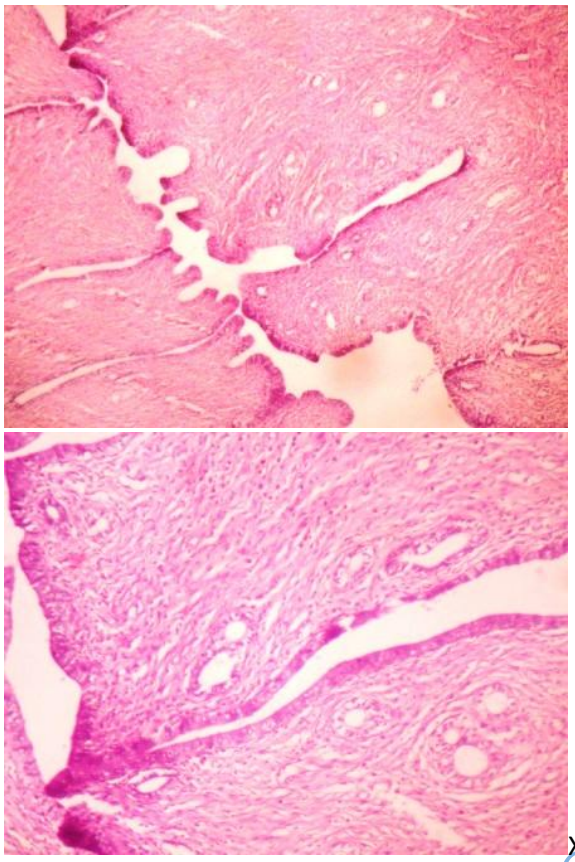
Plate 4.1: Photomicrophs of Thin Sections of Uterus Tissues Harvested from the Control Group I

Plates show no significant lesion



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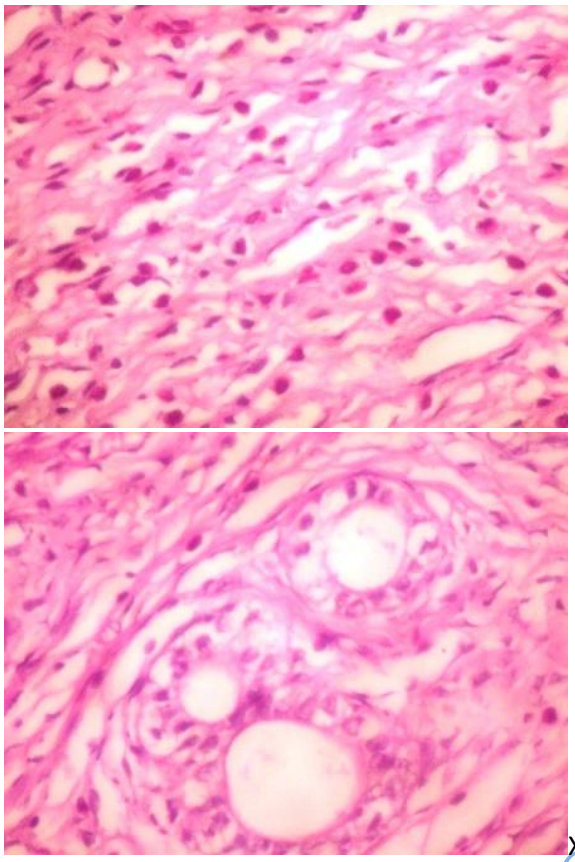

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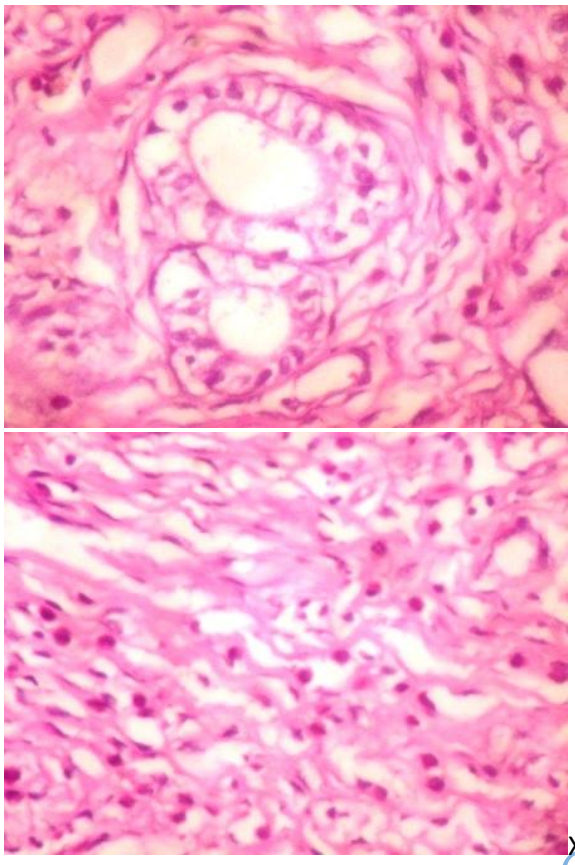

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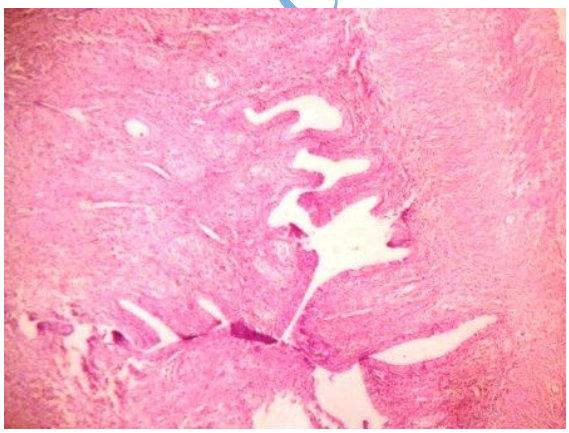
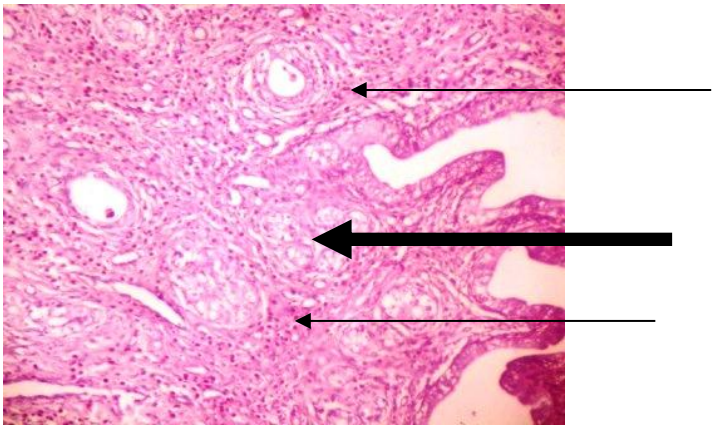
Plate 4.2: Photomicrophs of Thin Sections of Uterus Tissues Harvested from the Control Group II

Plates show no significant lesion





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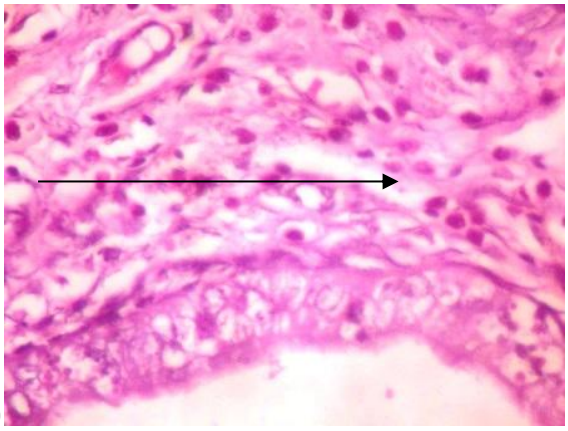

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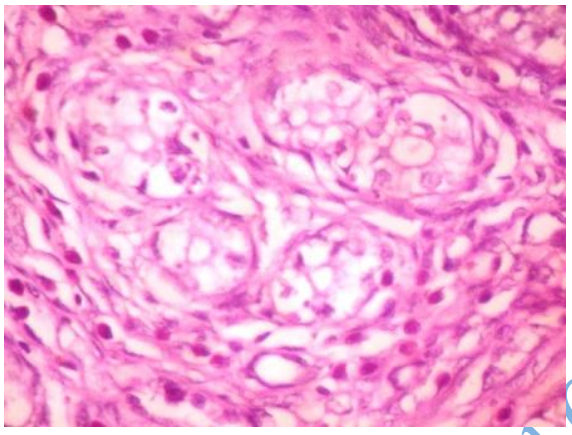

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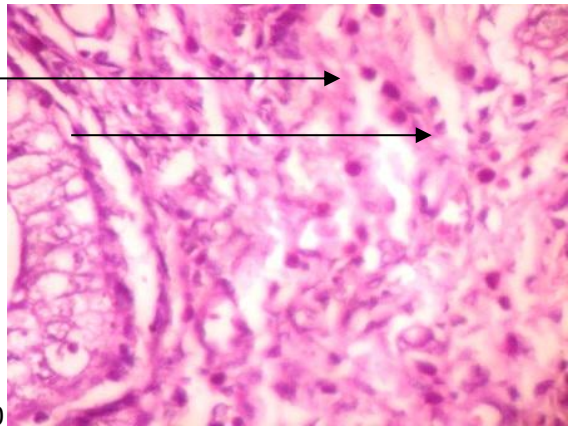

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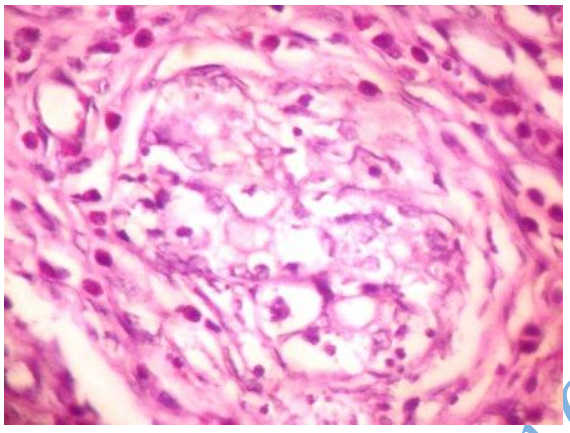

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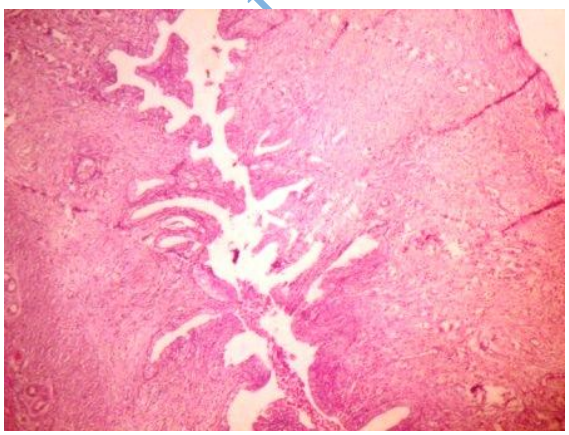
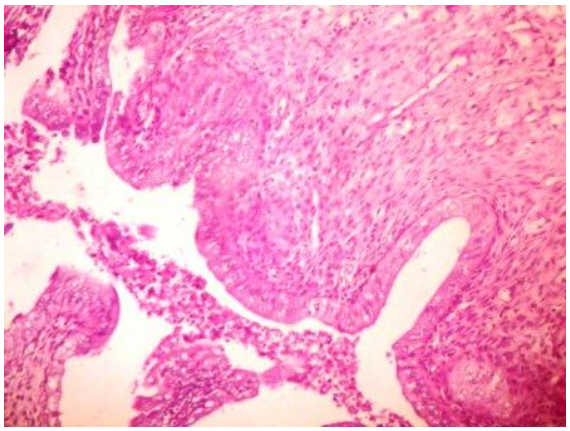
Plate 4.3: Photomicrophs of Thin Sections of Uterus Tissues Harvested from the Once Weekly Treated Group I

Plates show infiltration of mucosa by inflammatory cells (slender arrows) the degenerated uterine glands (black arrows).





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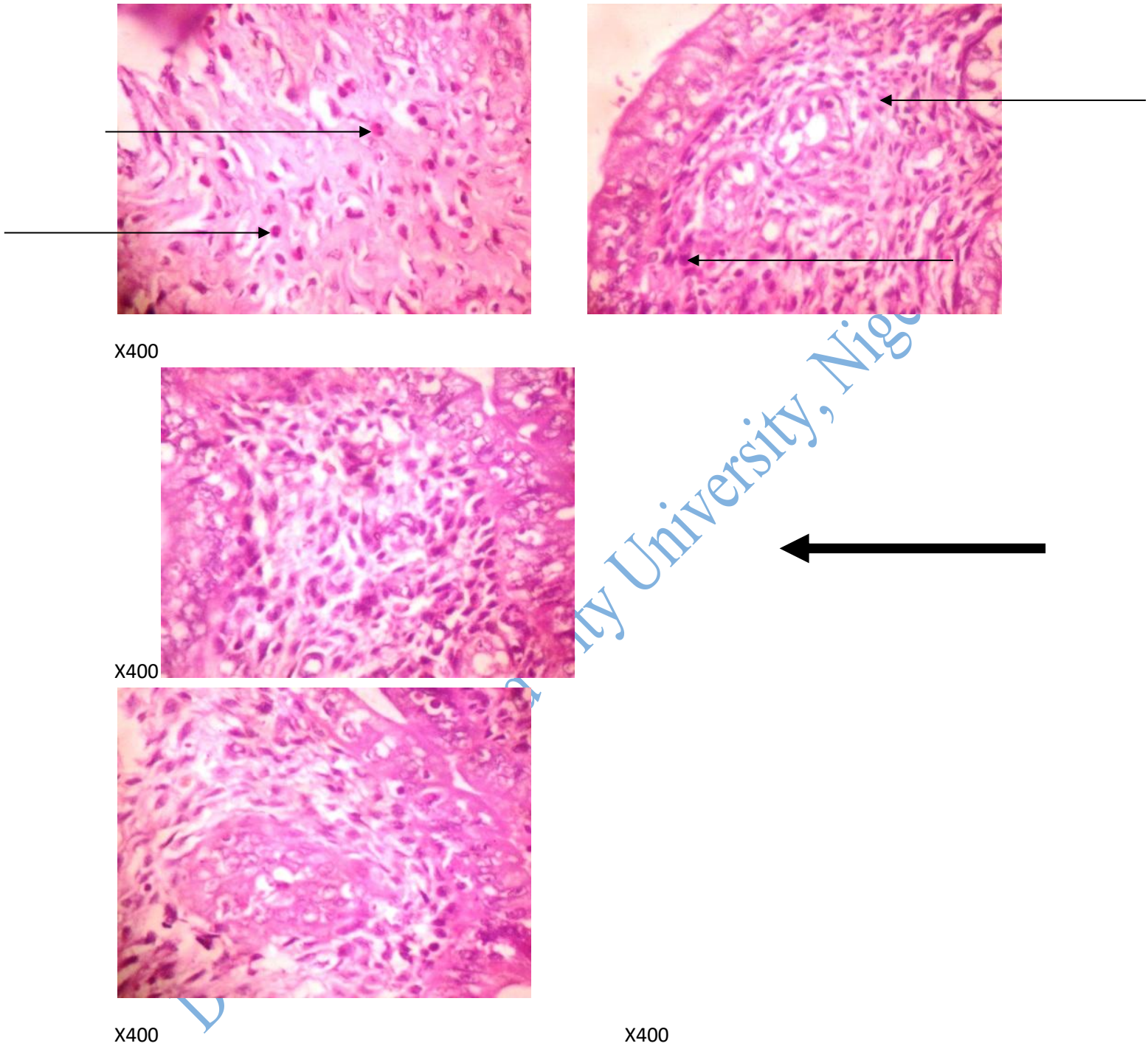
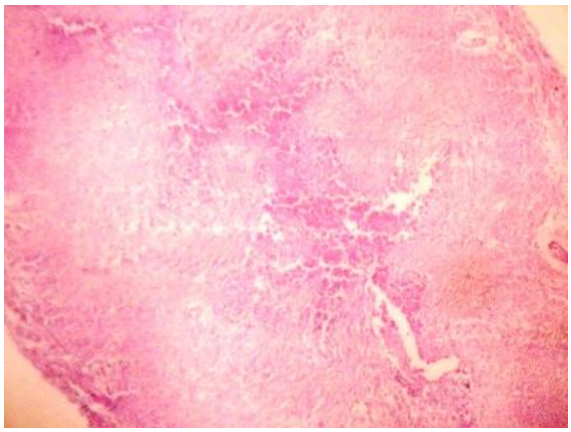


Plate 4.4: Photomicrophs of Thin Sections of Uterus Tissues Harvested from the Once Weekly Treated Group II

Plates show infiltration of mucosa by inflammatory cells (slender arrows) the hyperplastic uterine glands (black arrow).



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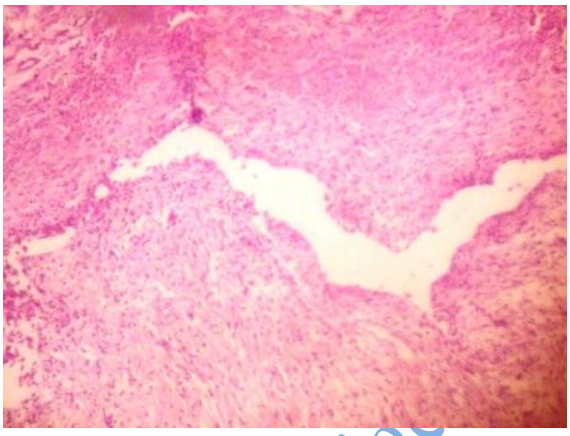

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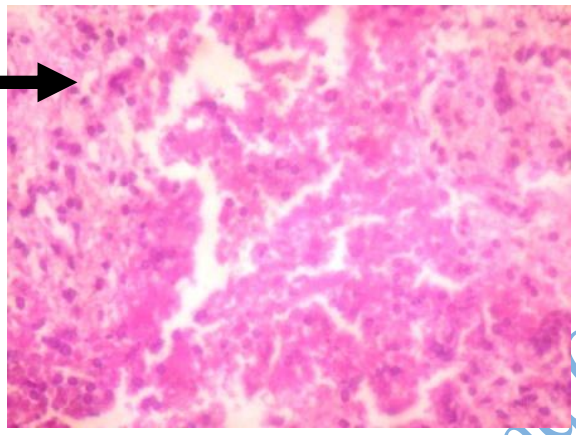

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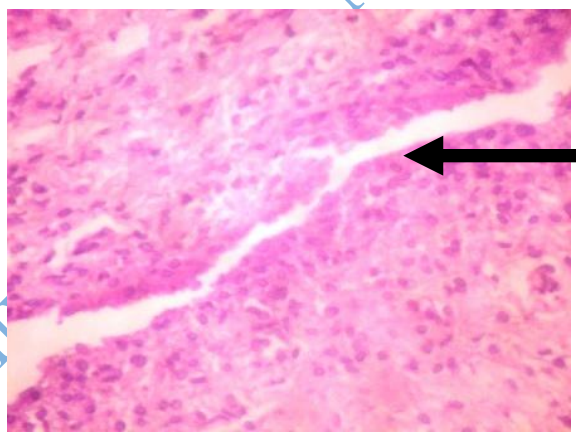

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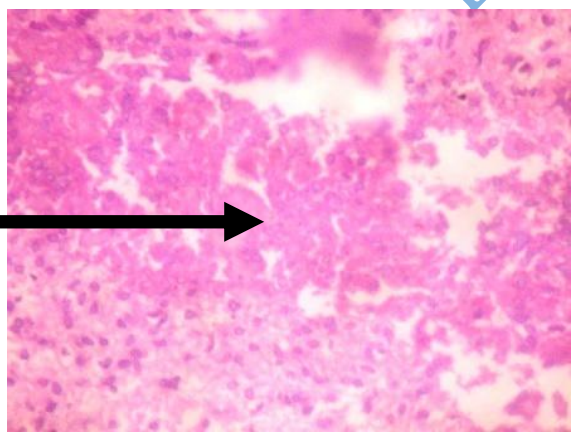


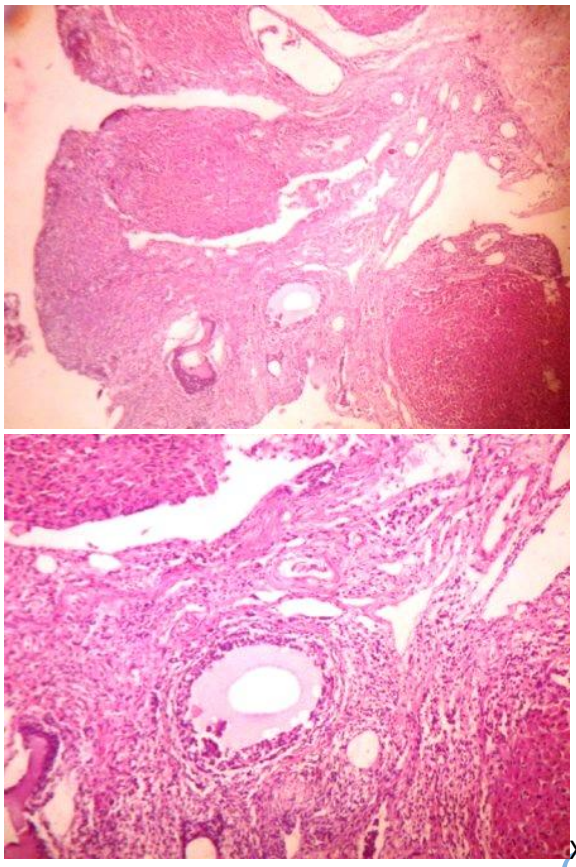
X400

Plate 4.5: Photomicrophs of Thin Sections of Uterus Tissues Harvested from the Twice Weekly Treated Group.

Plates show endometrial ulceration (black arrows)

4.6.2 Histopathological Examination of the Ovary

The photomicrograph of the ovary harvested from the treated groups was compared with that from control groups. The architecture of the control groups showed normal morphological architecture with atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as corpus luteum as shown in plates 4.6 and 4.7. Additionally, the photomicrographs of tissues harvested from rats treated with Postinor 2 once weekly and twice weekly did not demonstrate any visible signs of histological aberrations in the ovary (plates 4.8 to 4.11).



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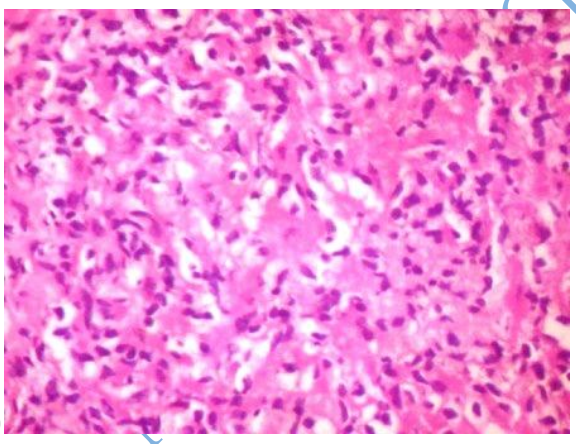

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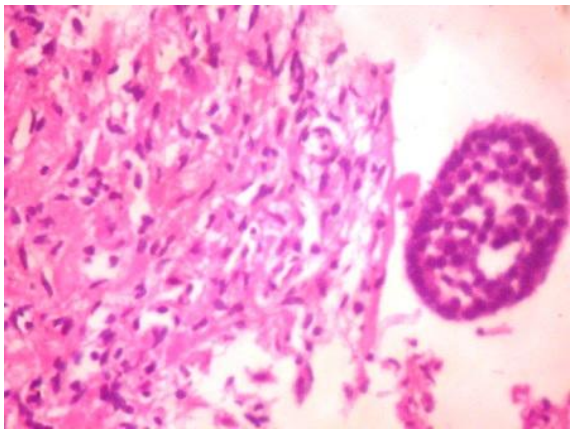

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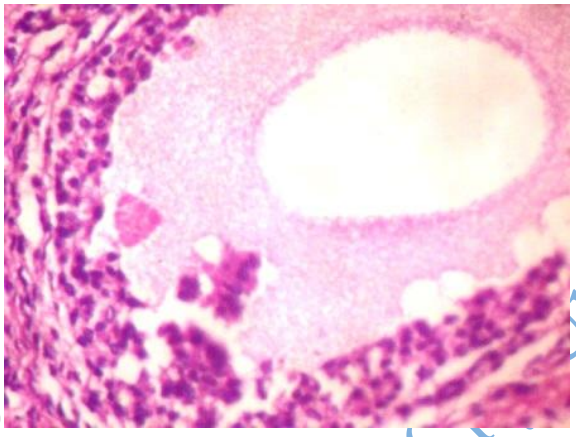

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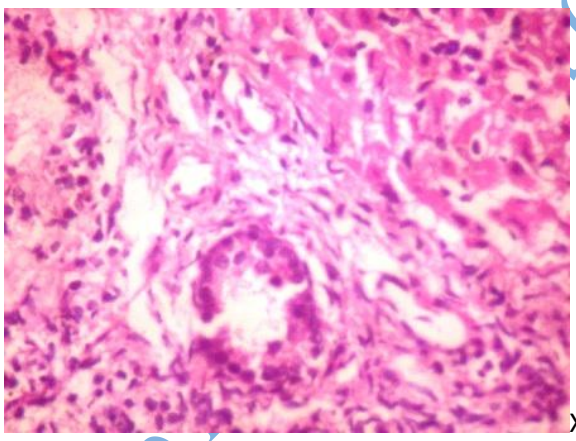
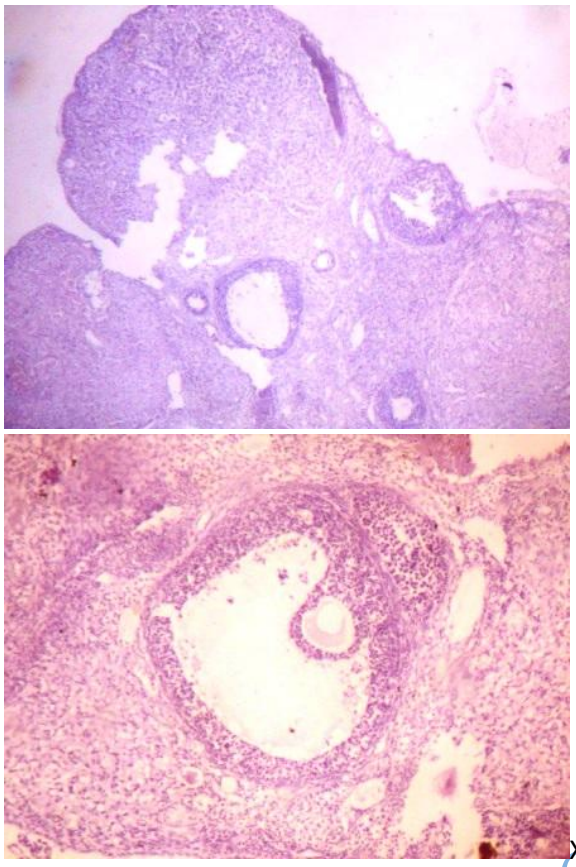


X40

X100

Plate 4.6: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Control Group I

Plates show atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary



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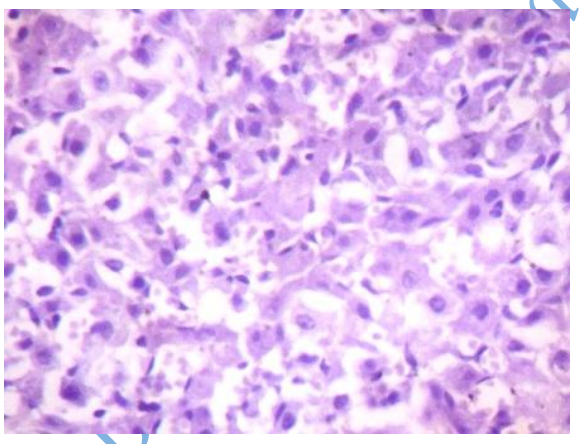

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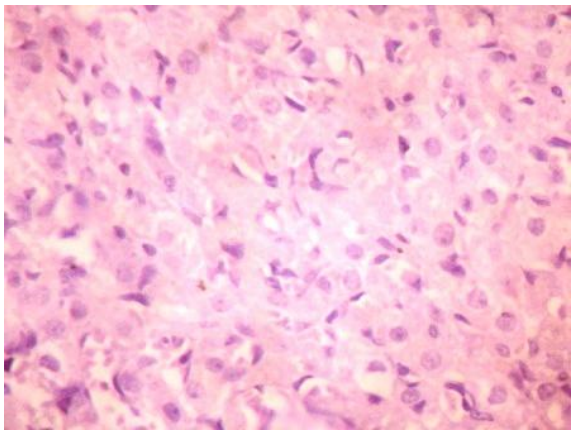

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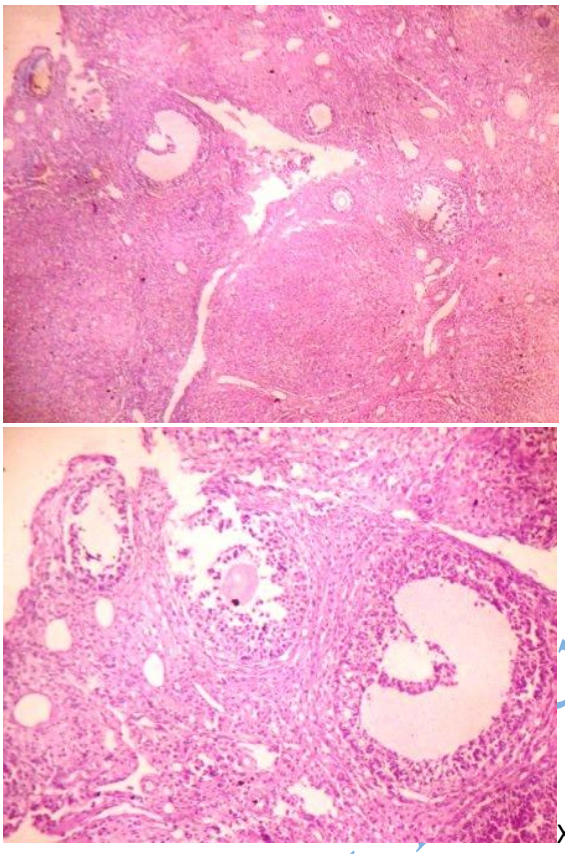
Plate 4.7: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Control Group II

Plates show atretic follicles at different stages of maturation (primordial. Primary, secodary antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary



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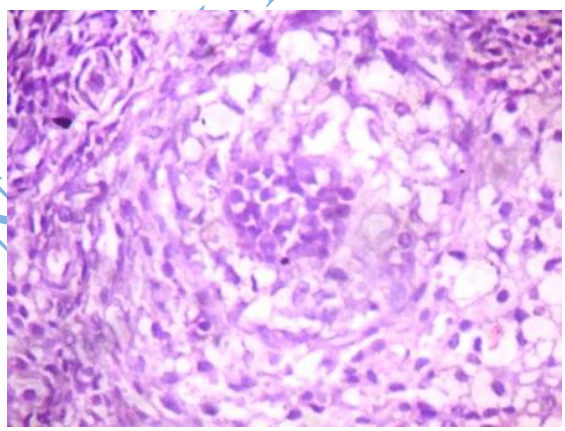

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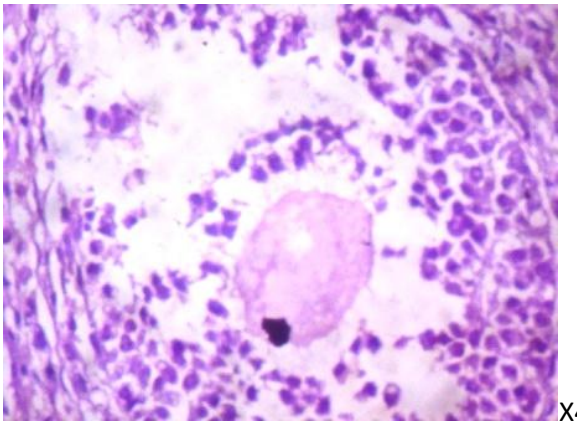

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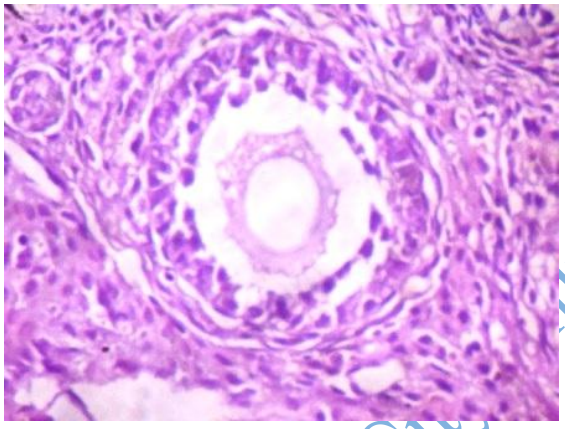

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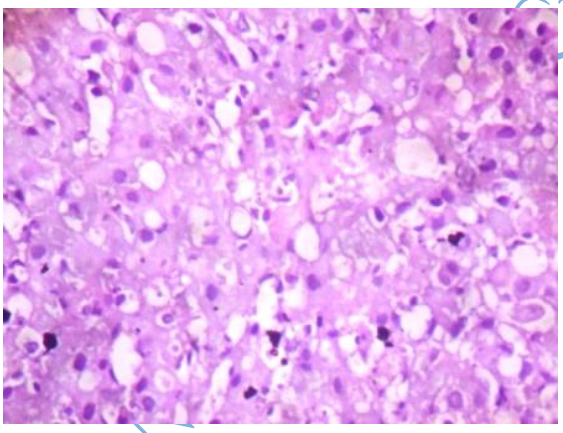

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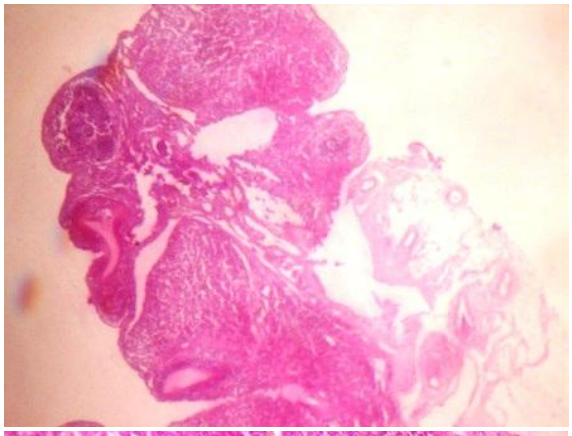
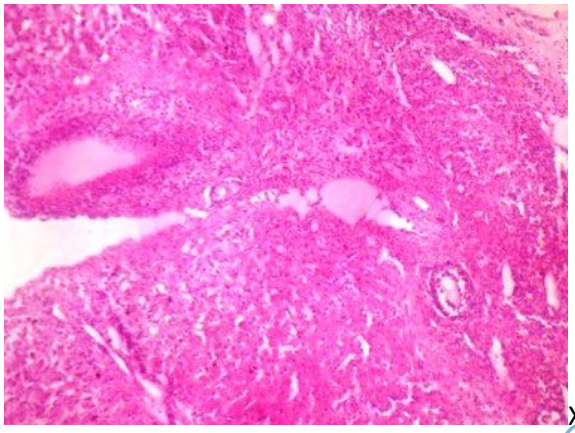
Plate 4.8: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Once Weekly treated Group I

Plates show atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary





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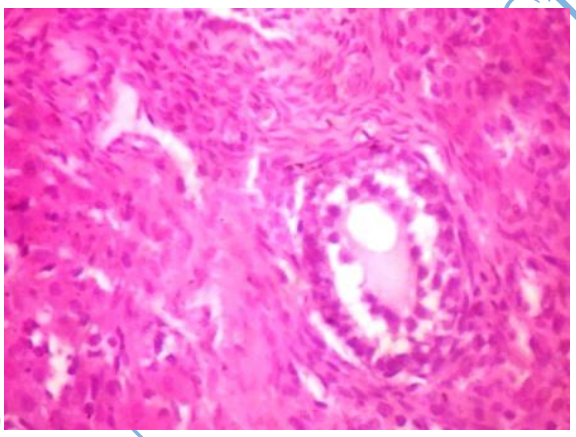

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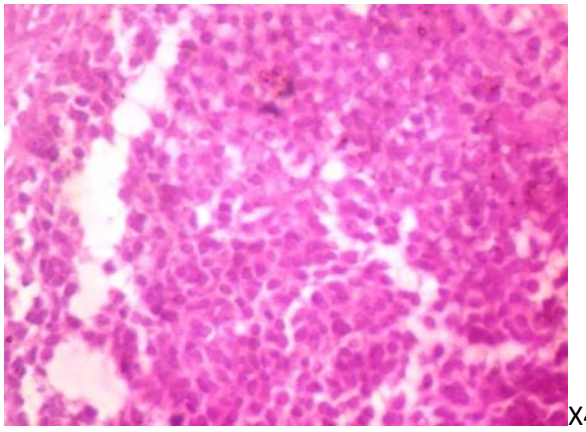

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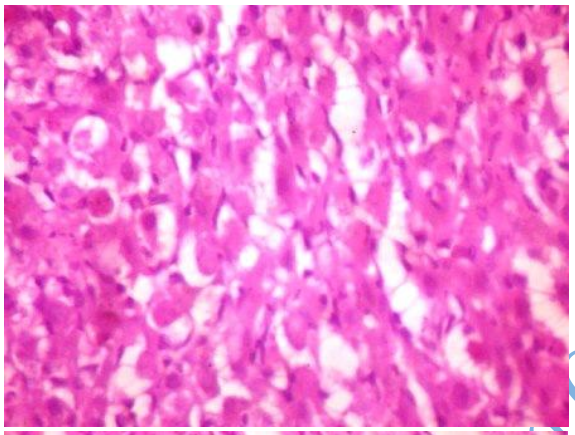
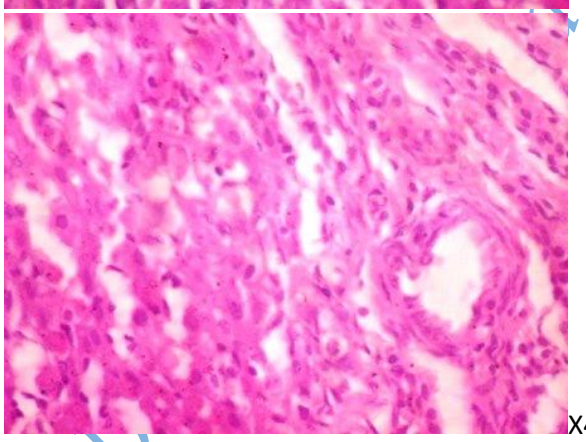


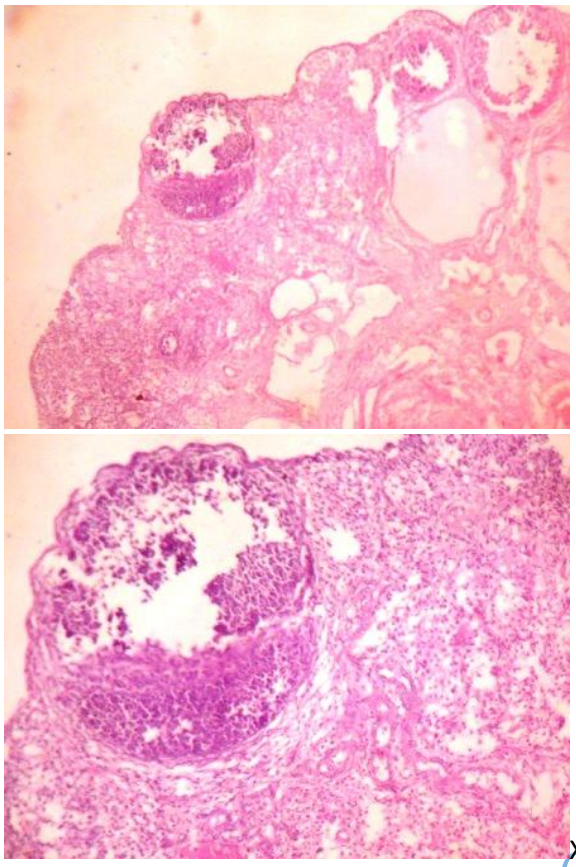


X400

X400

Plate 4.9: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Once Weekly treated Group II

Plates show few atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary



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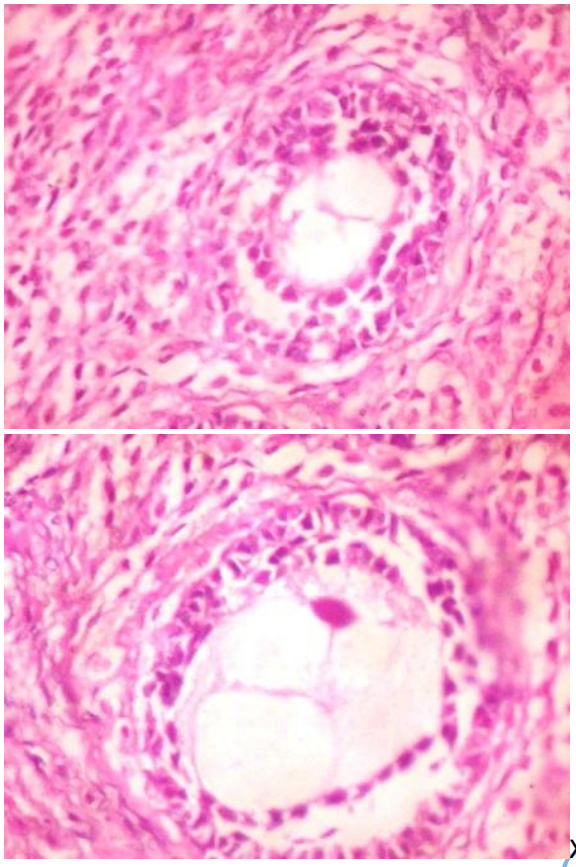

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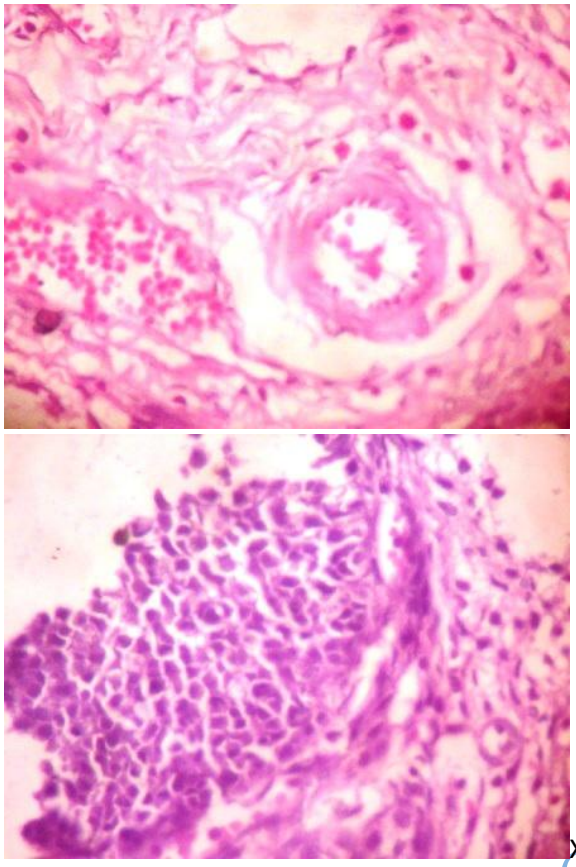
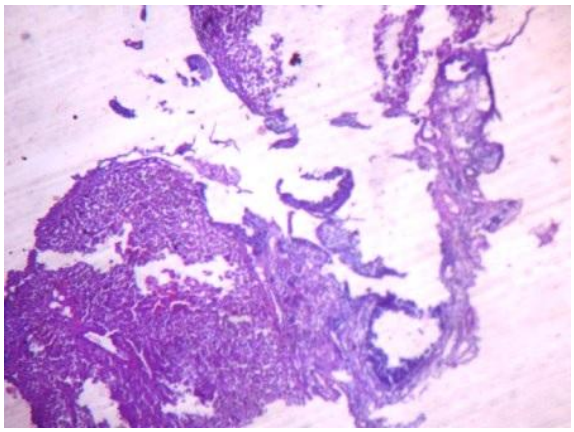


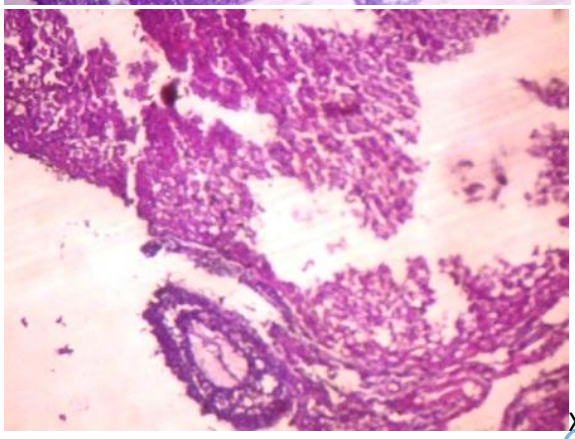
X400

X400

Plate 4.10: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Twice Weekly Treated Group I

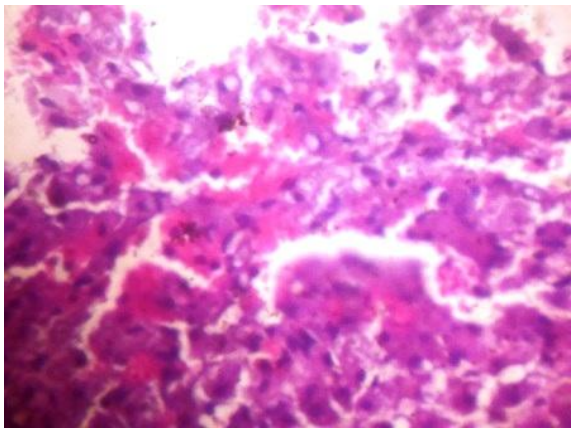
Plates show atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary

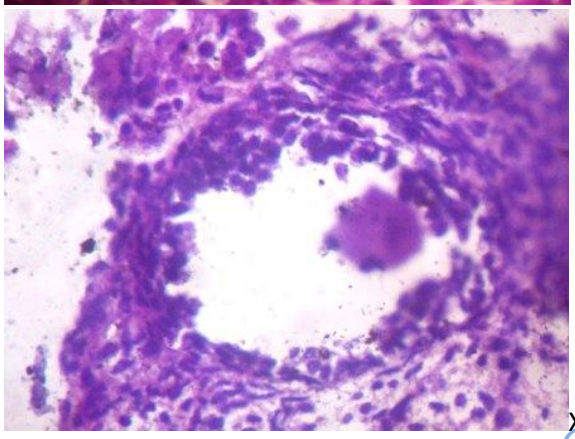




X40

X100





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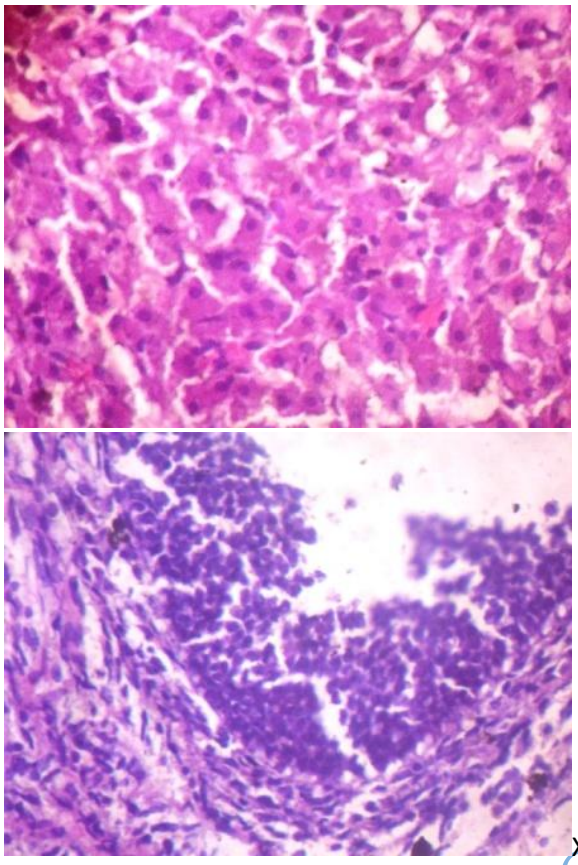


X400

X400

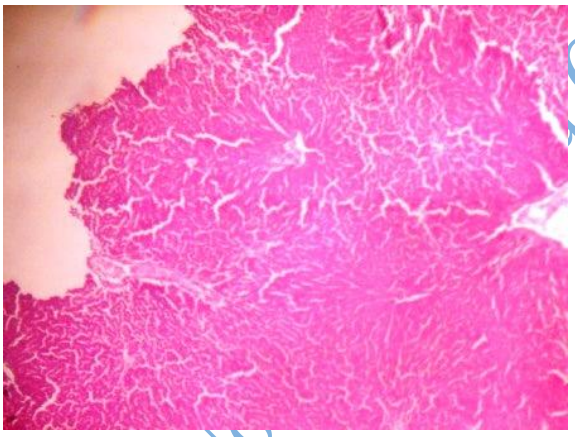
Plate 4.11: Photomicrophs of Thin Sections of Ovary Tissues Harvested from the Twice Weekly Treated Group II

Plates show very few atretic follicles at different stages of maturation (primordial, primary, secondary and antral follicles) as well as copus luteum. The overall feature is keeping with a normal morphology of the ovary

4.6.3 Histopathological Examination of the Liver

The photomicrographs of the liver harvested from the control group showed mild focal periportal and disseminated infiltration by inflammatory cells and mild disseminated infiltration of zone 2 by inflammatory cells (Plates 4.12 and 4.13). Also, photomicrographs of the liver showed that treatment of rats with Postinor-2, once weekly resulted in cogestion as well as mild focal periportal and disseminated infiltration by inflammatory cells (Plate 4.14 and plate 4.15). Whereas plate 4.16 and plate 4.17 showed marked disseminated congestion, mild disseminated

periportal infiltration by inflammatory cells and lymphoid aggregate in zone 2 and the focal area of hepatic nodules noted in animals treated with Postinor 2 twice weekly



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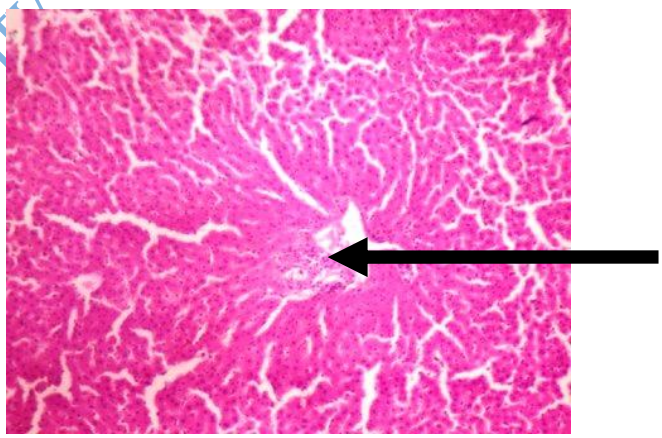

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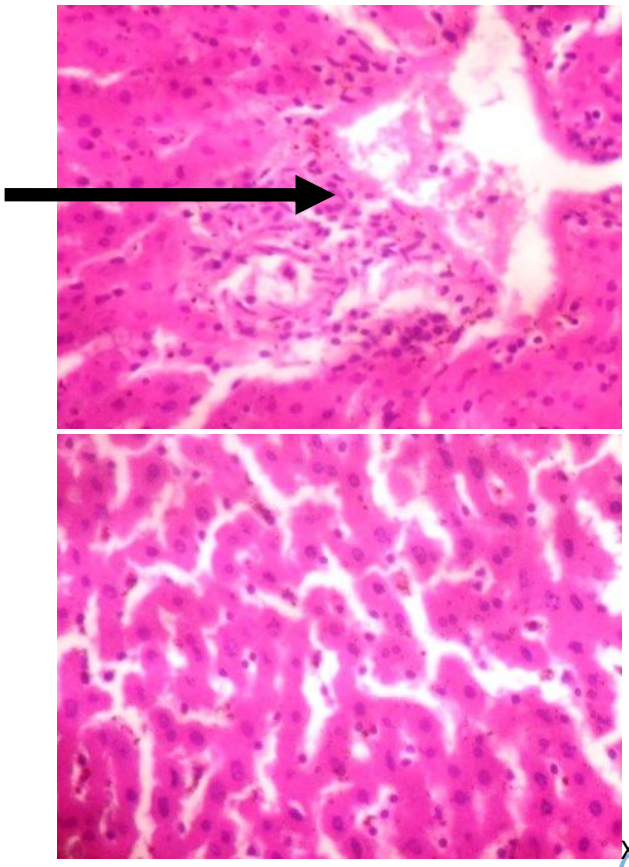

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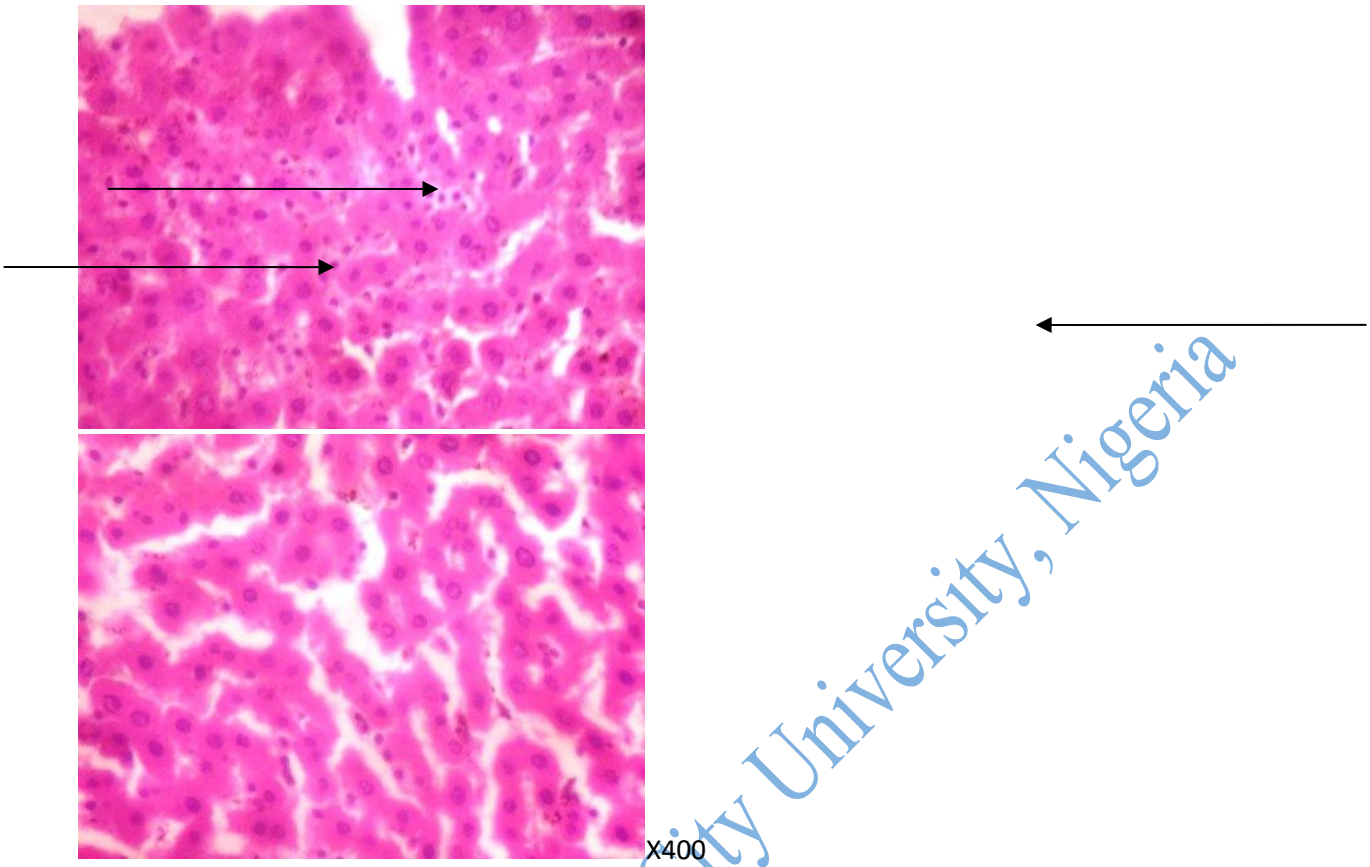
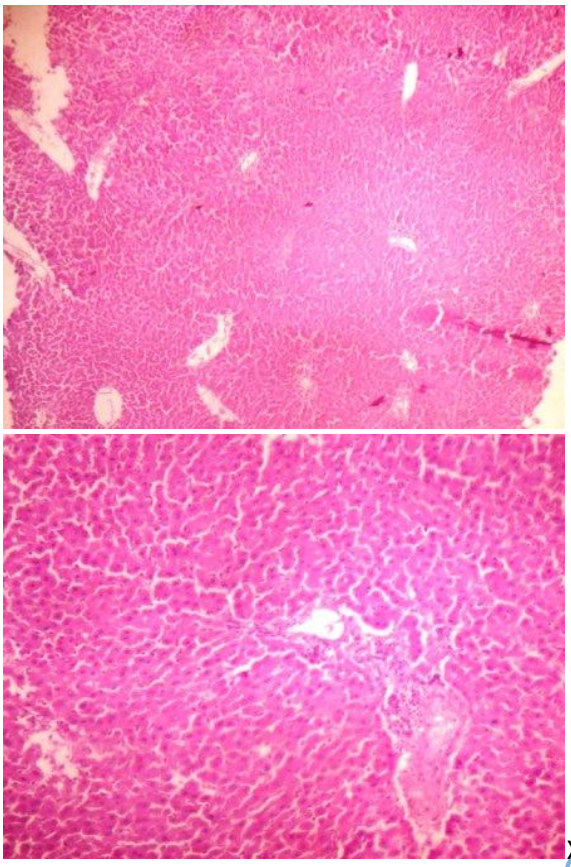


Plate 4.12: Photomicrophs of Thin Sections of Liver Tissues Harvested from Control Group I

Plates show mild focal periportal infiltration by inflammatory cells (black arrows) and mild disseminated infiltration of zone 2 by inflammatory cells (slender arrows).



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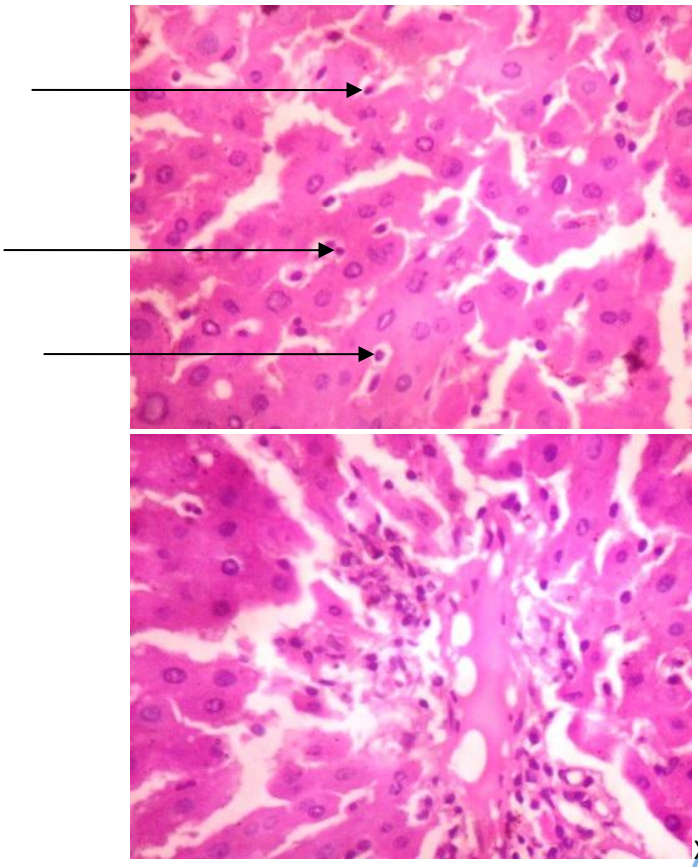
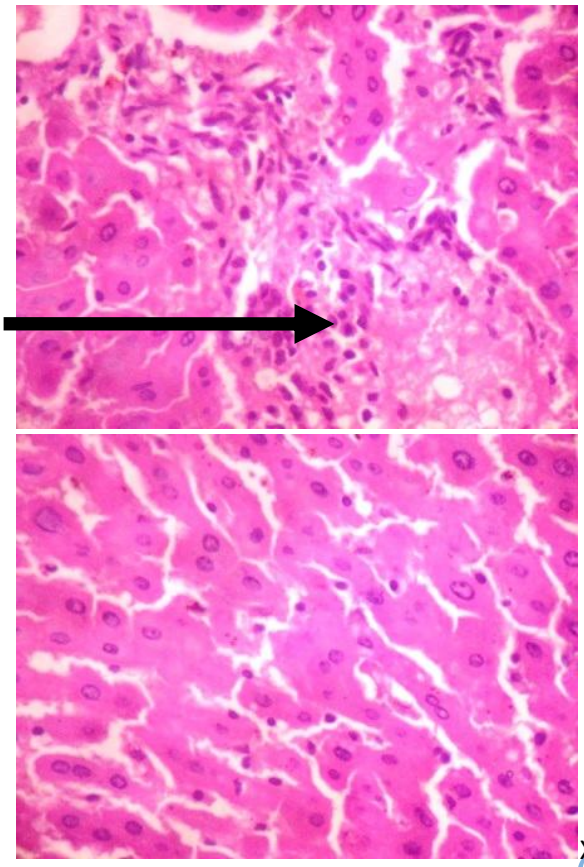


X400

X400

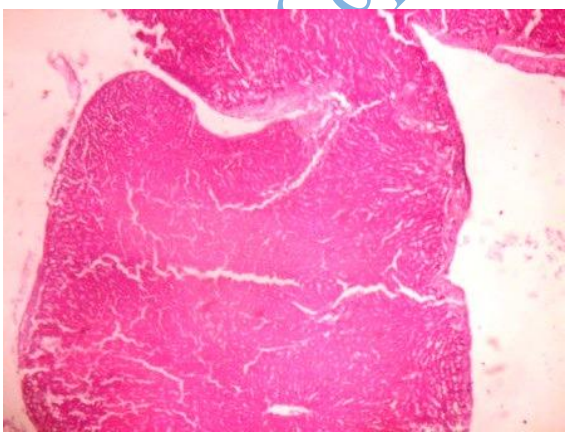


X400

X400

Plate 4.13: Photomicrophs of Thin Sections of Liver Tissues Harvested from Control Group II

Plates show mild focal periportal infiltration by inflammatory cells (black arrows) and mild disseminated infiltration of zone 2 by inflammatory cells (slender arrows).



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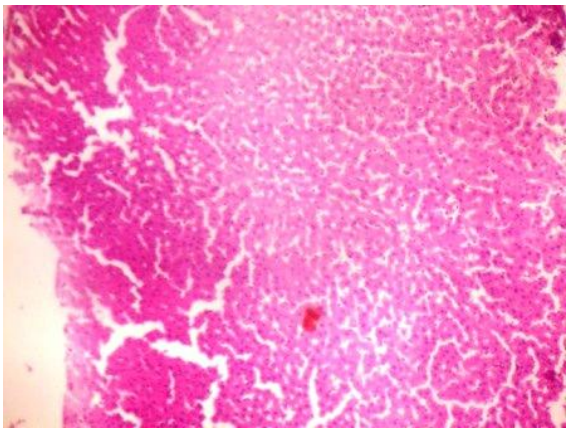

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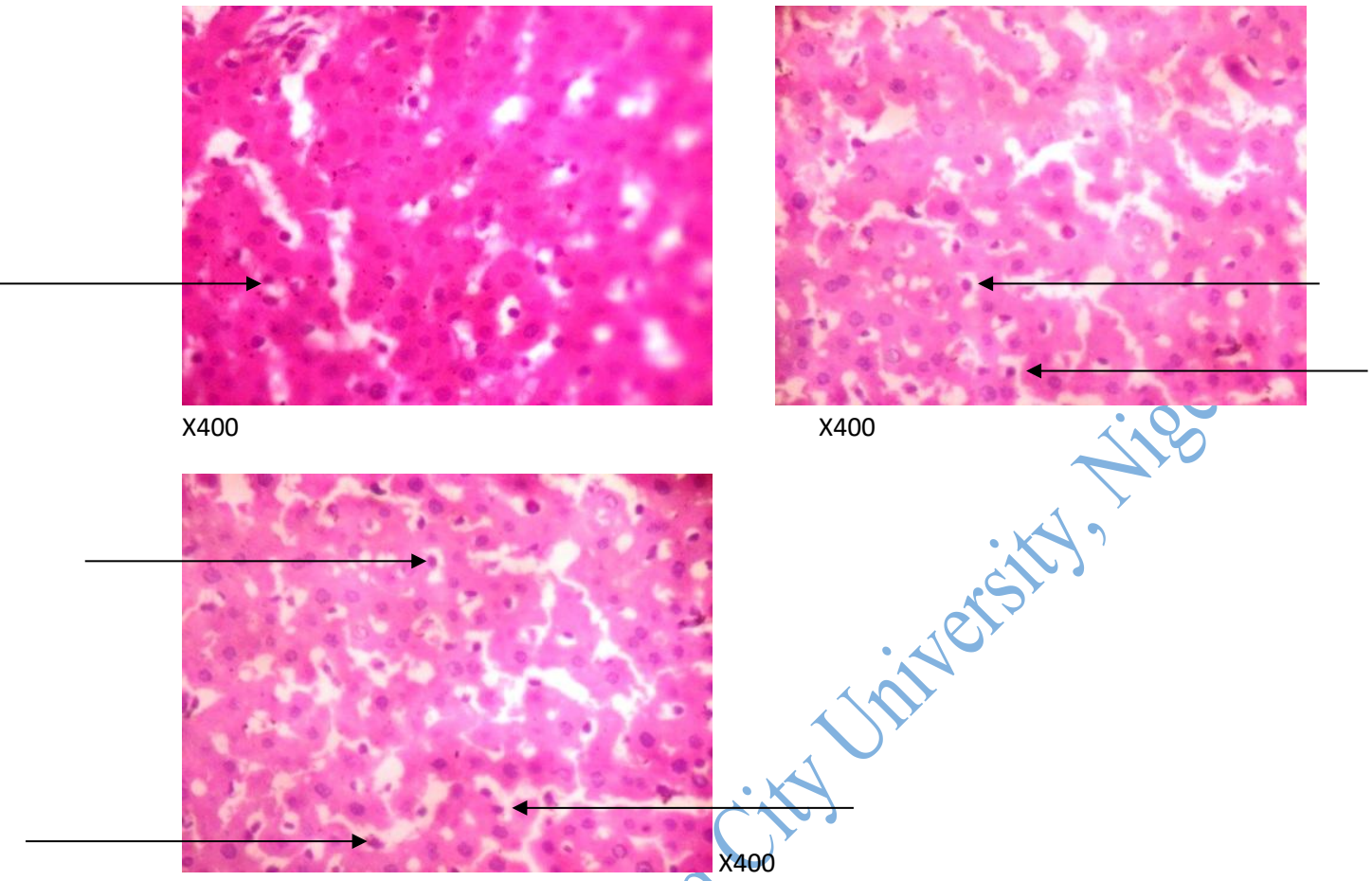
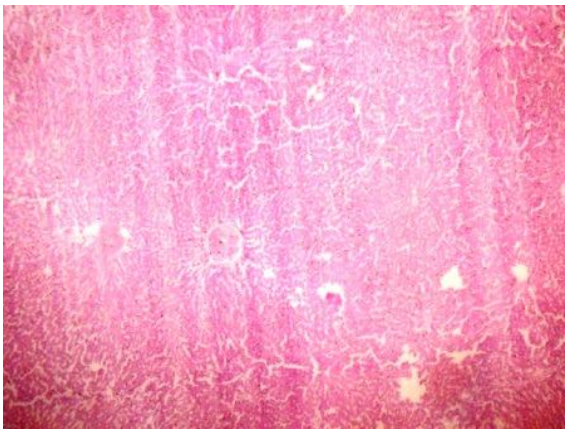


Plate 4.14: Photomicrophs of Thin Sections of Liver Tissues Harvested from Once Weekly Treated Group I

Plates show infiltration of zone 2 by inflammatory cells (slender arrows).



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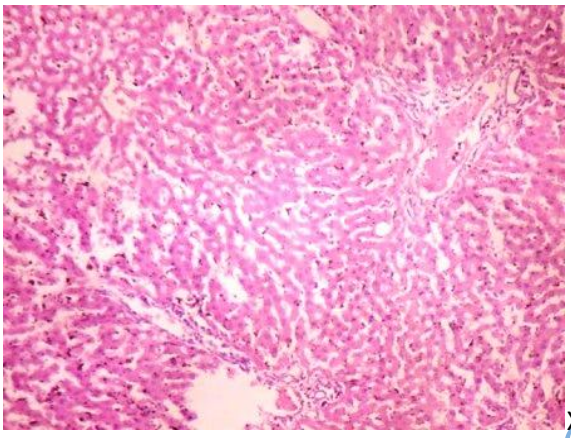

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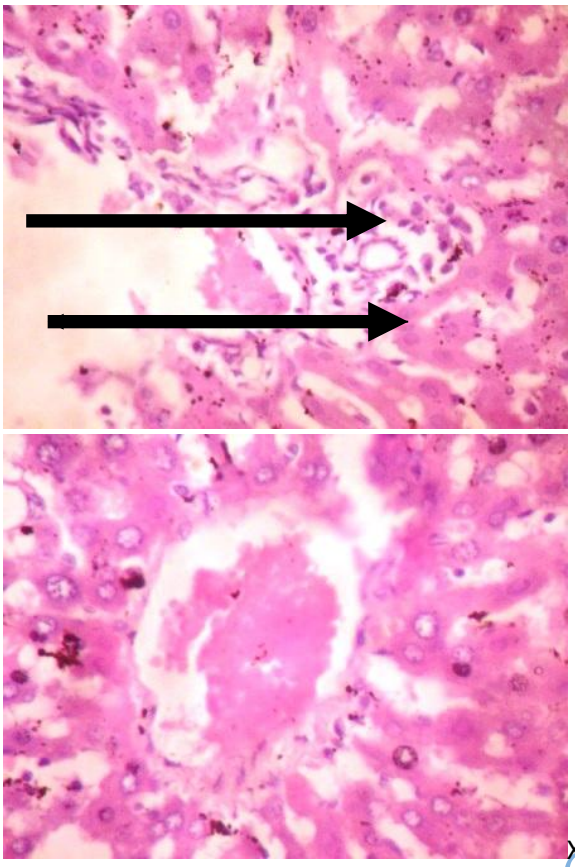

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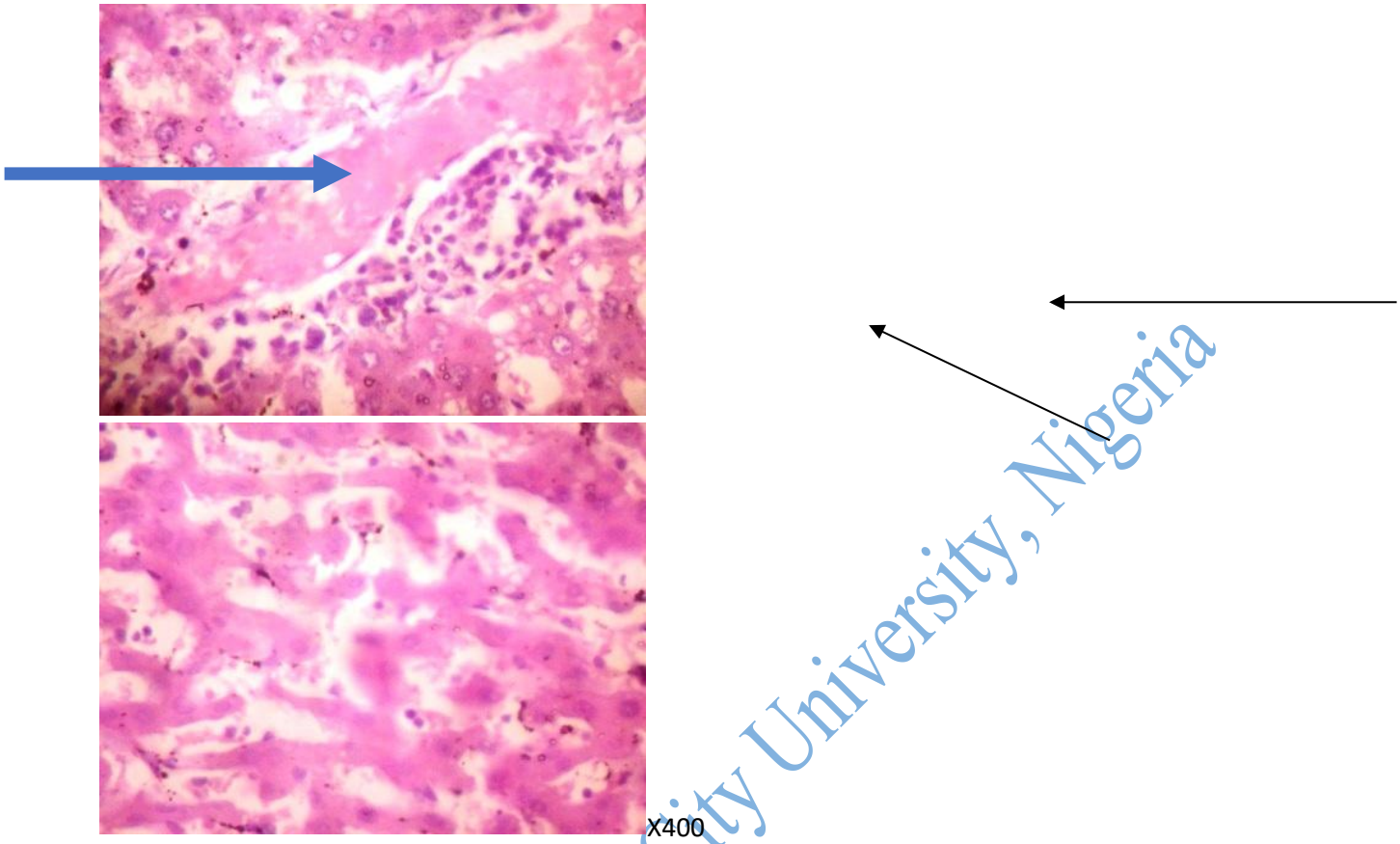
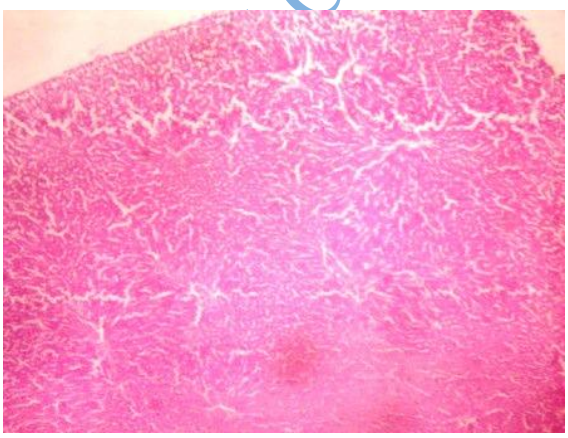


X400

X400

Plate 4.15: Photomicrophs of Thin Sections of Liver Tissues Harvested from Once Weekly Treated Group II

Plates show cogestion (Blue arrows), mild disseminated periportal infiltration by inflammatory cells (black arrows) and very mild disseminated infiltration of zone2 by inflammatory cells (slender arrows).



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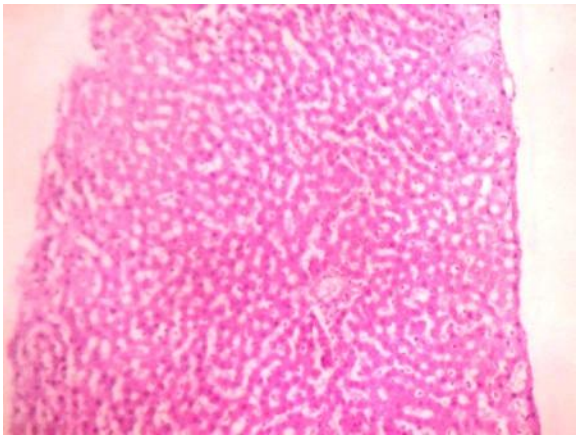

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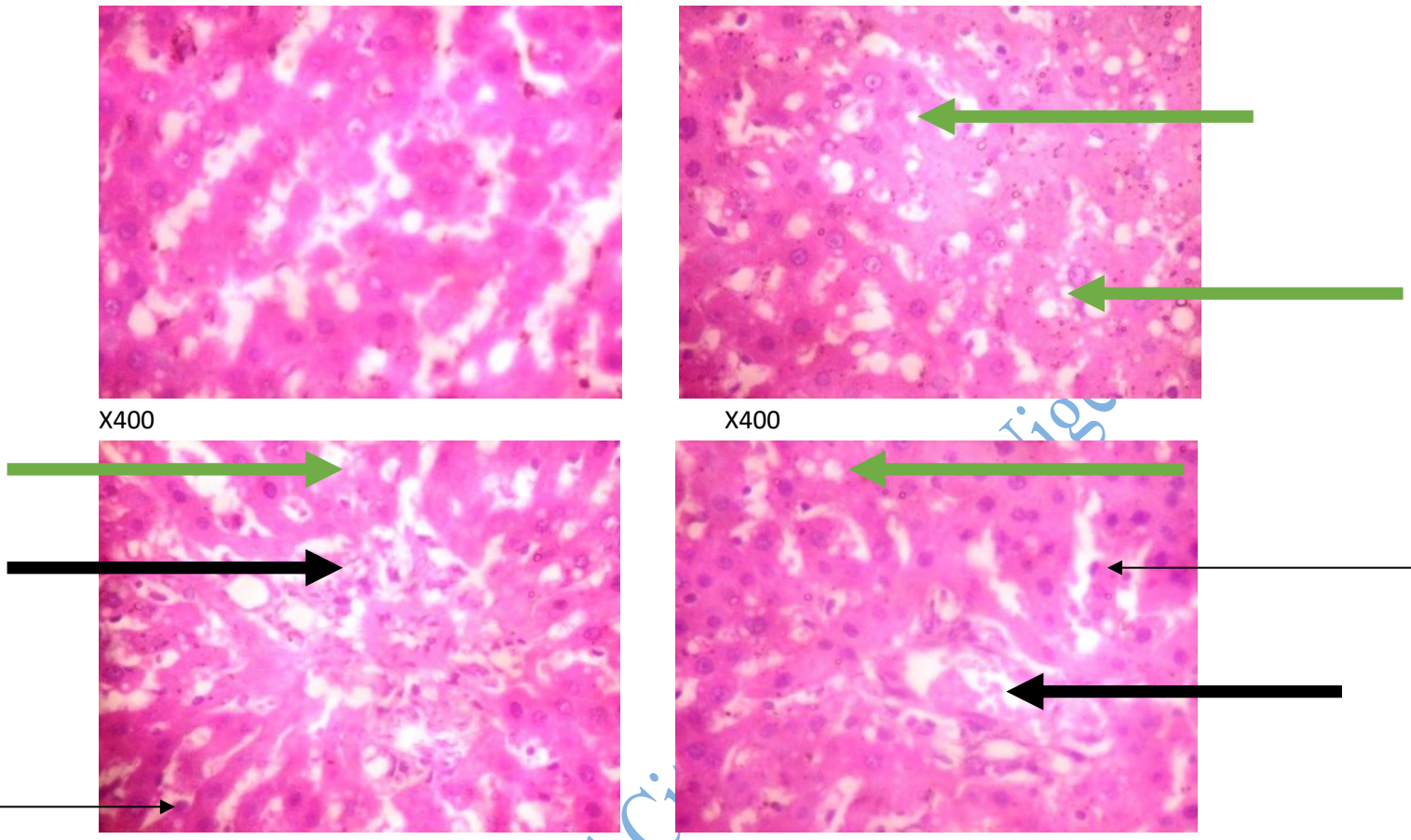
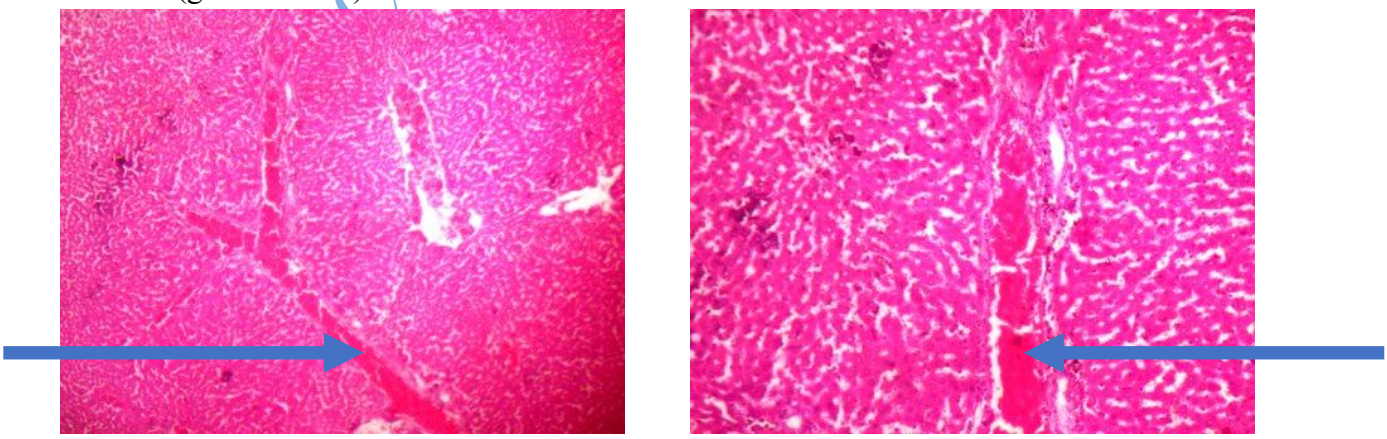


X400 X400

X400 X400

Plate 4.16: Photomicrophs of Thin Sections of Liver Tissues Harvested from Twice Weekly Treated Group I

Plates show mild disseminated infiltration of zone 2 by inflammatory cells (slender arrows), very mild periportal infiltration by inflammatory cells (black arrows) and disseminated microvesicular steatosis (green arrows).



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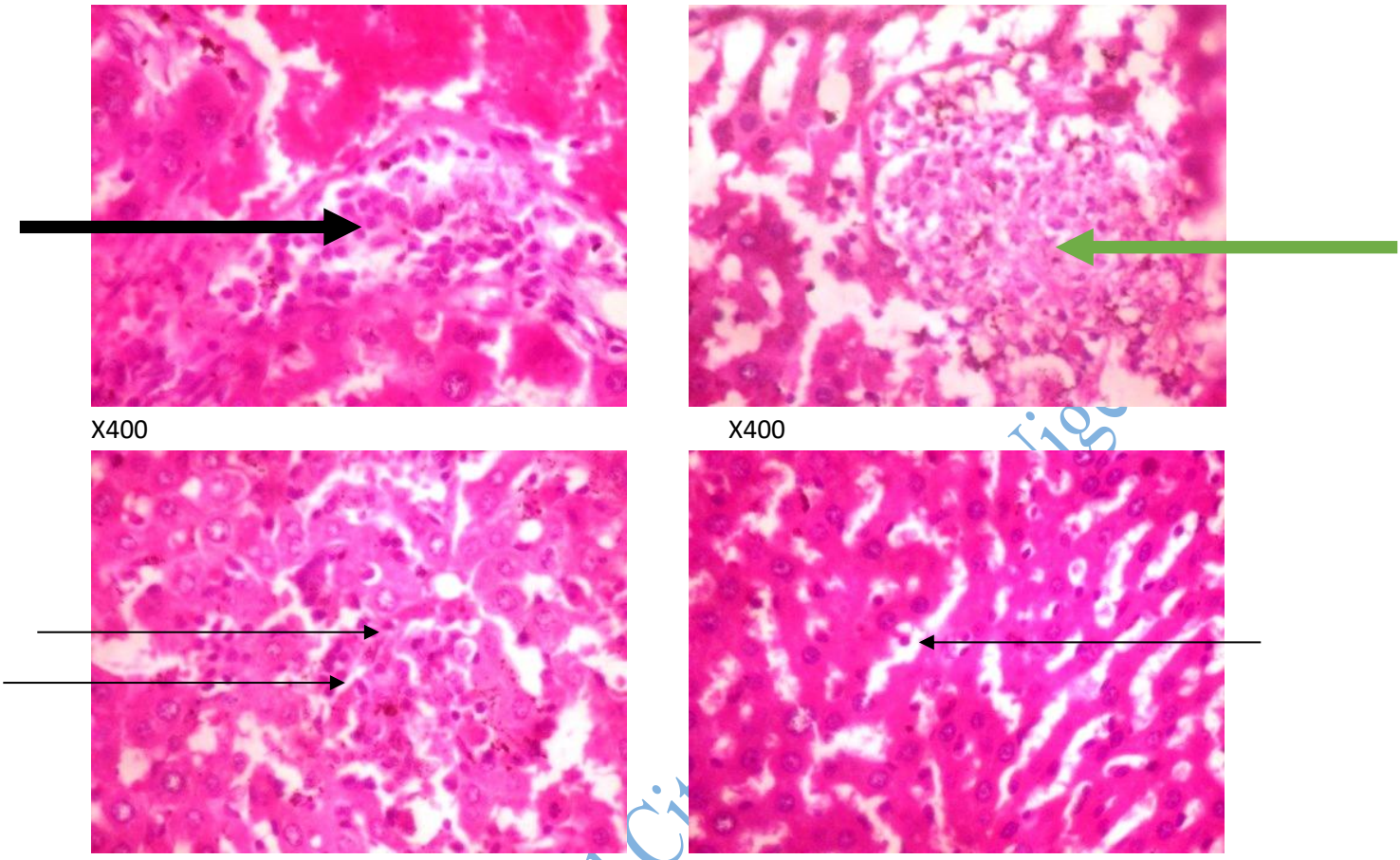


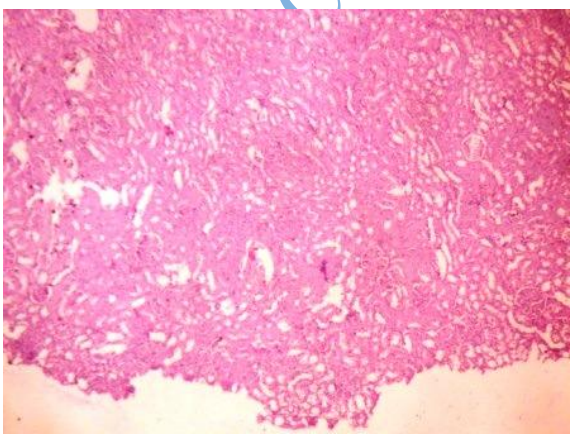
Plate 4.17: Photomicrophs of Thin Sections of Liver Tissues Harvested from Twice Weekly Treated Group II

Plates show marked disseminated congestion (blue arrows), mild disseminated periportal infiltration by inflammatory cells (black arrows), mild disseminated infiltration of zone 2 by inflammatory cells and lymphoid aggregate (slender arrows) and focal area of hepatic nodules (green arrow).

4.6.4 Histopathological Examination of the Kidney

The photomicrograph of the kidney harvested from the treated groups was compared with that from control groups. Tissues from control group showed normal morphological architecture with no significant lesion as shown in plates 4.18 and 4.19. However, the photomicrographs from tissues harvested from rats treated with Postinor 2 once weekly showed extensive tubular necrosis, mild disseminated tubular necrosis, glomerular messangialisation and focal periglomerular inflammation (plates 4.20 and 4.21). Meanwhile, Plates 4.22 and 4.23 from twice

weekly treated group showed marked to severe disseminated tubular necrosis, glomerular messagialisation and necrosis.



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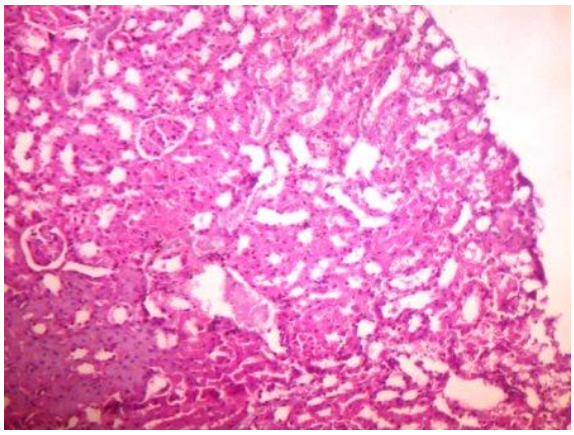

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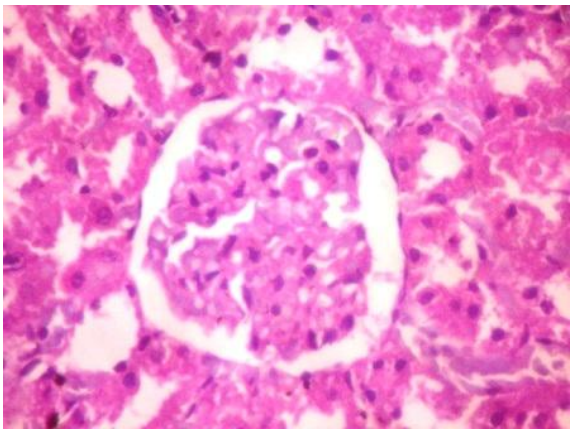

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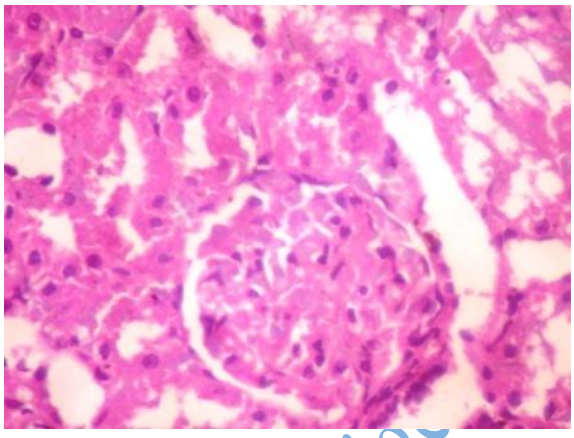

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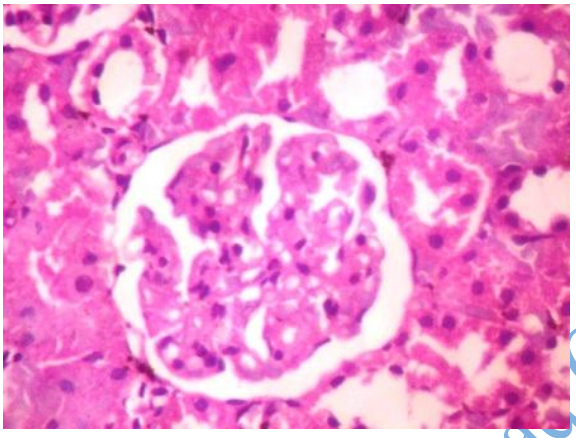

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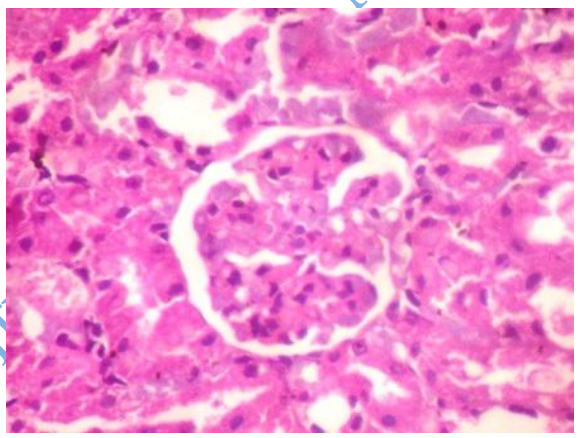

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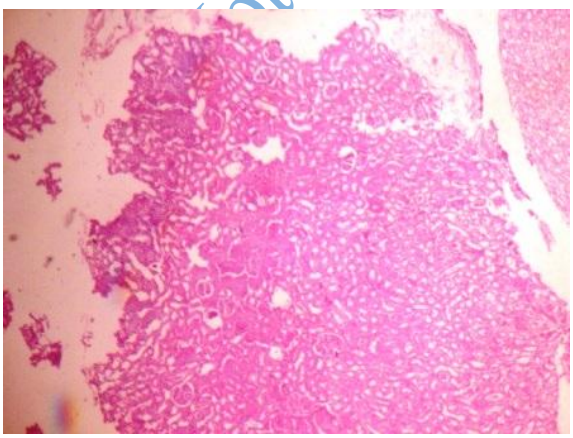
Plate 4.18: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Control Group I

Plates show no significant lesion.



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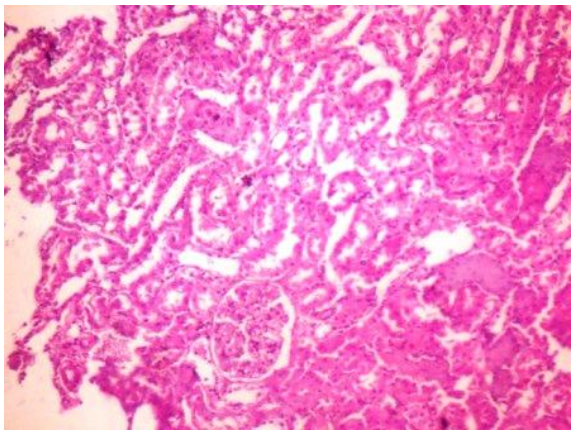

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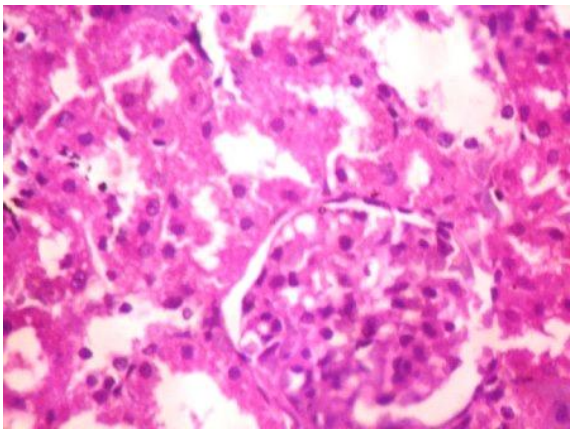

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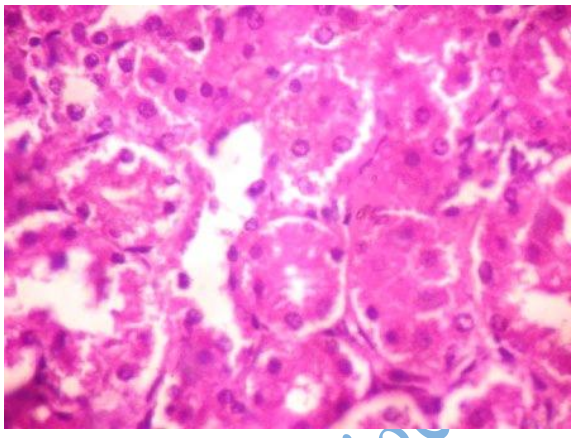

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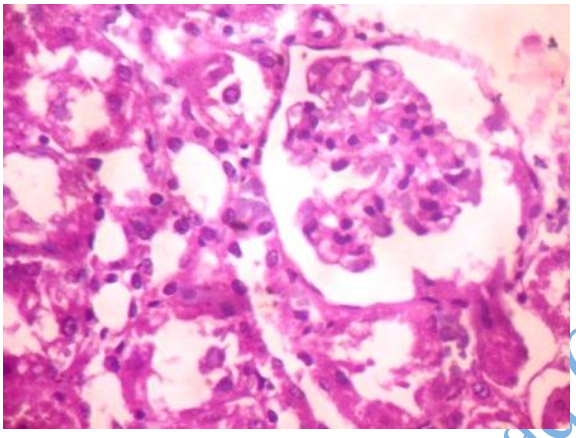


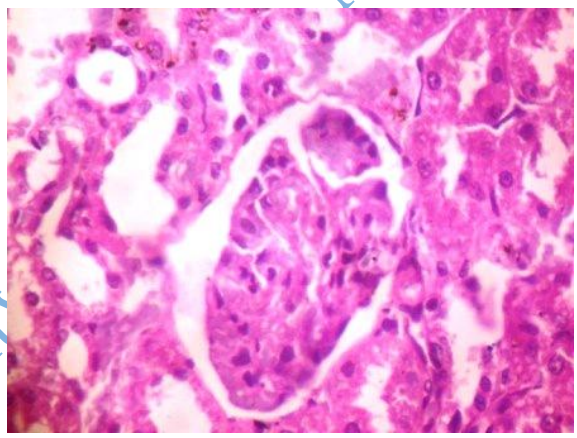
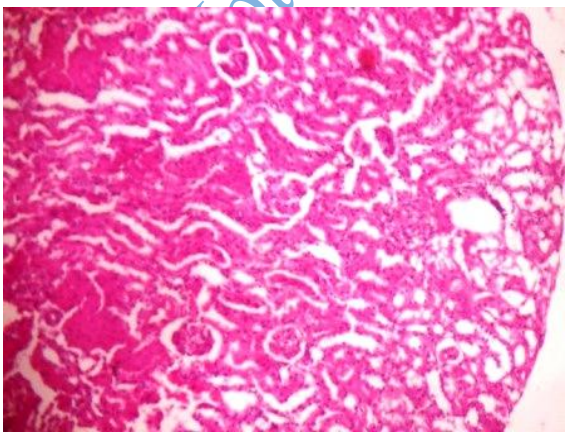


Plate 4.19: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Control Group II

Plates show no significant lesion.



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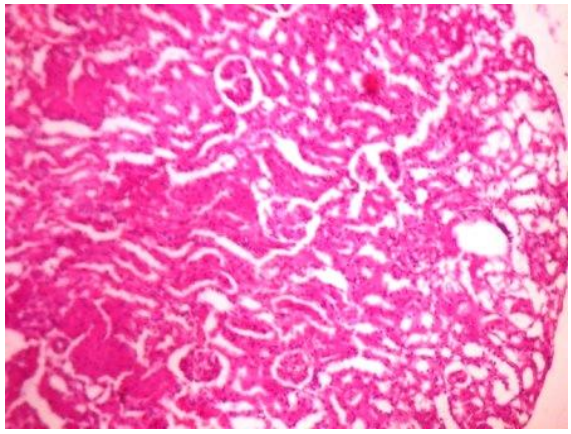

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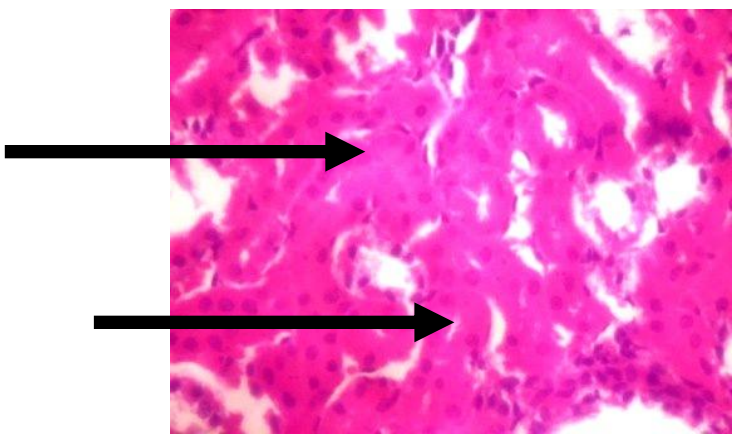

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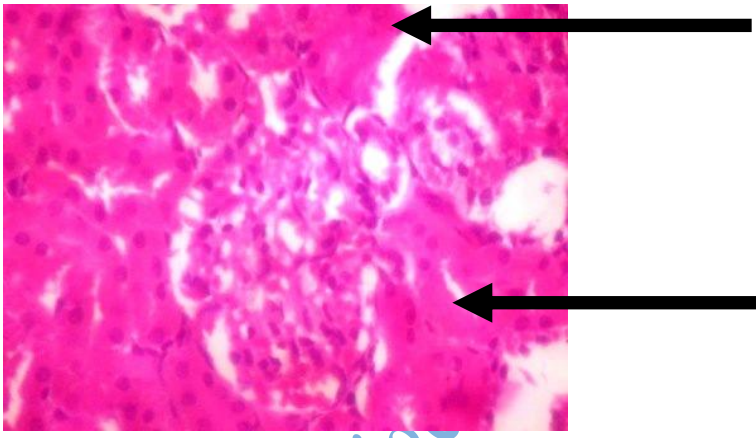

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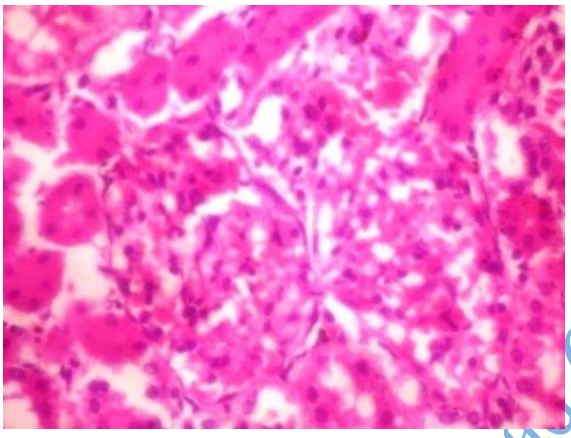

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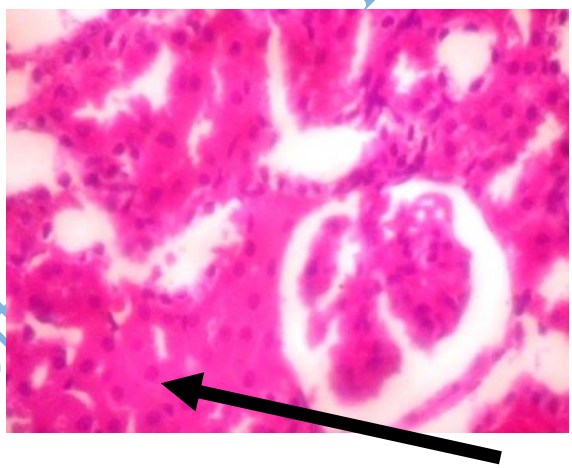

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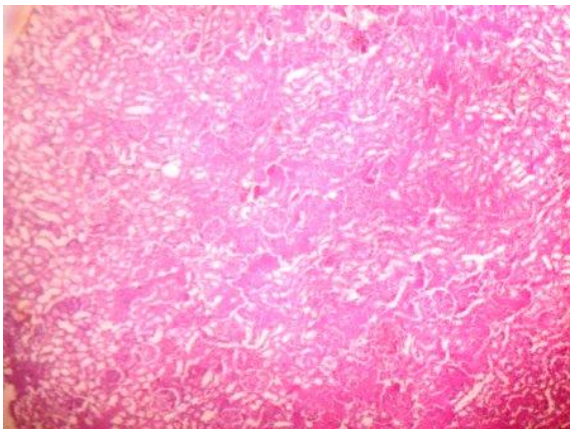
Plate 4.20: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Once Weekly Treated Group I

Plates show extensive tubular necrosis (black arrows).



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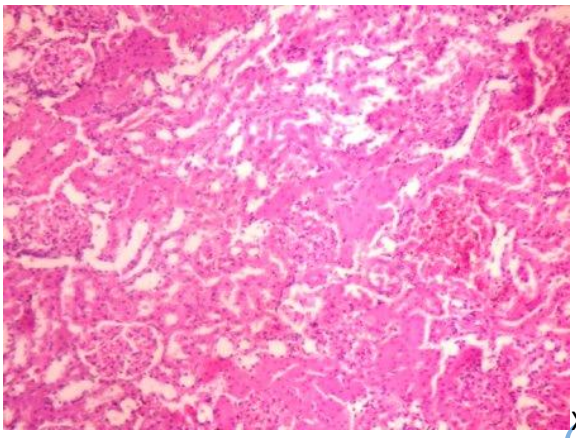

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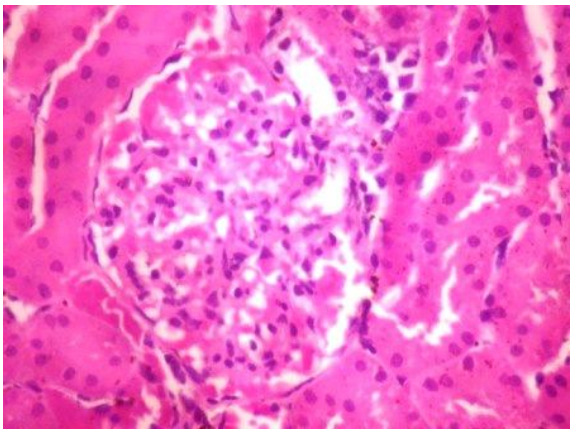

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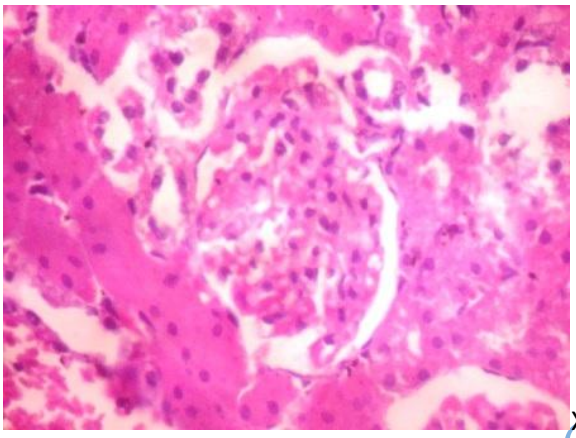

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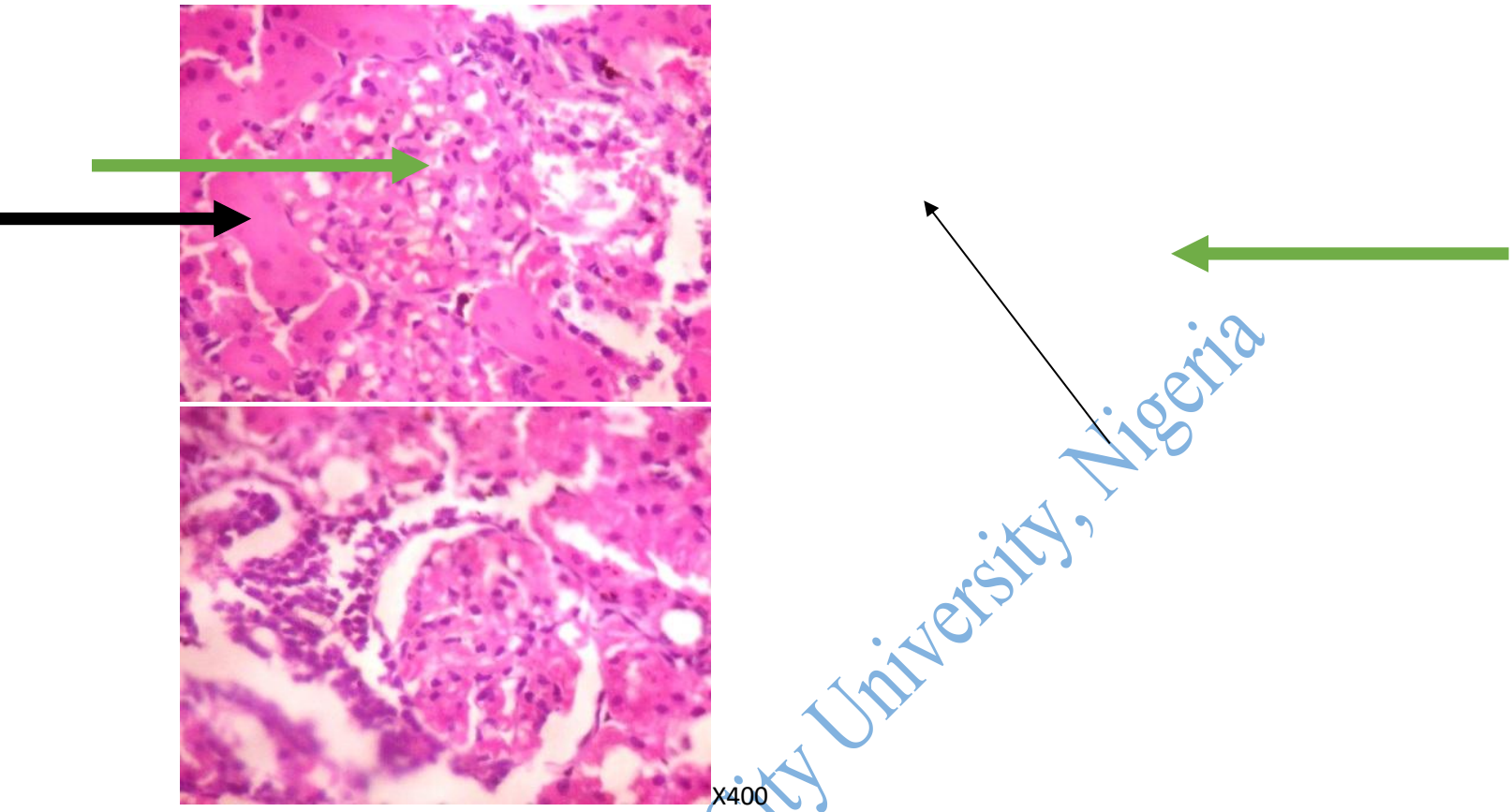
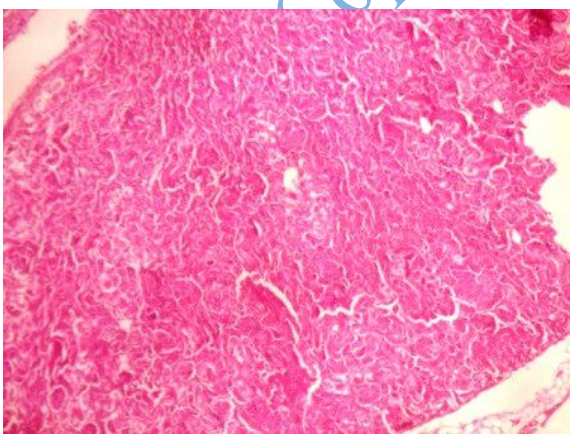


X400

X400

Plate 4.21: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Once Weekly Treated Group II

Plates show mild disseminated tubular necrosis (black arrows), glomerular messangialisation (green arrows) and focal periglomerular inflammation (slender arrows).



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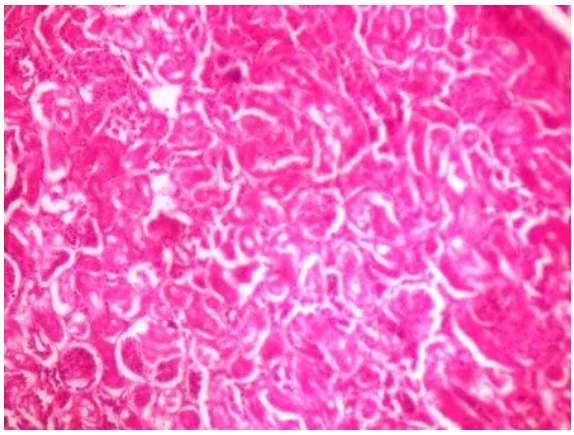

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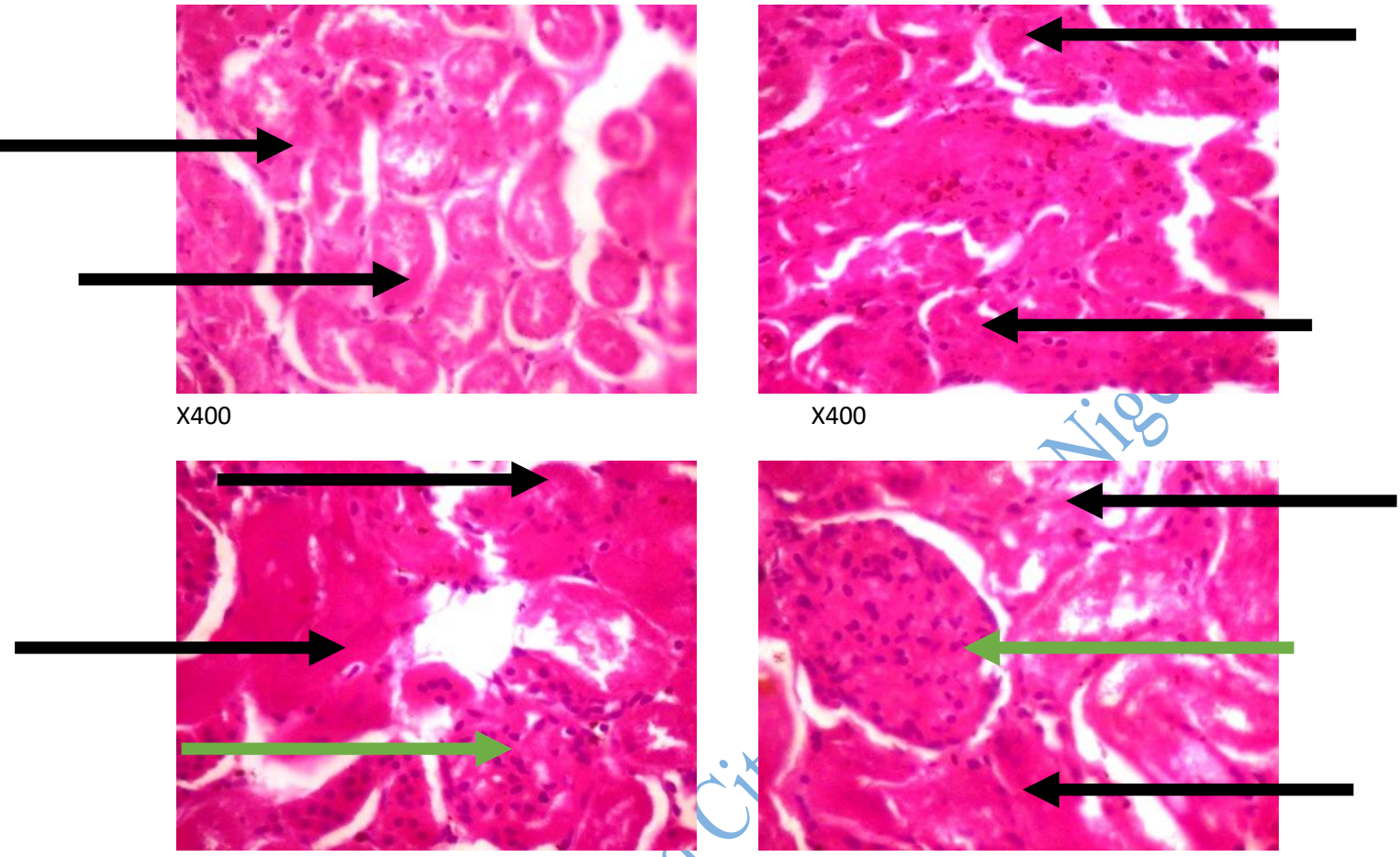
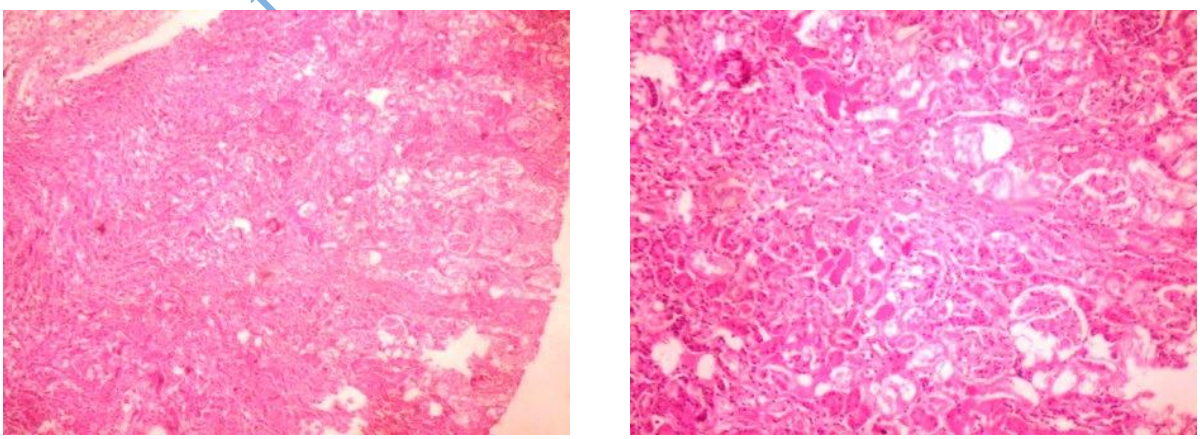


X400 X400

X400 X400

Plate 4.22: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Twice Weekly Treated Group I

Plates show marked to severe disseminated tubular necrosis (black arrows) and glomerular messagialisation/necrosis (green arrows).



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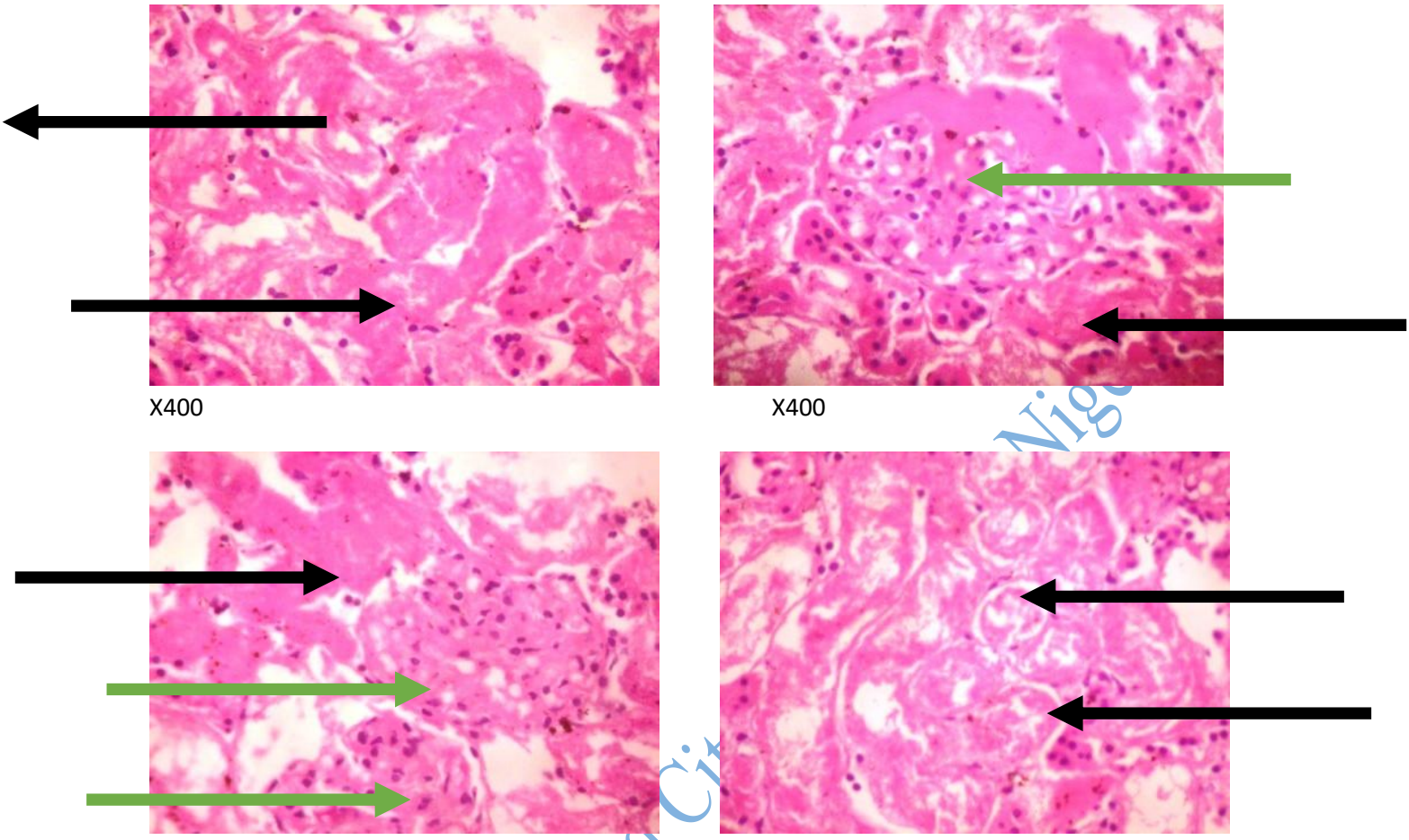

Plate 4.23: Photomicrophs of Thin Sections of Kidney Tissues Harvested from Twice Weekly Treated Group II

Plates show marked to severe disseminated tubular necrosis (black arrows) and glomerular messagialisation/necrosis (green arrows).

4.7 Effect of Postinor 2 Intake on Progesterone (P4) Level in the Serum of Wistar Rats

The concentration of progesterone in the serum of Wistar rats treated with Postinor 2 Once weekly and twice weekly was compared with the control group. The concentration of P4 was significantly lower in the serum of rats treated once weekly (46.9 ± 0.109) and twice weekly (47.1 ± 0.022) only at 60 days of treatment ($p < 0.05$) but no significance was observed in the concentration of P4 in any other month when compared with control as shown in figure 4.12

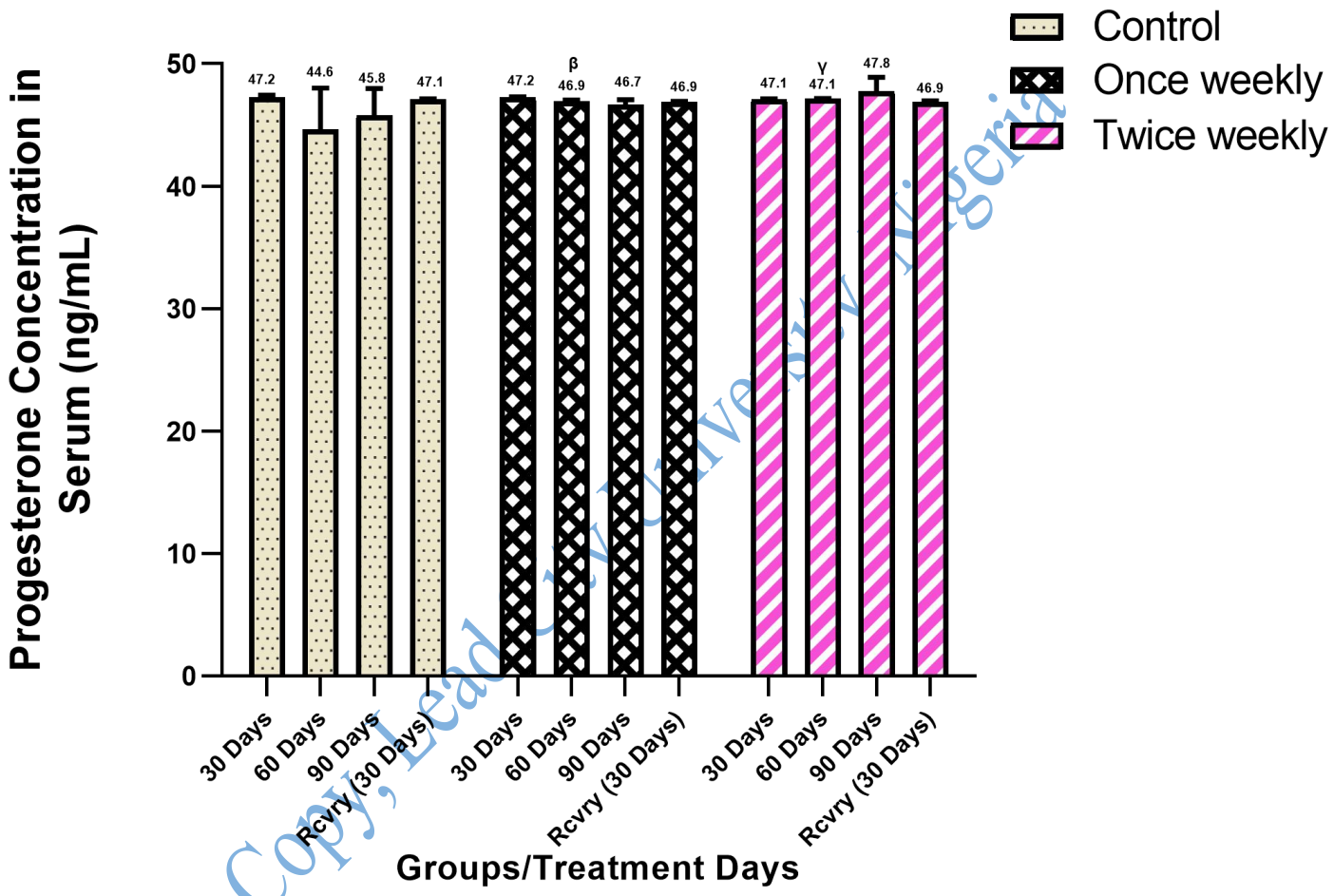

Figure 4.12: Effect on Postinor 2 Intake on the Serum Concentration of Progesterone

Values were expressed as mean ± standard deviation (SD), n = 5. **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly is compared with control ($p < 0.05$).

4.8 Effect of Postinor 2 Intake on Estrogen (E_2) Level in the Serum of Wistar Rats

Figure 4.13 showed the effct of Postinor 2 intake on E2 concentration. At the end of first 30 days of treatment, the concentration of E2 was significantly lower in twice weekly (2356.2 ± 25.4) treated groups than in both once weekly (2595.8 ± 32.4) and control in control group (2696.7 ± 52.4) ($p < 0.05$).

At the end of the 60 days of treatment, the concentration of E2 in the twice weekly group had dropped to 2215.1 ± 54.3 which was still significantly lower than in once weekly (2441.6 ± 59.2) and control (2595.5 ± 47.7) groups ($p < 0.05$). Additionally, when the concentration of E2 in the control was compared with once weekly treated group, there was a significant difference ($p < 0.05$).

By 90 days of treatment, the concentration of E2 had dropped further in twice weekly treated group (1789.5 ± 102.6). The reduction also took place in once weekly (1981.4 ± 46.8) but not as much as in twice weekly and both were significantly lower than the control (2281.2 ± 82.1) and once weekly treated group ($p < 0.05$).

After treatment had stopped for 30 days (30 days recovery period), the concentration of E2 increased in twice weekly treated group by 14 % (1928.6 ± 1.3) which was still significantly lower than in both once weekly treated group (2203.6 ± 9.0) and control (2281.3 ± 82.8) ($p < 0.05$) even though the concentration of E2 in once weekly had also increased by 5 %.

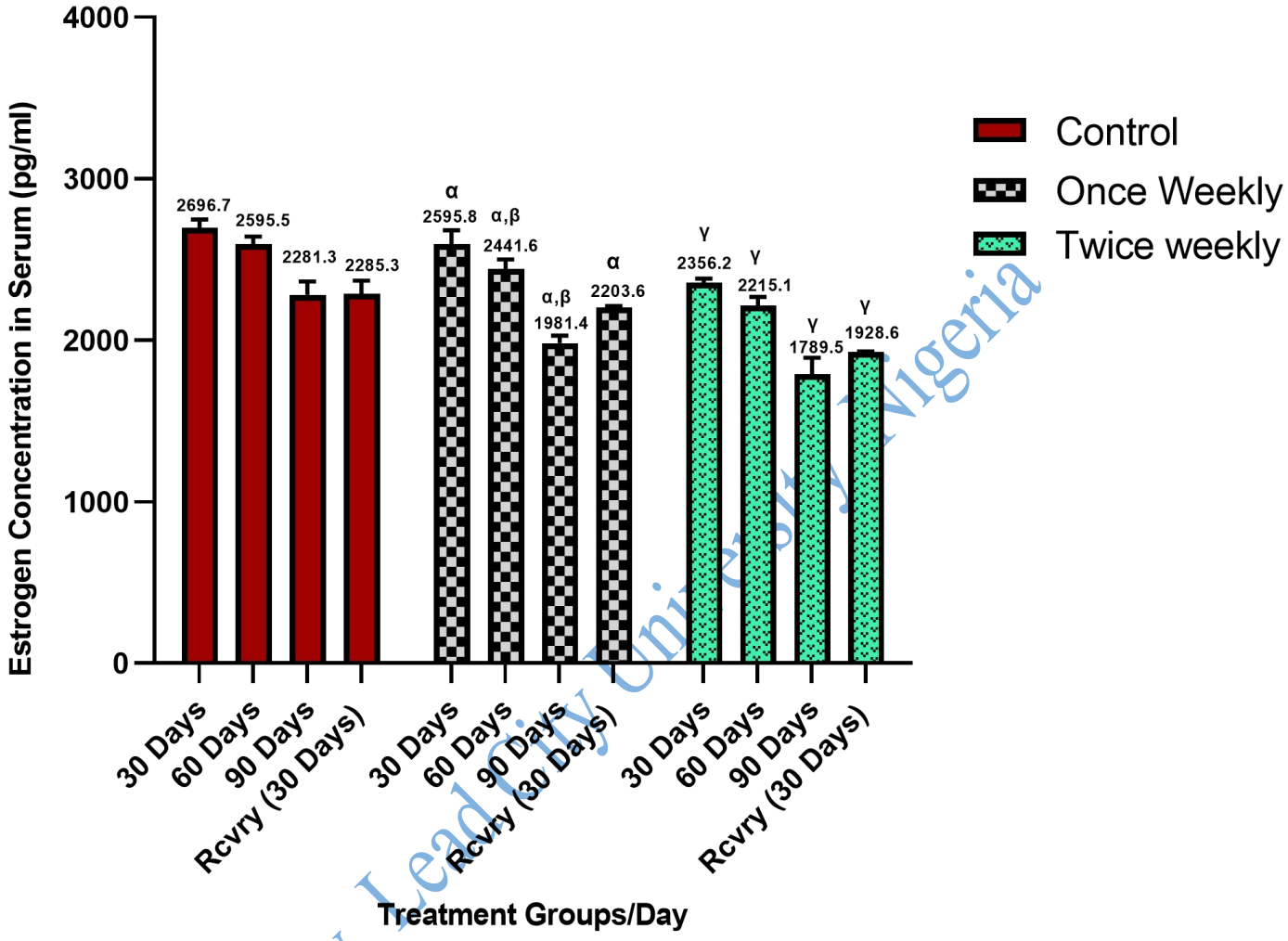

Figure 4.13: Effect of Postinor 2 Intake on the Serum Concentration of Estrogen

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.9 Effect of Postinor 2 Intake on Serum Concentration of Luteinizing Hormone (LH)

The effect on Postinor 2 intake on LH concentration was also assessed in the treated groups and compared with control as shown in figure 4.14. It was observed at the end of the first month of treatment that the concentration of LH was within the same range across all groups ($p > 0.05$). However, at the end of the second month, the concentration of LH had significantly increased in the group treated twice weekly from 68.0 ± 1.7 to 144.1 ± 0.8 which is above a 100 % while there was a decrease in that of control and once weekly treated groups from 63.5 ± 4.1 and 64.2 ± 0.9 to 56.9 ± 6.2 and 53.1 ± 1.3 respectively ($p < 0.05$). There existed no significant difference between once weekly and control groups ($p > 0.05$).

90 days of treatment further increased the concentration of LH in twice weekly group to 160.1 ± 1.5 which was still significantly higher than what was observed in both control (68.3 ± 0.9) and once weekly (58.3 ± 3.1) groups ($p < 0.05$).

Recovery was observed in LH concentration as a halt in the treatment for 30 days brought about a 19% decrease in the concentration of LH in twice weekly treated groups to 108.1 ± 3.2. Despite the decrease it was still significantly higher than the concentration LH in control (64.8 ± 4.9) and once weekly (51.7 ± 1.0) groups ($p < 0.05$). Also, the control group had a significantly higher concentration of LH than the once weekly group ($p < 0.05$).

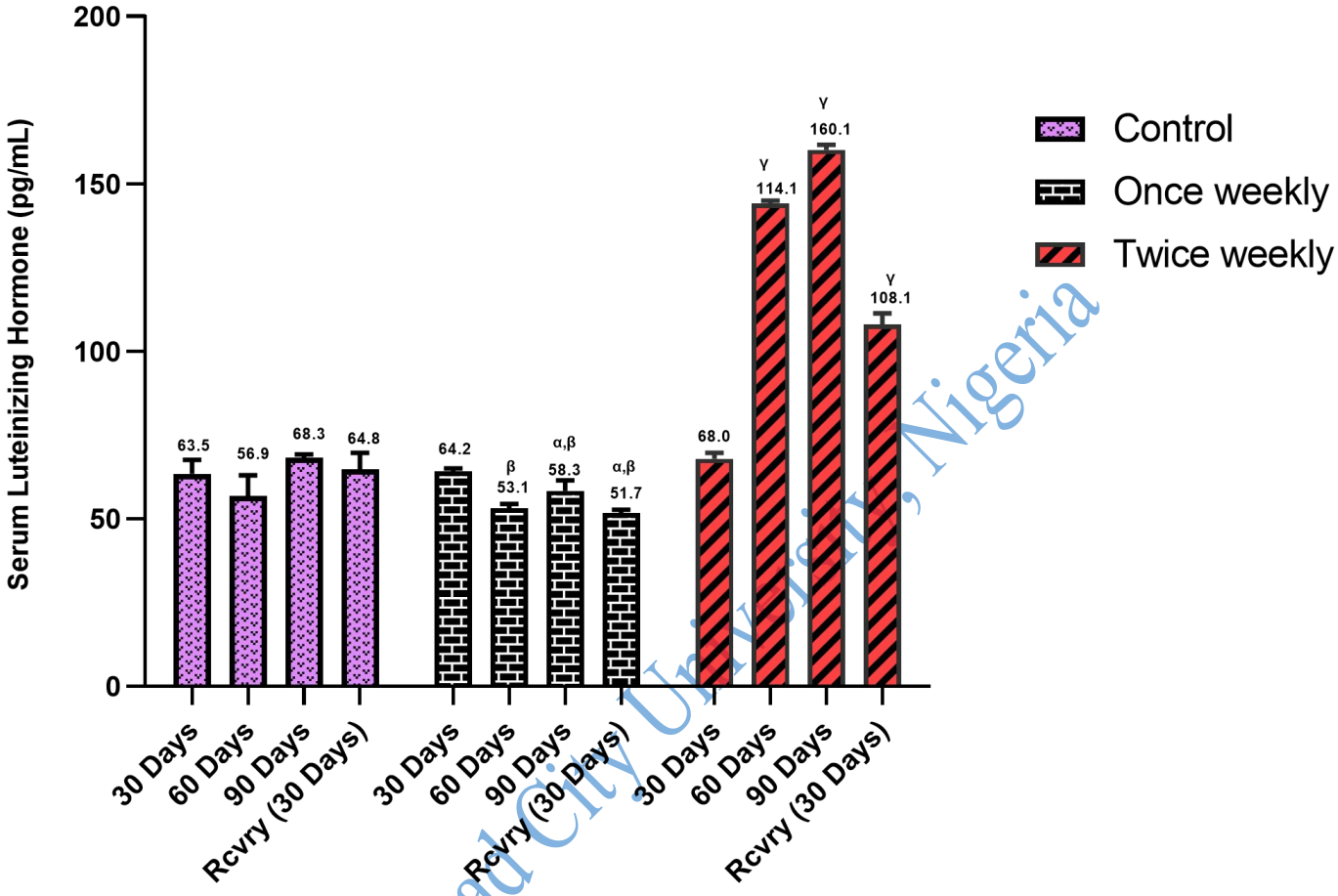

Figure 4.14: Effect of Postinor 2 Intake on the Serum Concentration of Luteinizing Hormone

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.10 Effect of Postinor 2 Intake on the Serum Concentration of Follicle Stimulating Hormone (FSH) of Wistar Rats

The effect of Postinor 2 on the concentration of FSH was also determined in the treated groups and compared with control as shown in figure 4.15. The first 30 days showed a marked elevation in the concentration of FSH in the twice weekly group (9.4 ± 0.5) than in both once weekly (6.2 ± 0.8) and control (6.0 ± 0.6). This elevated concentration was significant when compared with once weekly and control ($p < 0.05$). Conversely, the difference in the concentration between once weekly and control was not significant ($p = 0.0004$).

By 60 days, the control group concentration of FSH (5.9 ± 0.4) was significantly lower than in twice weekly (10.1 ± 0.5) ($p < 0.05$) and lower but again insignificant when compared with once weekly group (6.7 ± 0.4) ($p = 0.0994$). The difference in once weekly and twice weekly treated groups was also significant ($p < 0.05$).

Furthermore, in the third month of treatment, FSH concentration in the twice weekly treated group had increased to 12.2 ± 0.7 which was 16.7 % higher than what was observed in once weekly (8.7 ± 0.4) and about 23 % higher than what was observed in control (7.6 ± 0.4). These differences were statistically significant ($p < 0.05$).

30 days recovery period decreased the concentration of FSH in twice weekly group by 18 % to 8.4 ± 0.8 which was still significantly higher than once weekly (7.9 ± 0.67). The control (7.5 ± 0.43) was significantly lower when compared with both once weekly and twice weekly ($p < 0.05$).

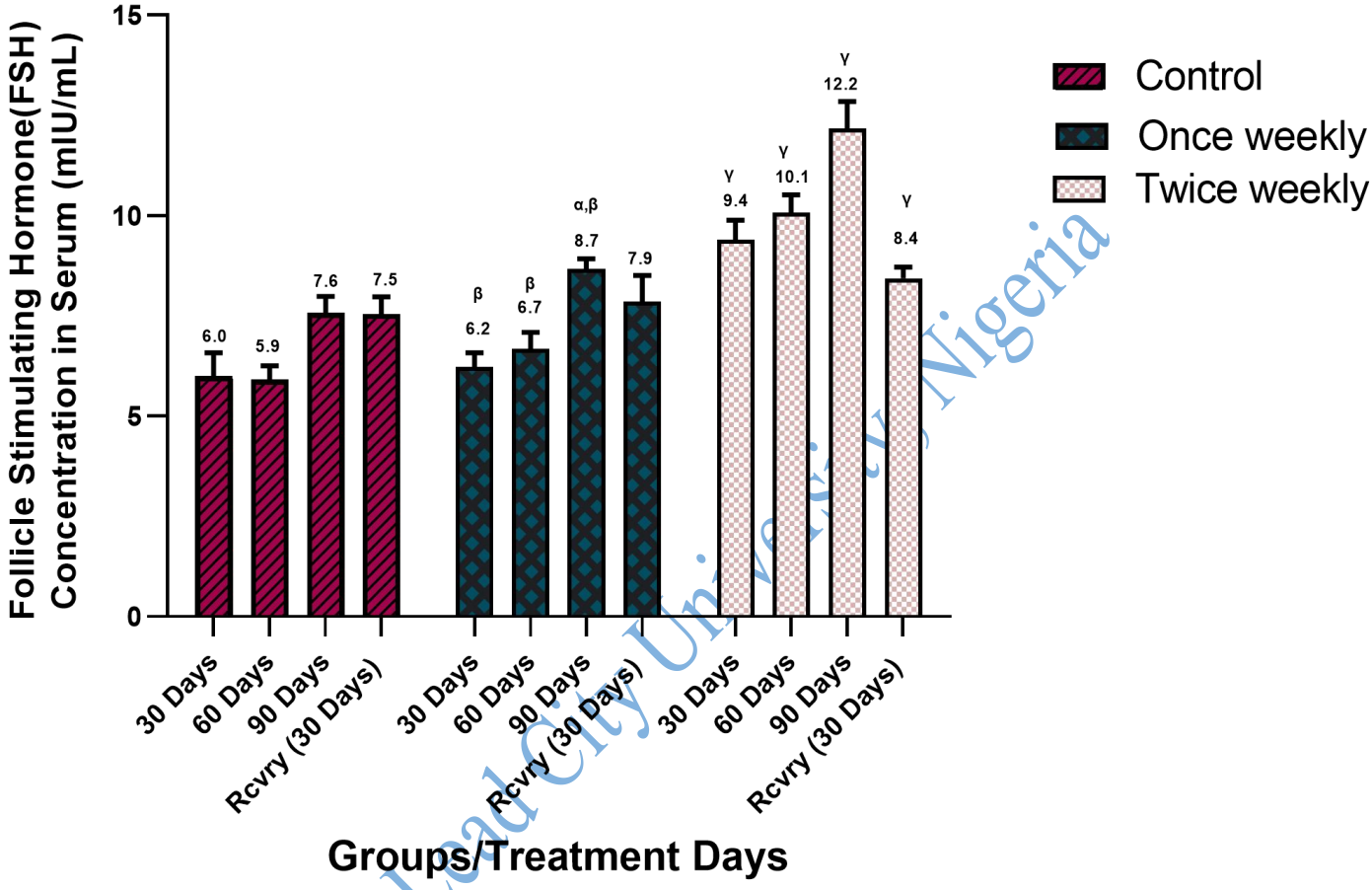

Figure 4.15: Effect of Postinor 2 Intake on the Serum Concentration of Follicle Stimulating Hormone

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.11 Effect of Postinor 2 Intake on the Serum Concentration of Progestagen Associated Endometrial Protein (PAEP) of Wistar Rats

As shown in figure 4.16, the concentration of PAEP was significantly lower in twice weekly treated group (1.7 ± 0.1) when compared with control (1.9 ± 0.1) and once weekly (1.8 ± 0.1) and was observed to keep decreasing with time and even in post treatment days ($p < 0.05$). The concentration of PAEP in once weekly was lower than in control but not significant ($p = 0.0683$). The decrease in the concentration of PAEP by the end of 60 days of treatment had reduced to (1.8 ± 0.1) in twice weekly group and to (1.6 ± 0.1) in once weekly. Meanwhile the concentration had increased in control group to (2.1 ± 0.1). A comparison among all the groups showed a significant difference between control and once weekly ($p = 0.0001$) as well as between control and twice weekly ($p < 0.05$). A significant difference was also observed ($p = 0.0002$) between once and twice weekly.

After 90 days of treatment, PAEP concentration in the control group (2.2 ± 0.1) was significantly higher than in both once weekly (1.8 ± 0.1) and twice weekly (1.3 ± 0.1) ($p < 0.05$). Consequently, a significant difference between once weekly and twice weekly ($p < 0.05$) was also observed.

There was recovery only in once weekly with observed increase in the concentration of PAEP to 1.8 ± 0.1 while it decreased further in twice weekly to (1.2 ± 0.1). When these were compared with the concentration of PAEP in control (2.1 ± 0.1), they were both significantly lower ($p < 0.05$). A significant difference was also observed ($p < 0.05$) between once and twice weekly.

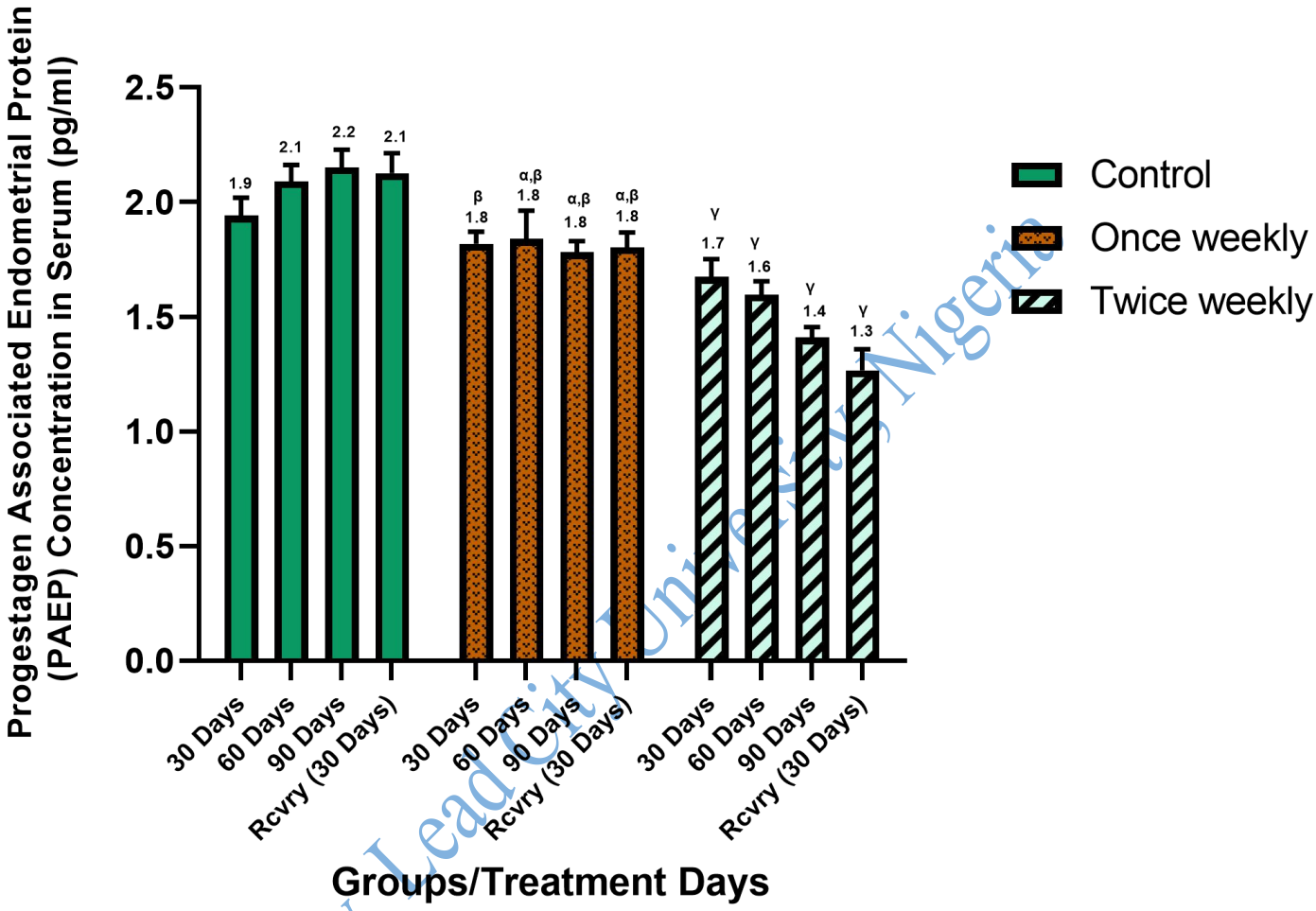

Figure 4.16: Effect of Postinor 2 Intake on the Serum Concentration of Progestagen Associated Endometrial Protein (PAEP)

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.12 Effect of Postinor 2 Intake on the Serum Concentration of Leukemia Inhibitory Factor (LIF)

The serum concentration of LIF in the treated group was also determined and compared with the control as illustrated in figure 4.17. The first 30 days of treatment did not show any difference in LIF concentration in once weekly while it was significantly lower in twice weekly. At 60 and 90 days, a significantly lower LIF than in the control was observed in once weekly group ($p < 0.05$), yet LIF in twice weekly group was significantly lower than both the control and once weekly groups. The post treatment period brought about a 3 % increase in the concentration of LIF in once weekly while the level of LIF was maintained in twice weekly, despite this, they were both significantly lower than control ($p < 0.05$).

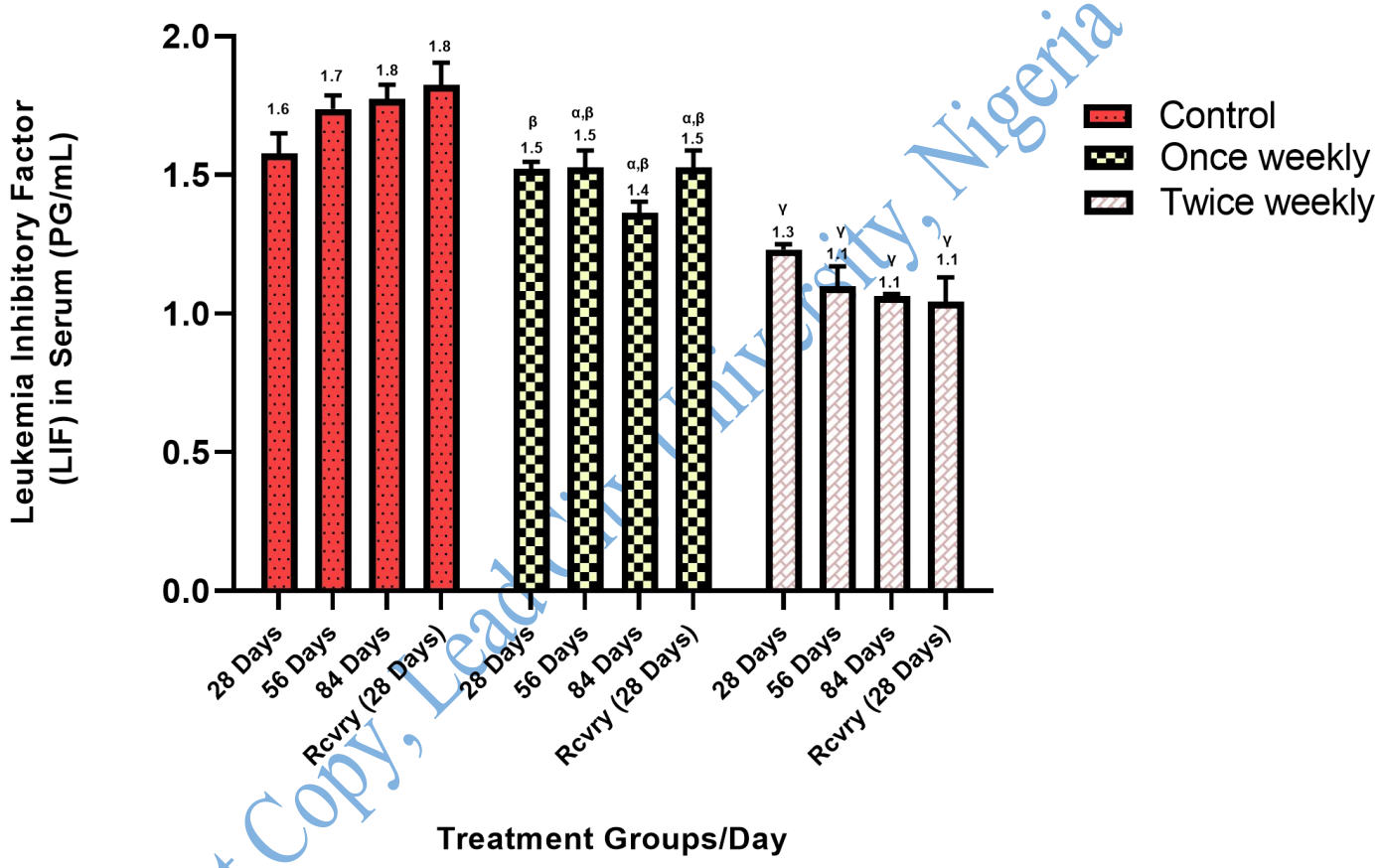

Figure 4.17: Effect of Postinor 2 Intake on the Serum Concentration of Leukemia Inhibitory Factor (LIF)

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.13 Effect of Postinor 2 Intake on the Serum Concentration of Prolactin (PRL)

The effect of treatment on the concentration of PRL is shown in figure 4. 18. At 30 days of treatment, the concentration of prolactin was significantly higher ($p < 0.05$) in control group (4.2 ± 0.2) when compared with twice weekly (3.2 ± 0.1) treated groups. The concentration of PRL was significantly lower in the twice weekly treated group compared to once weekly (4.1 ± 0.2) group ($p < 0.05$). Albeit, there is no significant difference in the concentration of control and once weekly ($p = 0.6122$).

At the end of the 60 days of treatment, the concentration of PRL in the twice weekly group had increased to 3.8 ± 0.3 which was still significantly lower than the control (4.4 ± 0.2) group ($p < 0.05$). Additionally, when the concentration of control was compared with once weekly treated group, there existed a difference that was not significant ($p > 0.05$). So also, was the difference between twice weekly and once weekly (4.2 ± 0.3).

The concentration of PRL decreased in twice weekly treated group (3.2 ± 0.1), by the end of the third month of treatment and was still significantly lower than both control (5.4 ± 0.1) and once weekly treated group which also had reduced to 3.9 ± 0.2 ($p < 0.05$). It was only at this time that the control group became significantly higher than once weekly in their concentration of PRL ($p < 0.05$).

After treatment had stopped for 30 days (30 days recovery period), the concentration of PRL slightly decreased in twice weekly treated group (3.2 ± 0.3) which was significantly lower than in once weekly where PRL had increased to 4.8 ± 0.3 ($p < 0.05$). Prolactin concentration was still significantly higher in the control group (5.7 ± 0.2) than in once weekly and twice weekly ($p < 0.05$).

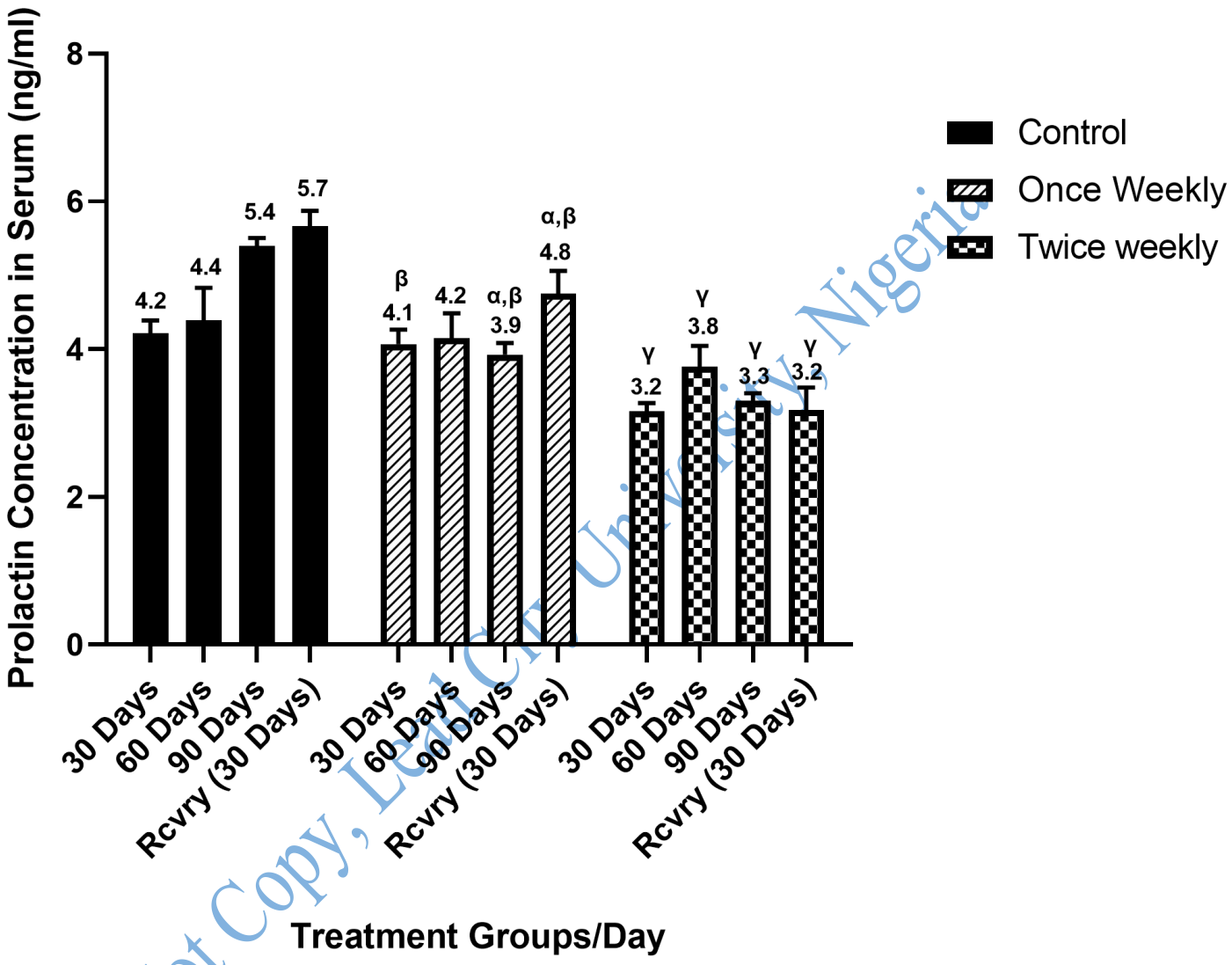

Figure 4.18: Effect of Postinor 2 Intake on the Serum Concentration of Prolactin

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.14 Effect of Postinor 2 Intake on the Concentration of Sex Hormone-Binding Globulin (SHBG) in the Serum

The effect on Postinor 2 intake on SHBG was also assessed and shown in figure 4.19. It was observed at the end of the first 30 days of treatment that the concentration of SHBG in the control group (1.3 ± 0.1) was similar to what was observed in once weekly (1.3 ± 0.1) but significantly higher than in twice weekly (1.2 ± 0.1) ($p = 0.0013$).

However, at the end of 60 days, the concentration of SHBG in once weekly has decreased to (1.2 ± 0.0), which was still insignificantly lower control group (1.3 ± 0.1) ($p = 0.4044$). However, SHBG in the control group was significantly higher than in twice weekly (0.8 ± 0.0) ($p < 0.05$). A comparison between once and twice weekly treated groups also showed a significant difference ($p < 0.05$).

By 90 days of treatment, the concentration of SHBG in control group (1.2 ± 0.0) was significantly higher than what was measured in both once weekly (1.1 ± 0.0) and twice weekly (0.8 ± 0.0) groups ($p < 0.05$). A comparison between once and twice weekly treated groups also illustrated a significant difference ($p < 0.05$).

A halt in the treatment for 30 days still showed SHBG in control (1.3 ± 0.0) to be significantly higher than both once weekly (1.0 ± 0.1) and twice weekly treated groups (0.8 ± 0.1) ($p < 0.05$) Additionally, SHBG concentration in once weekly was significantly higher than in twice weekly ($p < 0.05$).

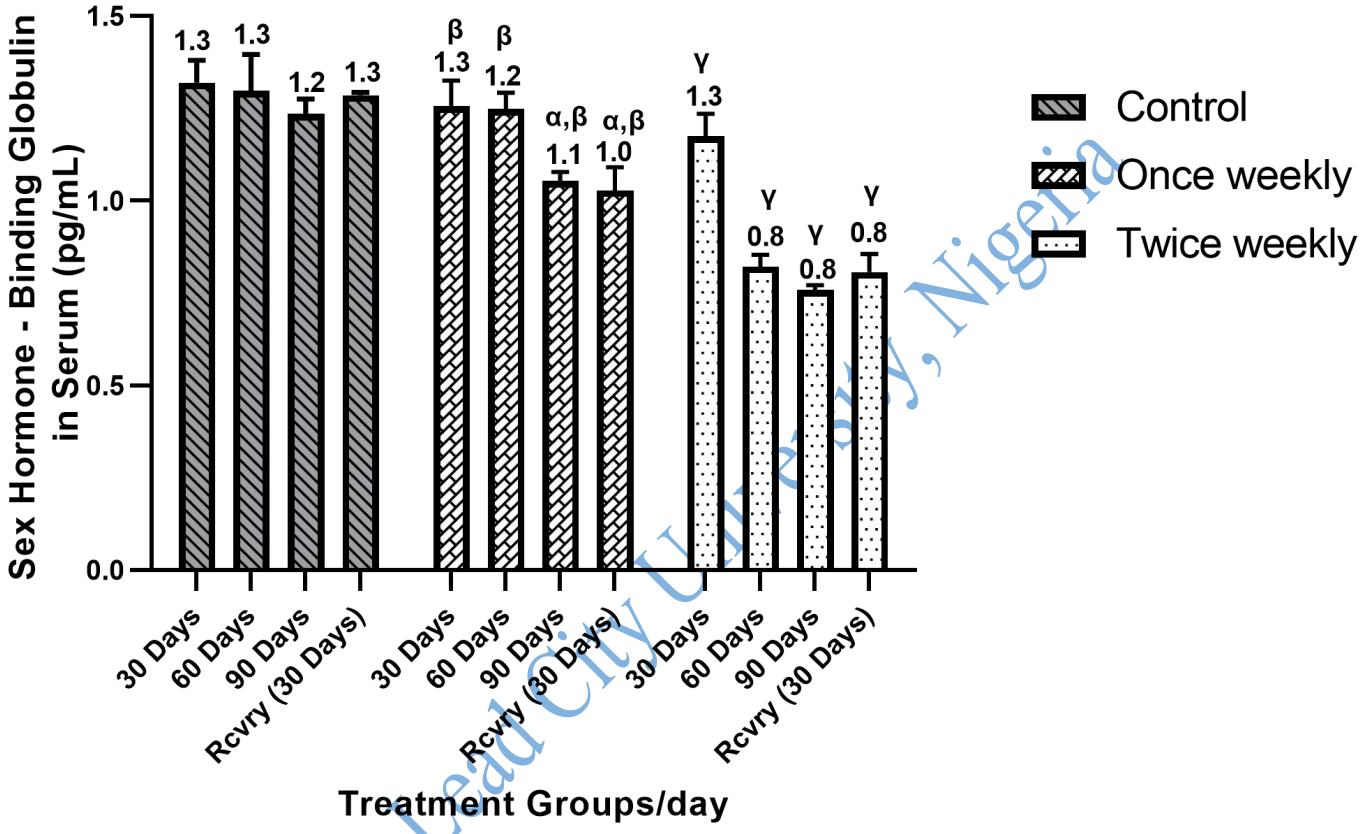

Figure 4.19: Effect of Postinor 2 Intake on the Serum Concentration of Sex Hormone-Binding Globulin

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.15 Effect of Postinor 2 Intake on the Concentration of Nuclear Factor -Kappa B (NF-kB) in the Serum and Reproductive Organs of Wistar Rats

The concentration of NF-kB was measured in the serum (figure 4.20a), uterus (figure 4.20b) and ovary (figure 4.20c) of the Wistar rats treated with Postinor 2 and compared with control. In the serum, treatment for the first 30 days showed a significantly higher concentration of NF-kB in twice weekly than what was seen in control group ($p < 0.05$) and in once weekly treated groups. At the end of the 60 days of treatment, the concentration of NF-kB in the twice weekly group had increased to 1.8 ± 0.3 which was higher significantly than the similar concentration observed in the control group and once weekly (1.7 ± 0.3 and 1.7 ± 0.4 respectfully) ($p < 0.05$).

The concentration of NF-kB had increased in twice weekly treated group (2.1 ± 0.1), by the end of the third month of treatment, and still higher than once weekly treated group (1.7 ± 0.2) and control (1.6 ± 0.2) ($p = 0.05$).

In the uterus and ovary, there was a dose- and time-dependent increase in NF-kB concentration. After treatment had stopped for 30 days (30 days recovery period), the concentration of NF-kB still significantly higher in twice weekly treated group than in once weekly where NF-kB had decreased to $1.5.0 \pm 0.3$ and control ($p < 0.05$). This is similar to what took place in the ovary except that NF-kB reduce more in once weekly than twice weekly. Meanwhile, there was no improvement in the level of NF-kB in the uterus.

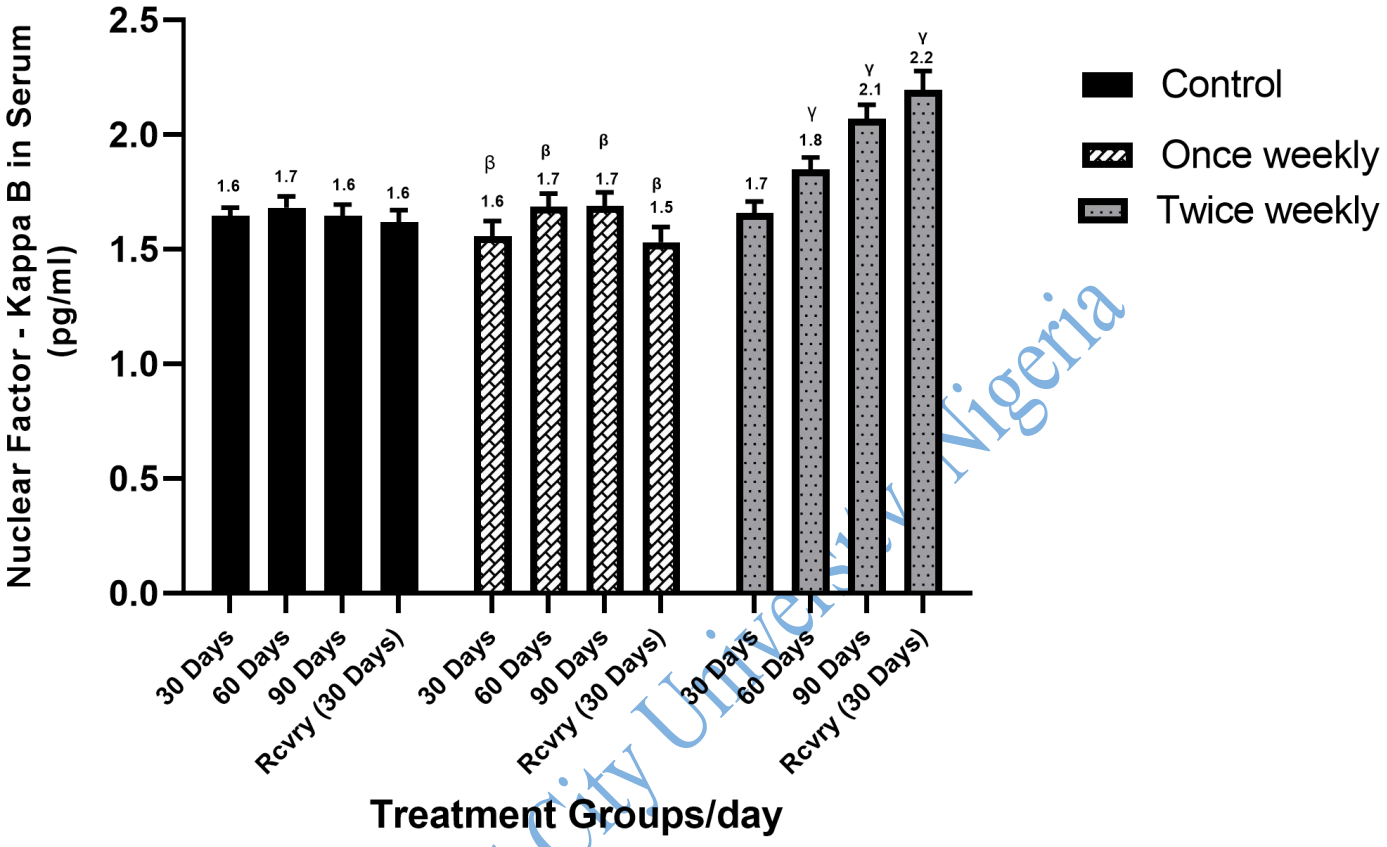

Figure 4.20a: Effect of Postinor 2 Intake on the Serum Concentration of Nuclear Factor - Kappa B

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

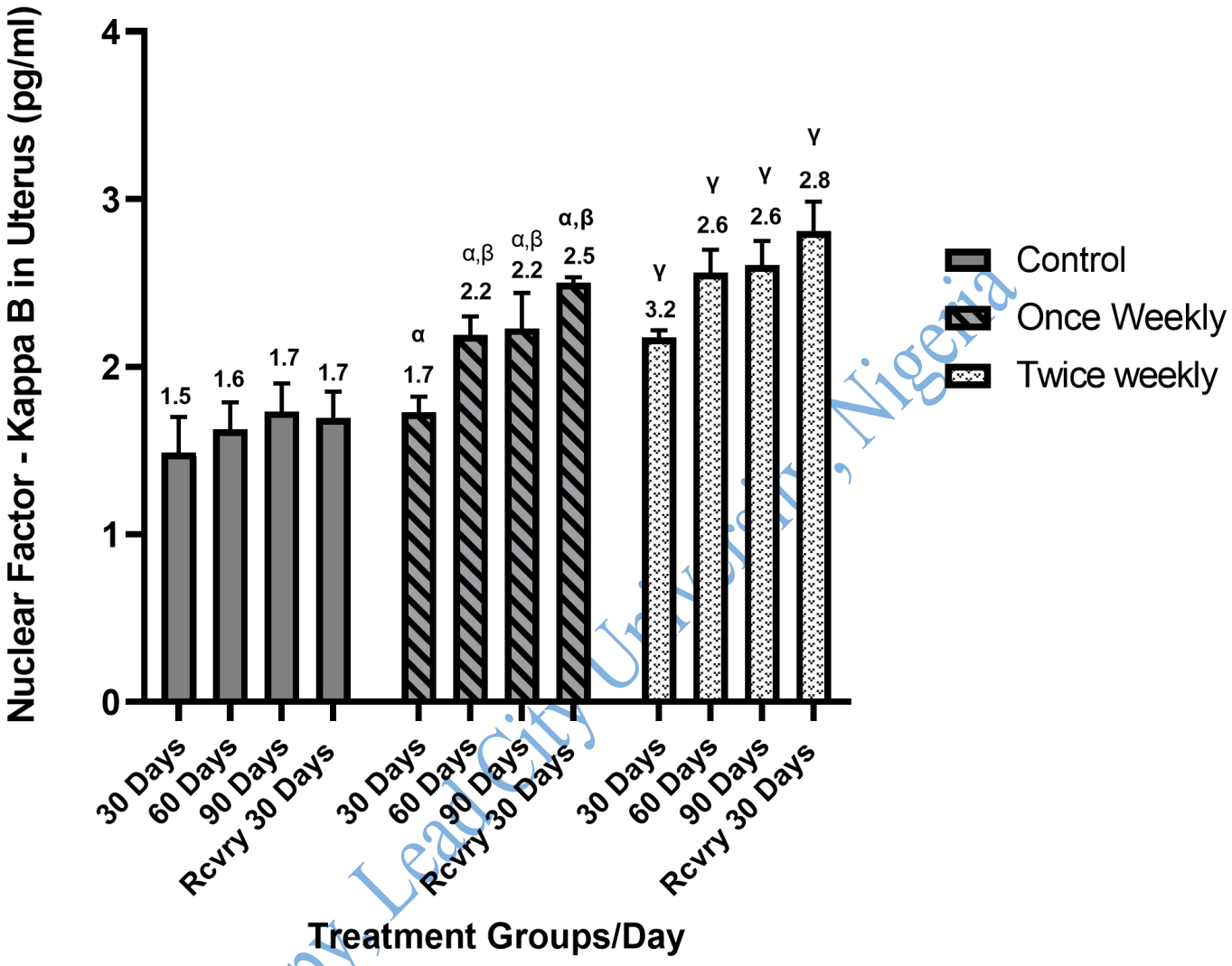

Figure 4.20b: Effect of Postinor 2 Intake on the Concentration of Nuclear Factor -Kappa B in the Uterus of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical

significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

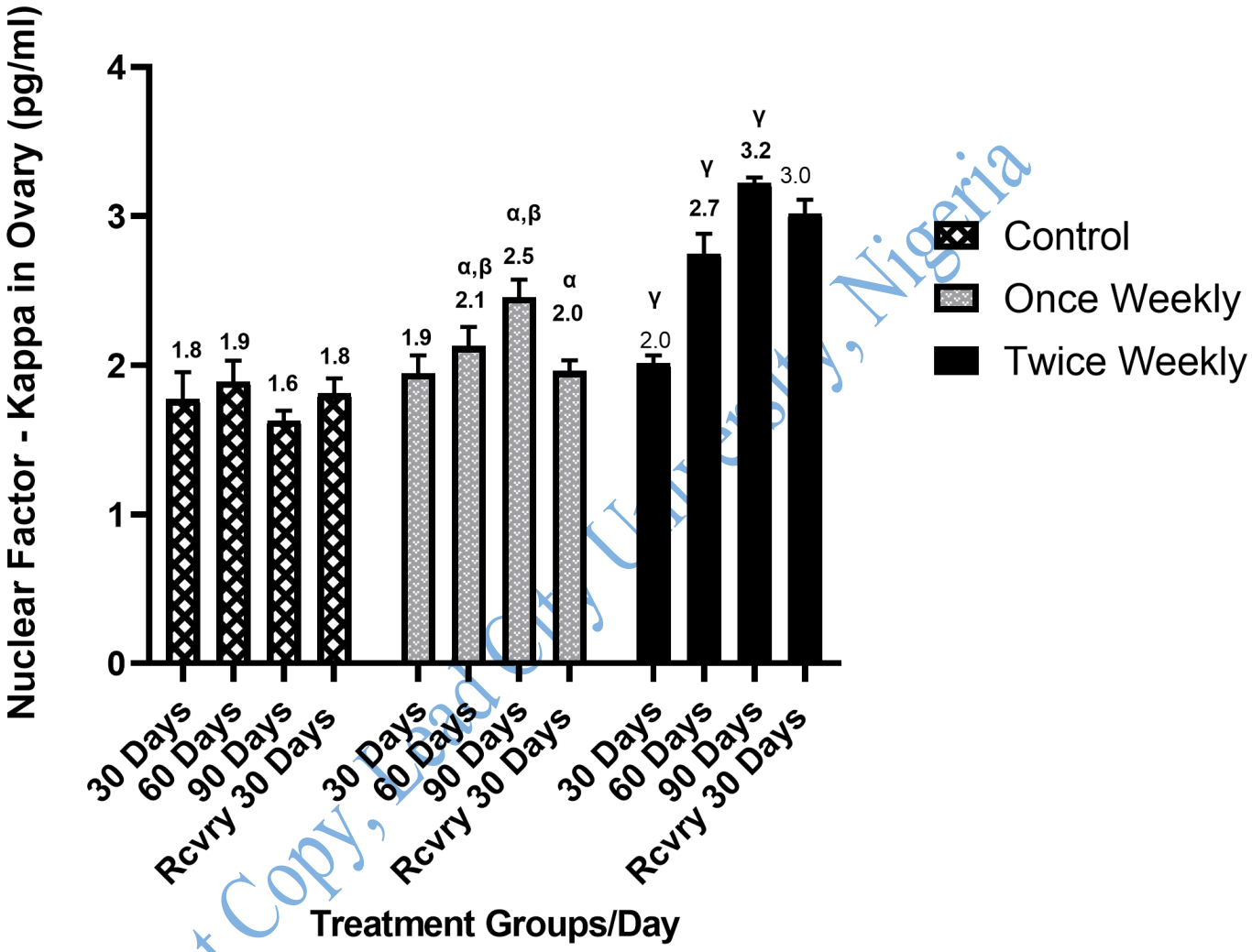

Figure 4.20c: Effect of Postinor 2 Intake on the Concentration of Nuclear Factor -Kappa B in the Ovary of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical

significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **y** showed significance when twice weekly was compared with control ($p < 0.05$).

4.16 Effect of Postinor 2 Intake on the Concentration of Tumor Necrosis Factor -Alpha (TNF-α) in the Serum and Reproductive Organs of Wistar Rats

As shown in figure 4.21a, after 30 days of treatment, there was no significance in the difference in the concentration of TNF-α when a comparison was done between the control group in the serum (3.5 ± 0.3) and both once weekly (3.7 ± 0.2) ($p = 0.2440$) and twice weekly (3.7 ± 0.1) ($p = 0.4683$). Furthermore, no significance was observed in the concentration of TNF-α in once weekly and twice weekly ($p = 0.8954$).

60 days of treatment showed the same pattern in the amount of TNF-α as was observed at the end of first month as no significance was observed across the groups. However, a different pattern was observed by the end of 90 days of treatment where TNF-α in the treated groups (once weekly 3.9 ± 0.1 and twice weekly 3.9 ± 0.1) were significantly higher than the control group ($p = 0.05$). When the concentration was compared between once weekly and twice weekly, it was not significantly different ($p = 0.8039$).

In the uterus and ovary (figure 21b and 21c), there was significantly higher concentration of TNF-α in the treated groups which was more pronounced in twice weekly group. The TNF-α in the ovary of once weekly was not significantly different from control throughout the period of experiment and and at post treatment. 30 days post treatment brought about a reduction the level of TNF- α in the serum and uterus unlike in the ovary.

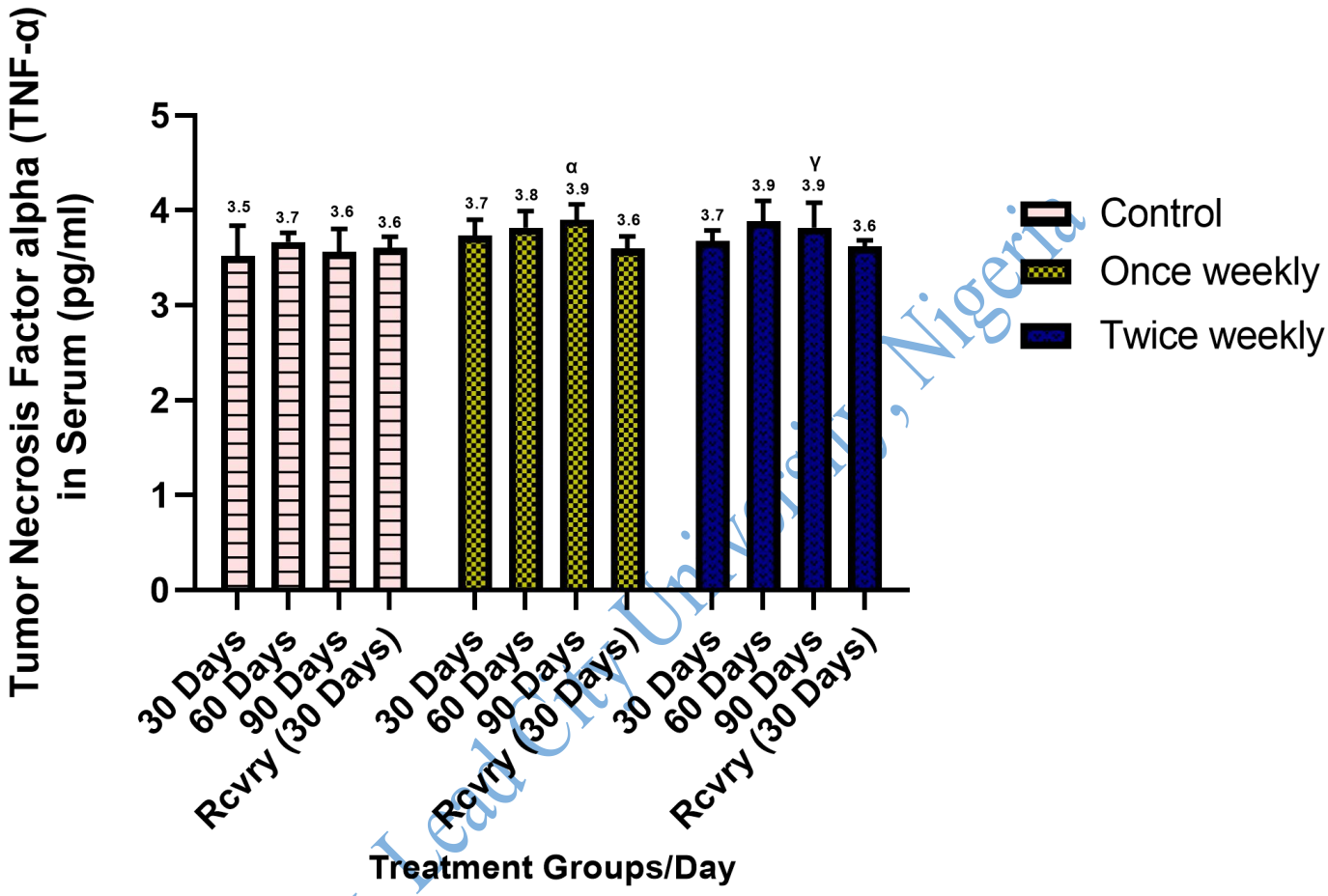

Figure 4.21a: Effect of Postinor 2 Intake on the Serum Concentration of Tumor Necrosis Factor-alpha

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

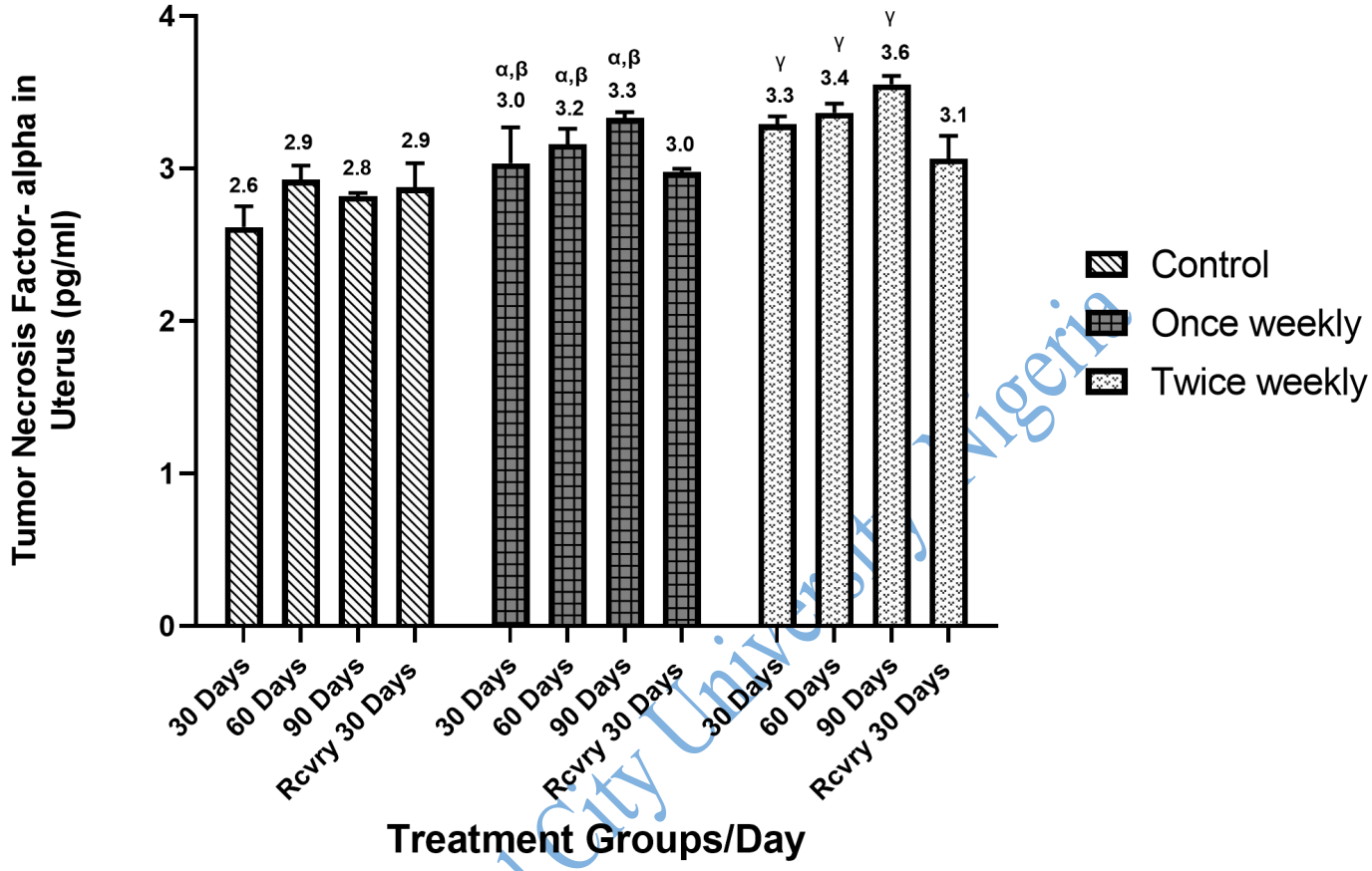

Figure 4.21b: Effect of Postinor 2 Intake on the Concentration of Tumor Necrosis Factor-alpha in the Uterus

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

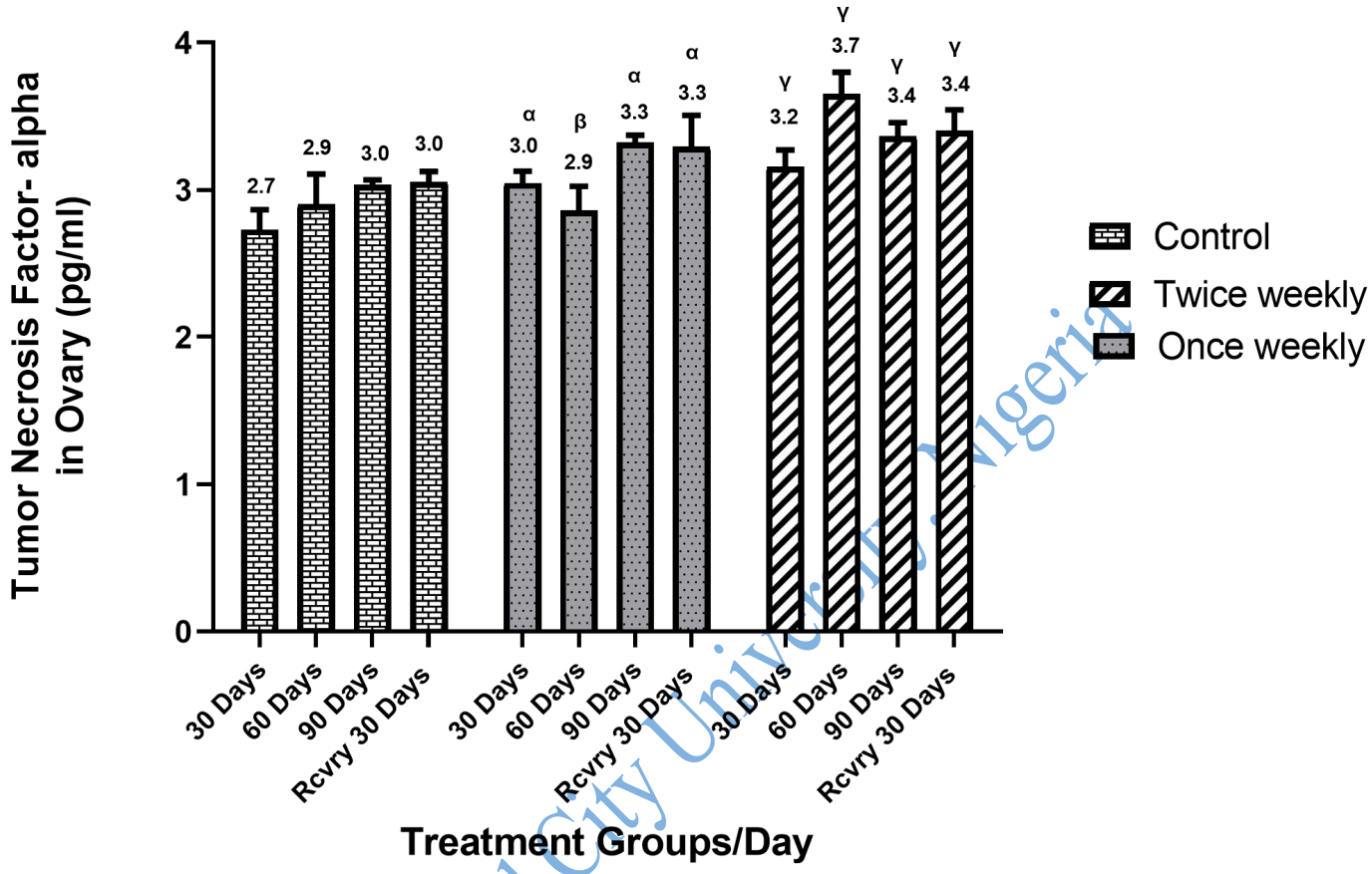

Figure 4.21c: Effect of Postinor 2 Intake on the Concentration of Tumor Necrosis Factor-alpha in the Ovary

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17 Effect of Postinor 2 Intake on Liver Function Indices

4.17.1 Effect on Postinor 2 Intake on Aspartate Transferase (AST) in the Serum and Liver of Wistar Rats

The effect of Postinor 2 on the activity of AST in serum was determined in the serum and liver and the results extrapolated from the standard curve shown in figure 4.22a. It was observed that there was a significant increase in level of AST in treated groups which was more pronounced in the twice weekly groups than in once weekly compared to control (Figure 4.22b). Although there was a little fluctuation in once weekly group, it was still higher than in control. For instance, the level dropped in the second month and rose again in the third month and dropped again after recovery period.

At the end of 30 days, twice weekly was higher than once weekly ($p = 0.0004$) and control ($p < 0.0001$). AST activity in once weekly was also significantly higher than control ($p = 0.0071$). Postinor 2 further increased the concentration of AST in treated groups to 140.3 ± 7.2 in twice weekly and 122.8 ± 7.3 in once weekly ($p < 0.05$) by 60 days of treatment which were higher than in control ($p < 0.05$) and $p = 0.0020$ respectively). Treatment for 90 days further significantly increased AST activity in twice weekly to 142.1 ± 4.0 which was significantly higher than both control and once weekly ($p < 0.05$).

After recovery period, the activity of AST in the serum of once weekly had dropped by 8% which was slightly higher than control but not significant ($p = 3391$). However, twice weekly maintained the same range and that was still signicantly higher than once weekly and control ($p < 0.05$).

Figure 4.22c shows the effect of Postinor 2 treatment on AST activity in the liver of Wistar rats and this showed a significant decrease in once weekly treated group and twice weekly throughout the treatment month ($p < 0.05$). An exception was observed in the first month where once weekly was insignificantly lower than control ($p = 0.0089$). There was only slight decrease in activity after 30 days post treatment

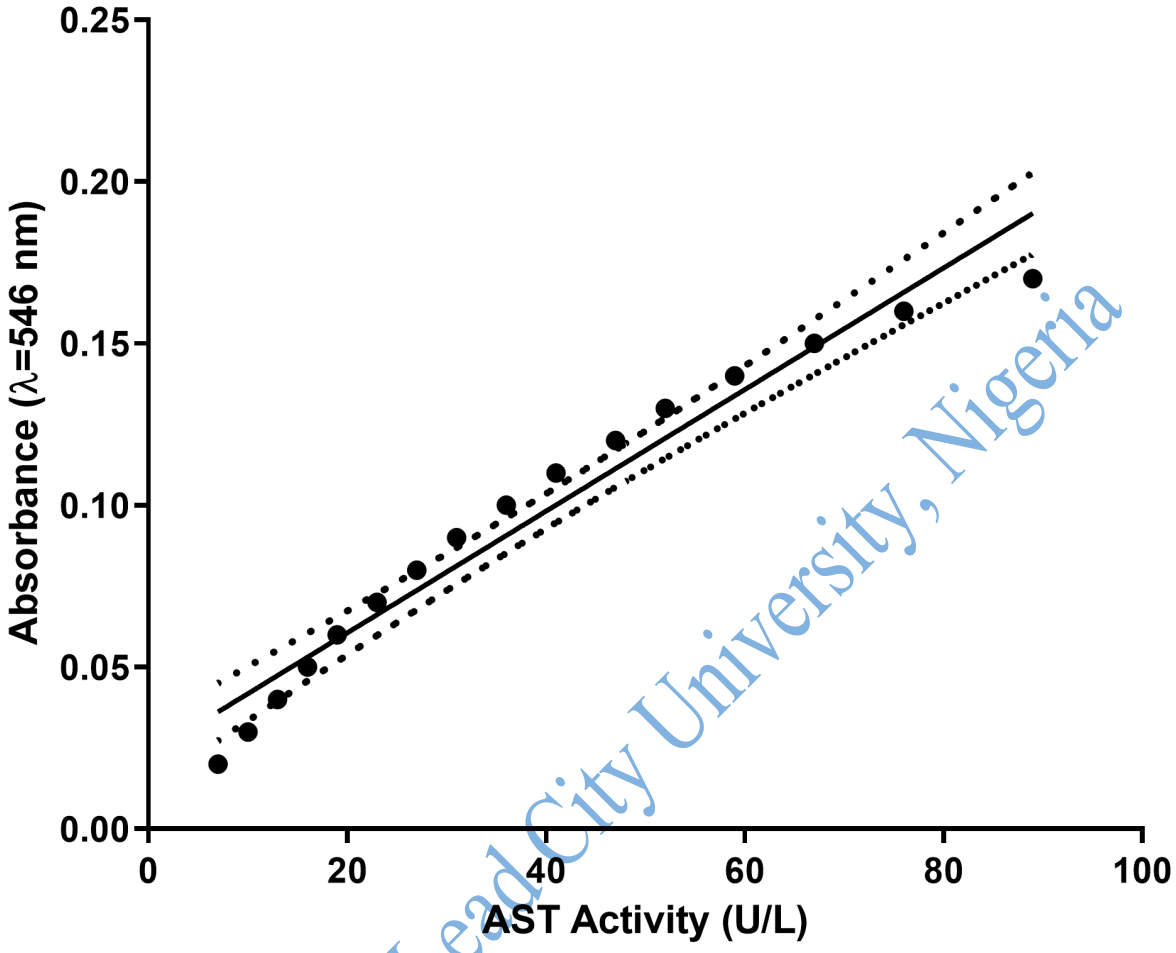

Figure 4.22a: Aspartate Aminotransferase (AST) Standard Curve

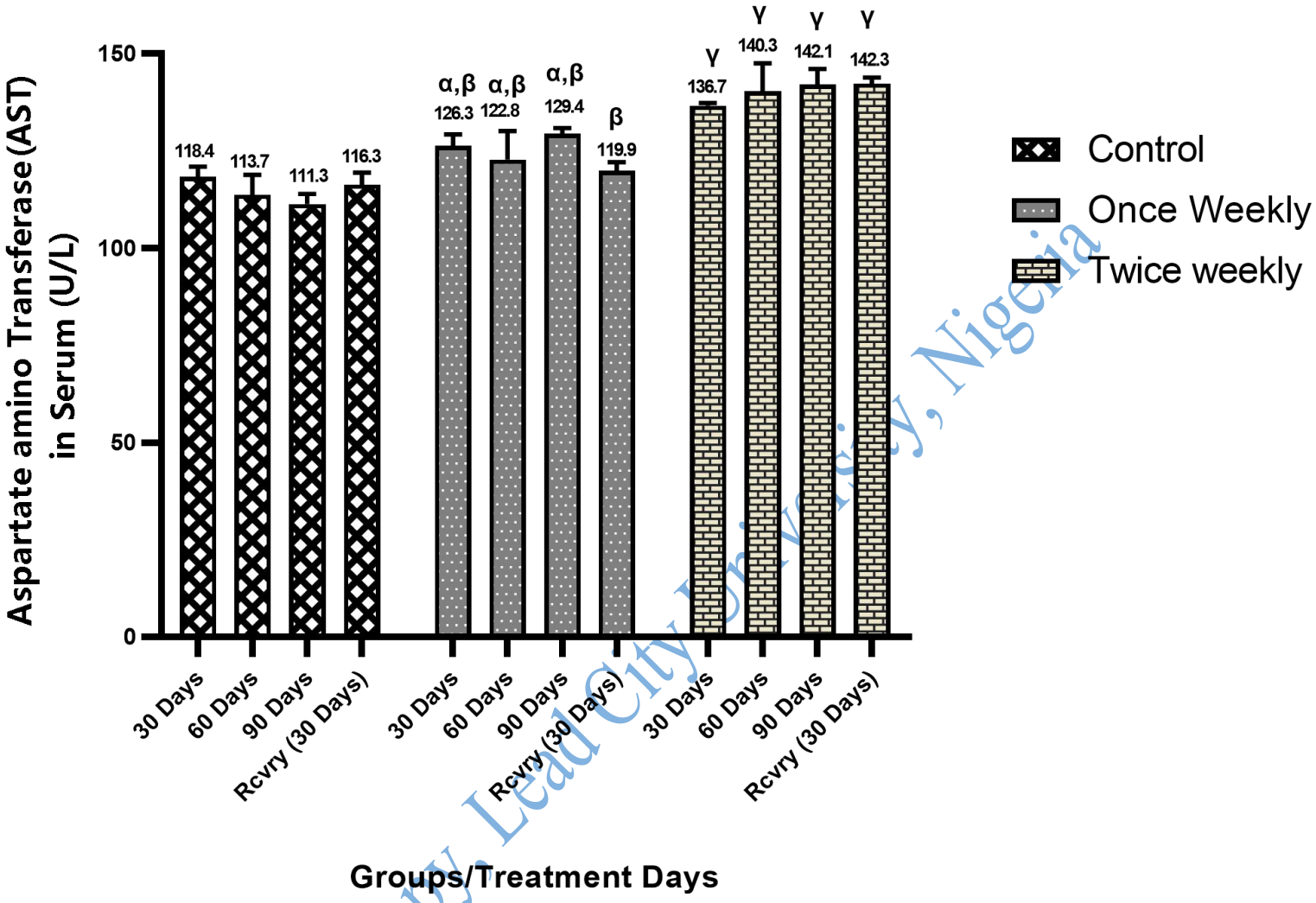

Figure 4.22b: Effect of Postinor 2 Intake on Aspartate Aminotransferase (AST) Activity in the Serum of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

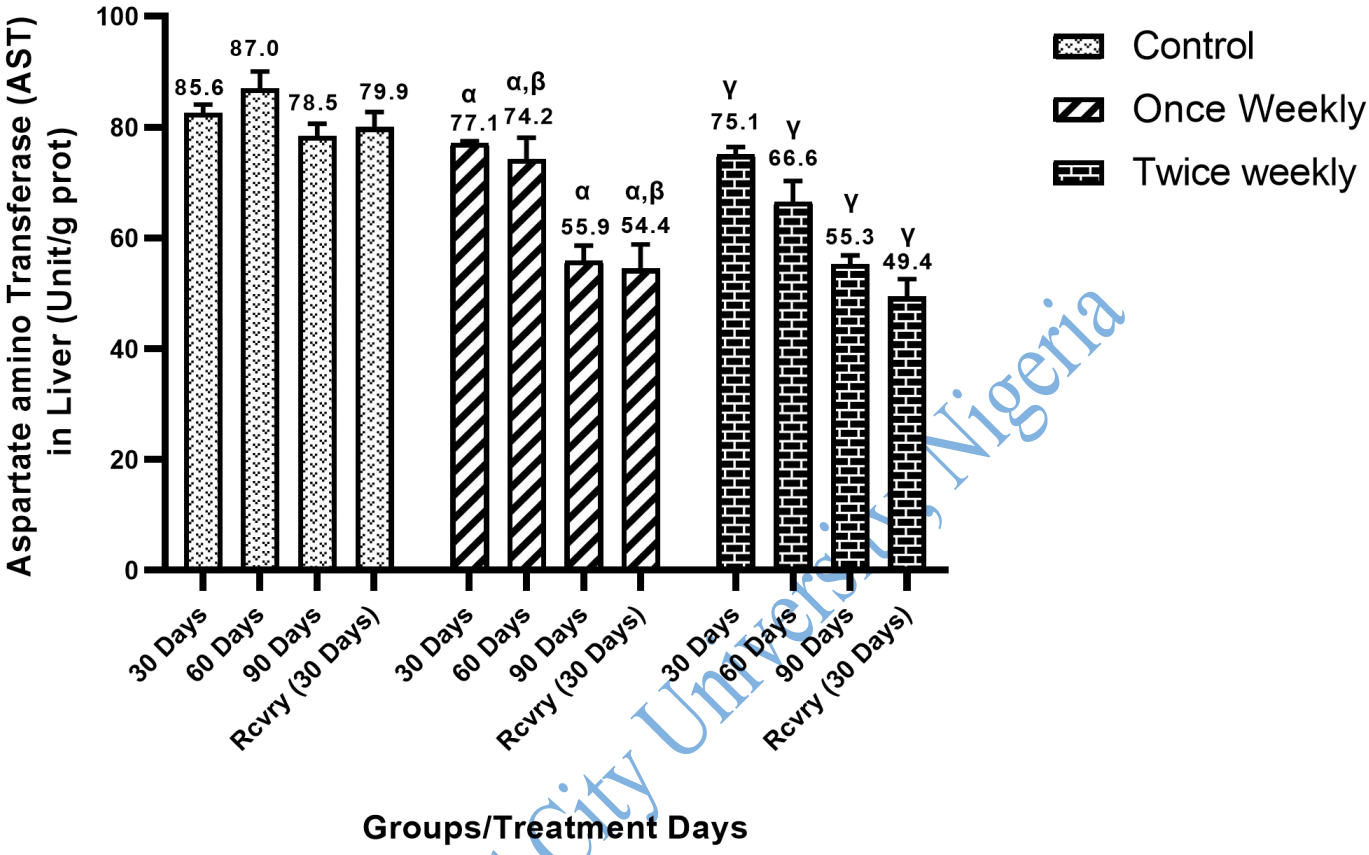

Figure 4.22c: Effect of Postinor 2 Intake on Aspartate Aminotransferase (AST) Activity in the Liver of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.2 Effect of Postinor 2 Intake on Alanine Aminotransferase (ALT) Activity in the Serum and Liver of Wistar Rats

The activity of ALT was determined in the serum and liver of Wistar rats and results were extrapolated from the standard curve shown in figure 4.23a. Postinor 2 intake also affected the activity of ALT in the serum of rats treated once weekly and twice weekly as shown in figure 4.23b. Treatment for 30 days presented a slight decrease in once weekly and a slight increase in twice weekly both of which were insignificant when compared with control ($p < 0.05$). Then by 60 days, both once weekly and twice weekly groups again increased insignificantly when compared with control. The increase became significant by 90 days of treatment with twice weekly quite elevated when compared with control ($p < 0.05$).

Figure 4.23c shows the effect of Postinor 2 intake on ALT activity in the liver of Wistar, there was significant decrease that was time dependent among the treated groups and control in all the treatment months ($p < 0.05$). The recovery period brought no improvement to the activity of ALT in the serum and in the liver of both treated groups as it still decreases significantly from each other and control ($p < 0.05$).

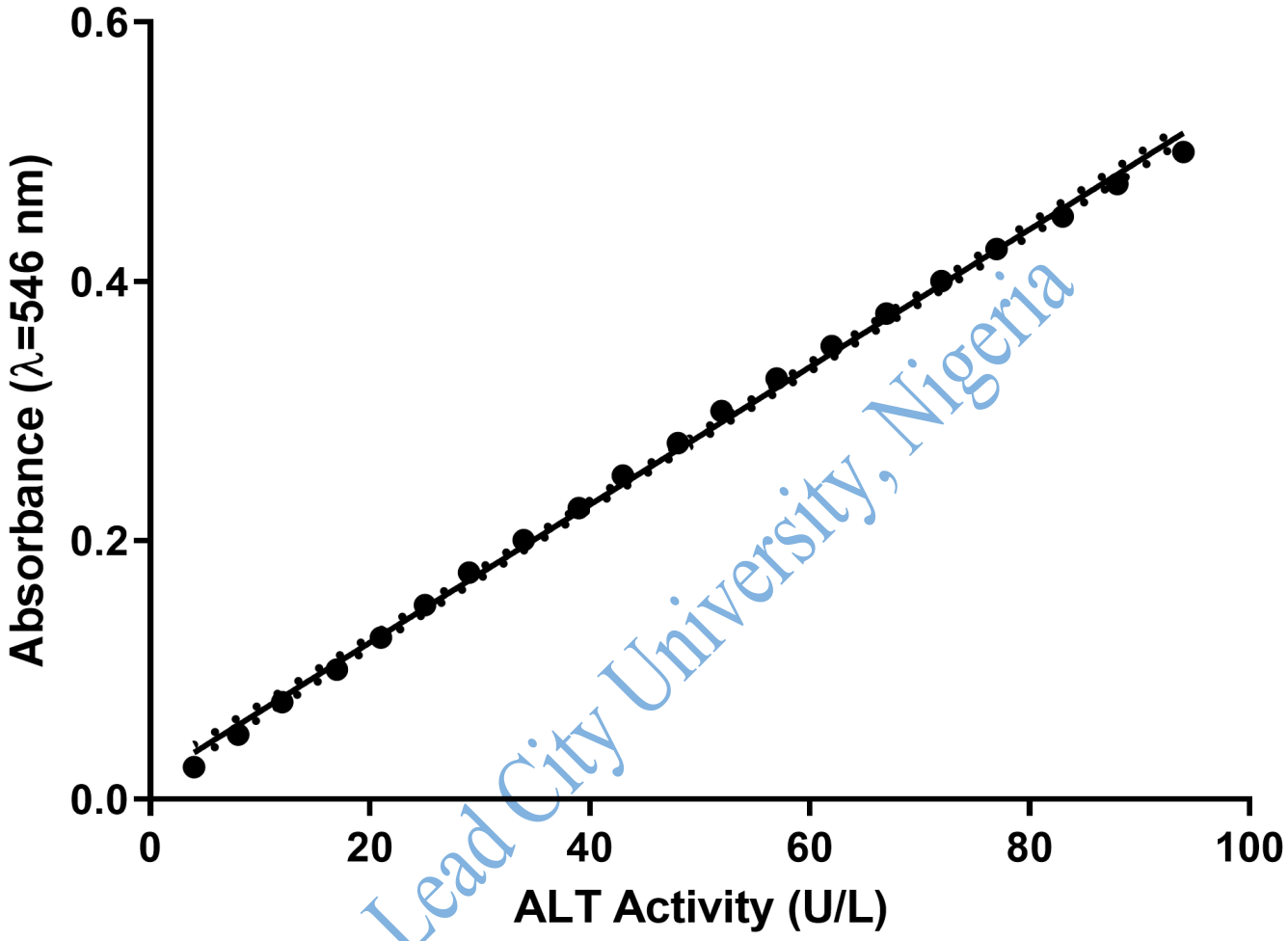

Figure 4.23a: Alanine Transaminase (ALT) Standard Curve

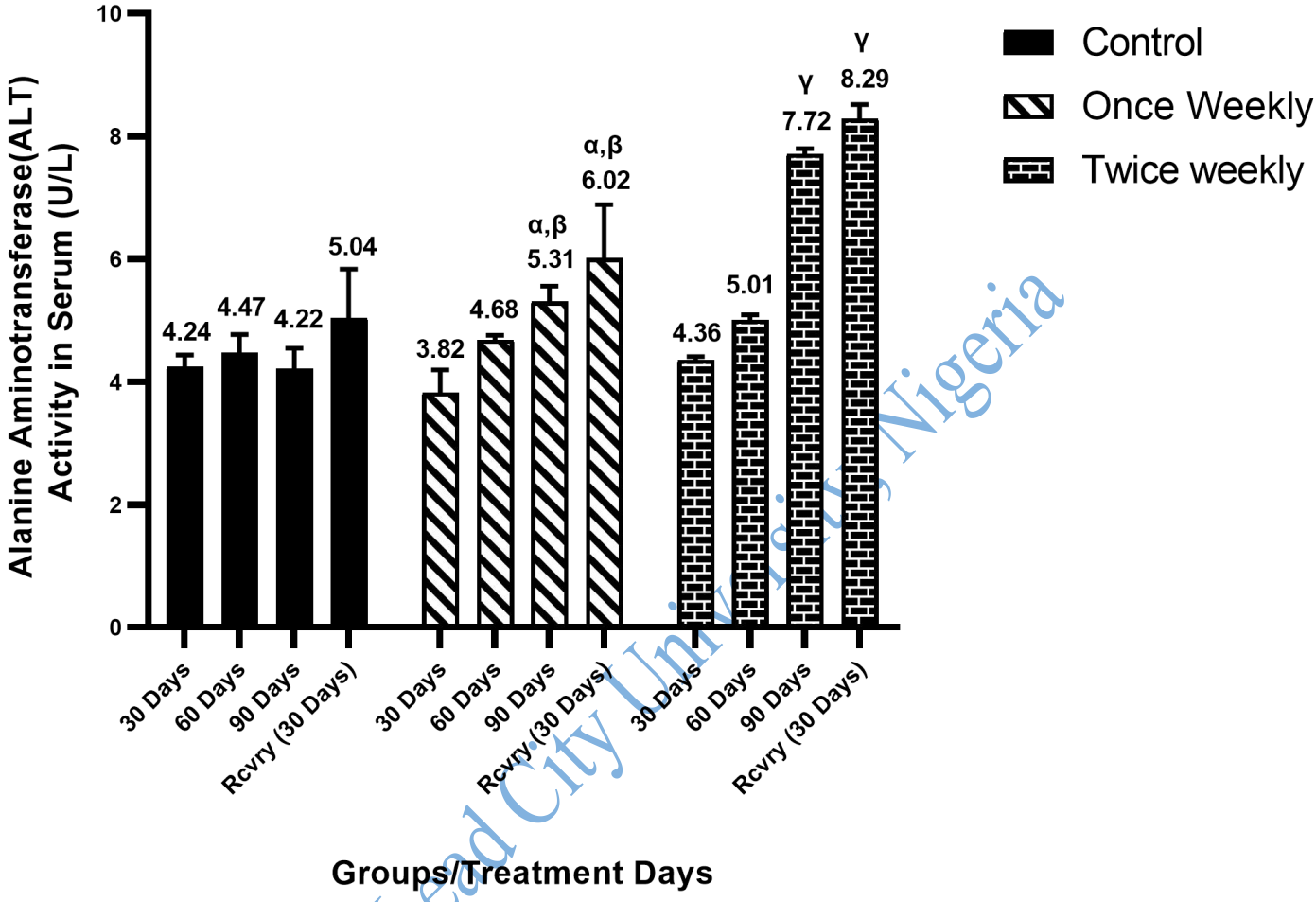

Figure 4.23b: Effect of Postinor 2 Intake on Alanine Transaminase (ALT) Activity in the serum of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

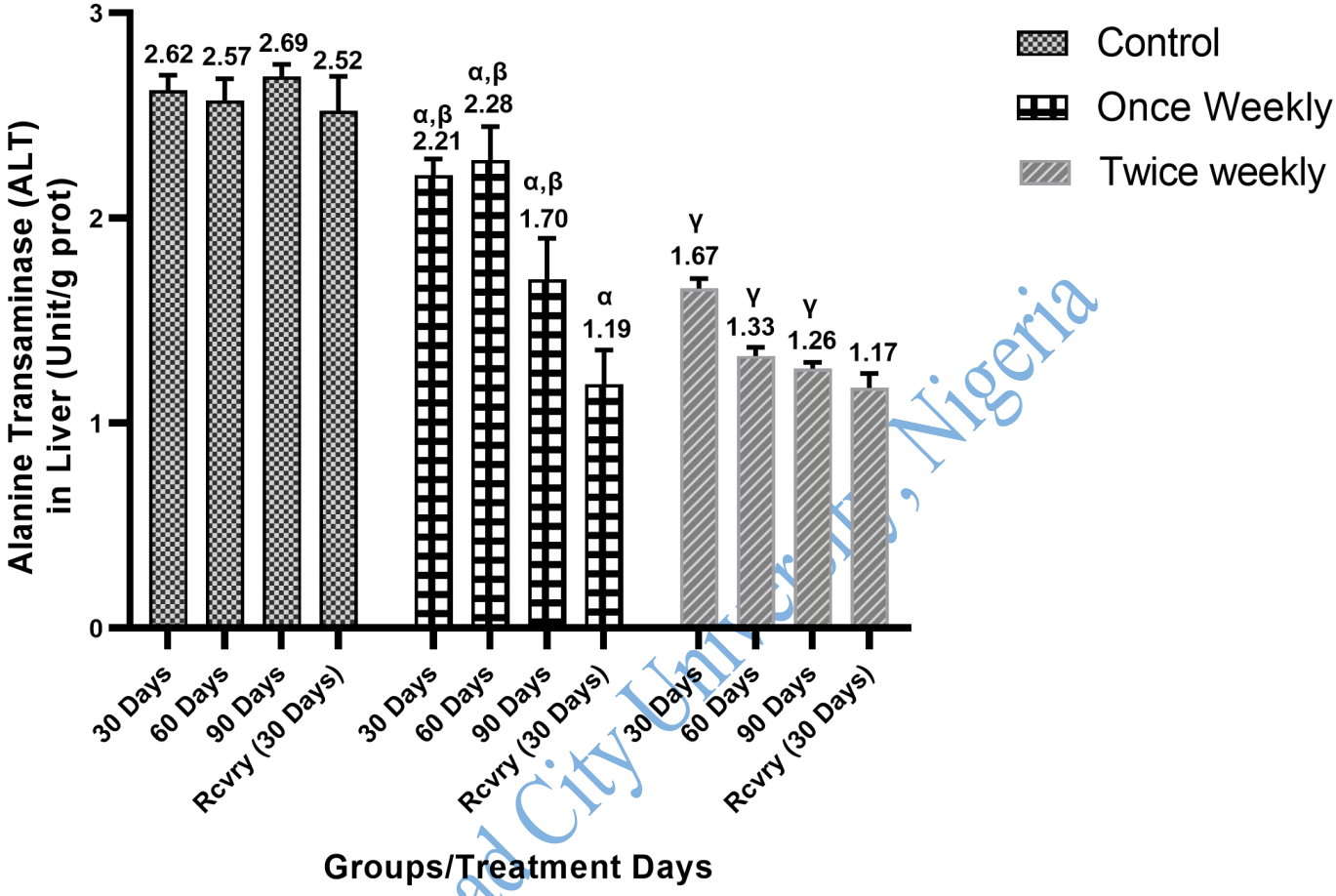

Figure 4.23c: Effect of Postinor 2 Intake on Alanine Transaminase (ALT) Activity in the Liver of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.3 Effect of Postinor 2 Intake on Aspartate Transferase (AST): Alanine Aminotransferase (ALT) Ratio in the Serum and Liver of Wistar Rats

The ratio of AST and ALT was determined in the serum (figure 4.24a) and in the kidney (figure 4.24b) to further evaluate the effect of Postinor 2 intake on the liver. In the serum, the twice weekly group presented a significantly higher values at 30 and 60 days which was 70 % and 53 % higher than in the control. At 90 days in twice weekly group, the ratio became symmetrical with the control and in once weekly it was lower by about 20 % than in control.

In the liver, both once weekly and twice weekly were higher than the control but once weekly dropped to the same value as control after post treatment period.

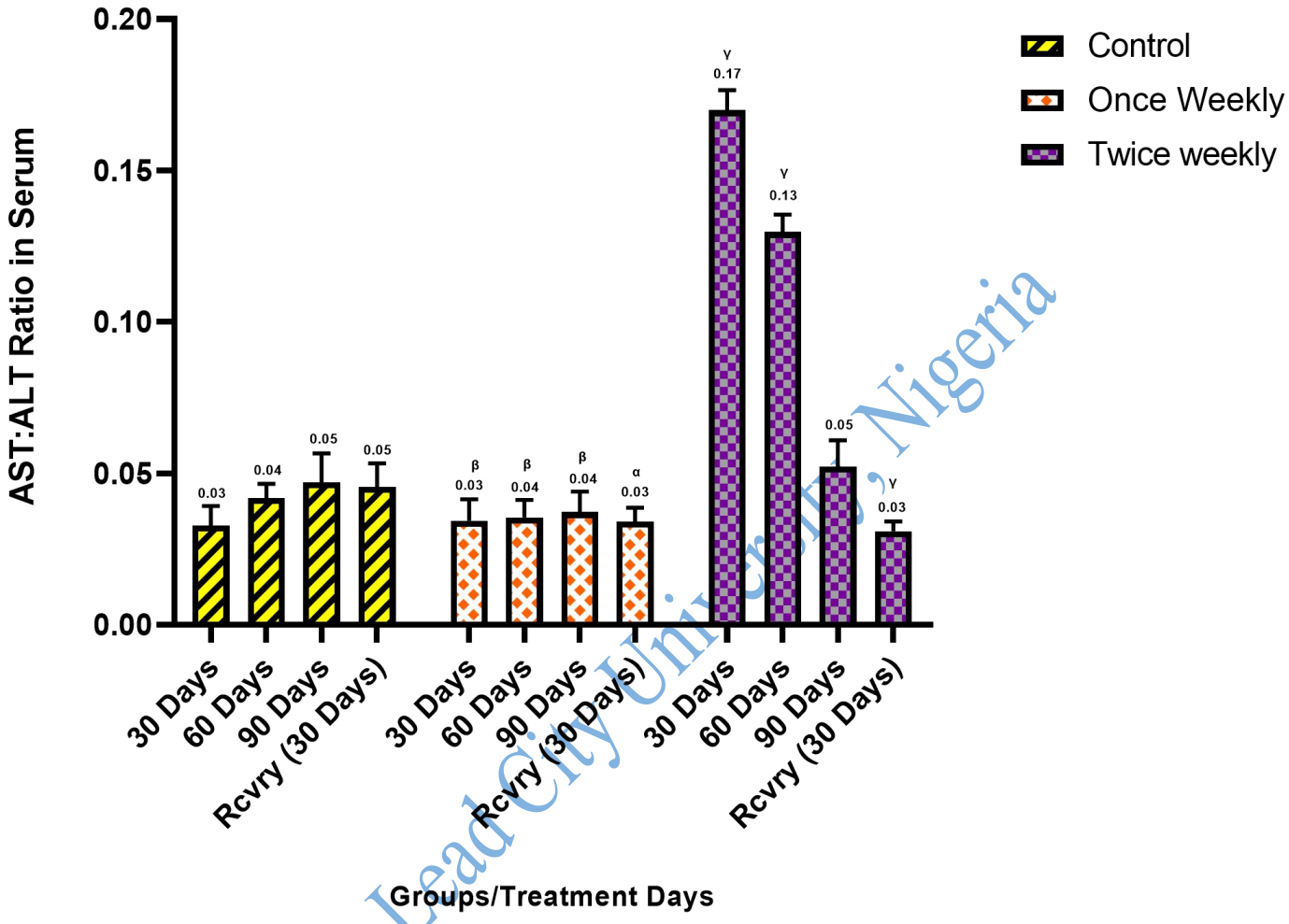

Figure 24a: AST: ALT Ratio in the Serum of Wistar Rats Treated with Postinor 2 Once Weekly, Twice Weekly and Control

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

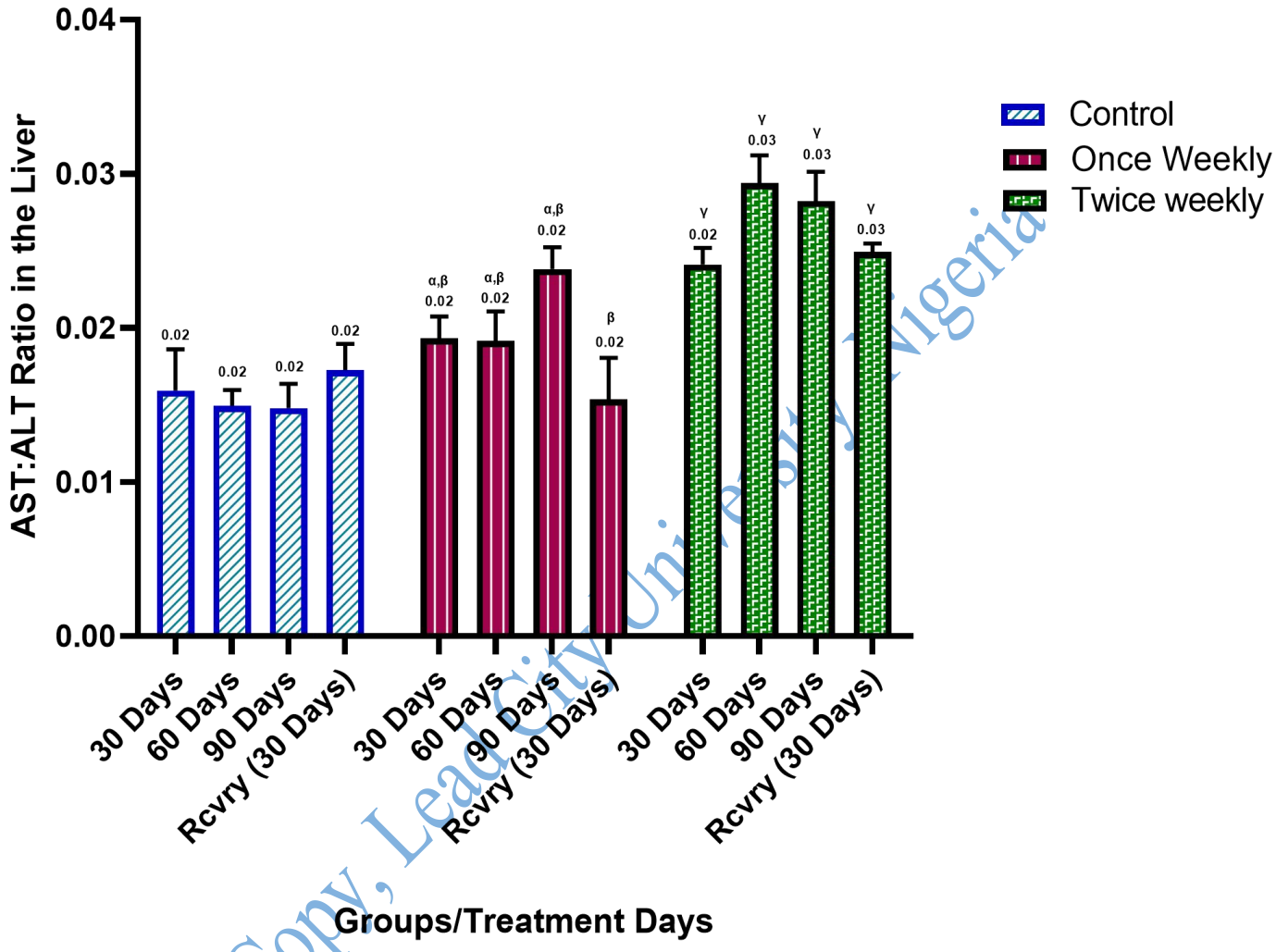

Figure 24b: AST: ALT Ratio in the Liver of Wistar Rats Treated with Postinor 2 Once Weekly, Twice Weekly and Control

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.4 Effect of Postinor 2 Intake on Alkaline Phosphatase (ALP) Activity in the Serum and Liver of Treated Rats Compared with Control

Figure 4.25a showed the effect of postinor-2 intake on the activity of ALP in the serum of rats treated once and twice weekly for a period 3 months and left for 1-month post treatment.

Compared to the control group, administration of Postinor-2 once weekly and twice weekly caused a slight but non-significant increase compared to the control group at the end of 30 days of treatment. The increase was significant in both once weekly and twice weekly for all the month of treatment and post treatment when comparison was done between them and also with control.

As shown in figure 4.25b, the liver of treated rats experienced a compromised activity of ALP which was significantly lower than control in the months of treatment. Compared to the control group, administration of Postinor-2 once weekly and twice weekly caused significant reduction in the liver when compared with control. The decrease was significant between each other and with control. This decrease in activity was time dependent and continued in the post treatment period to show no recovery.

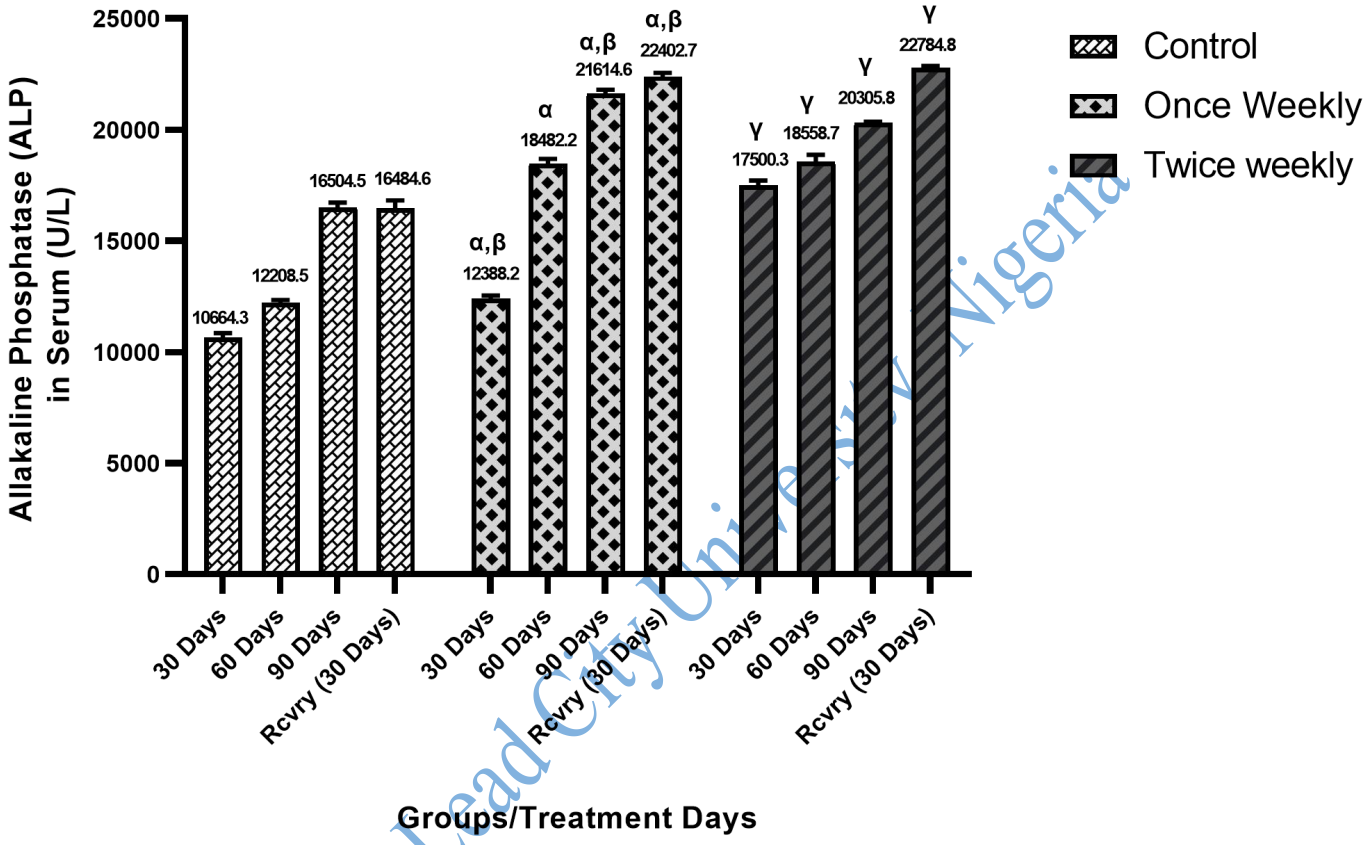

Figure 4.25a: Effect of Postinor 2 Intake on Alkaline Phosphatase (ALP) Activity in the Serum of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

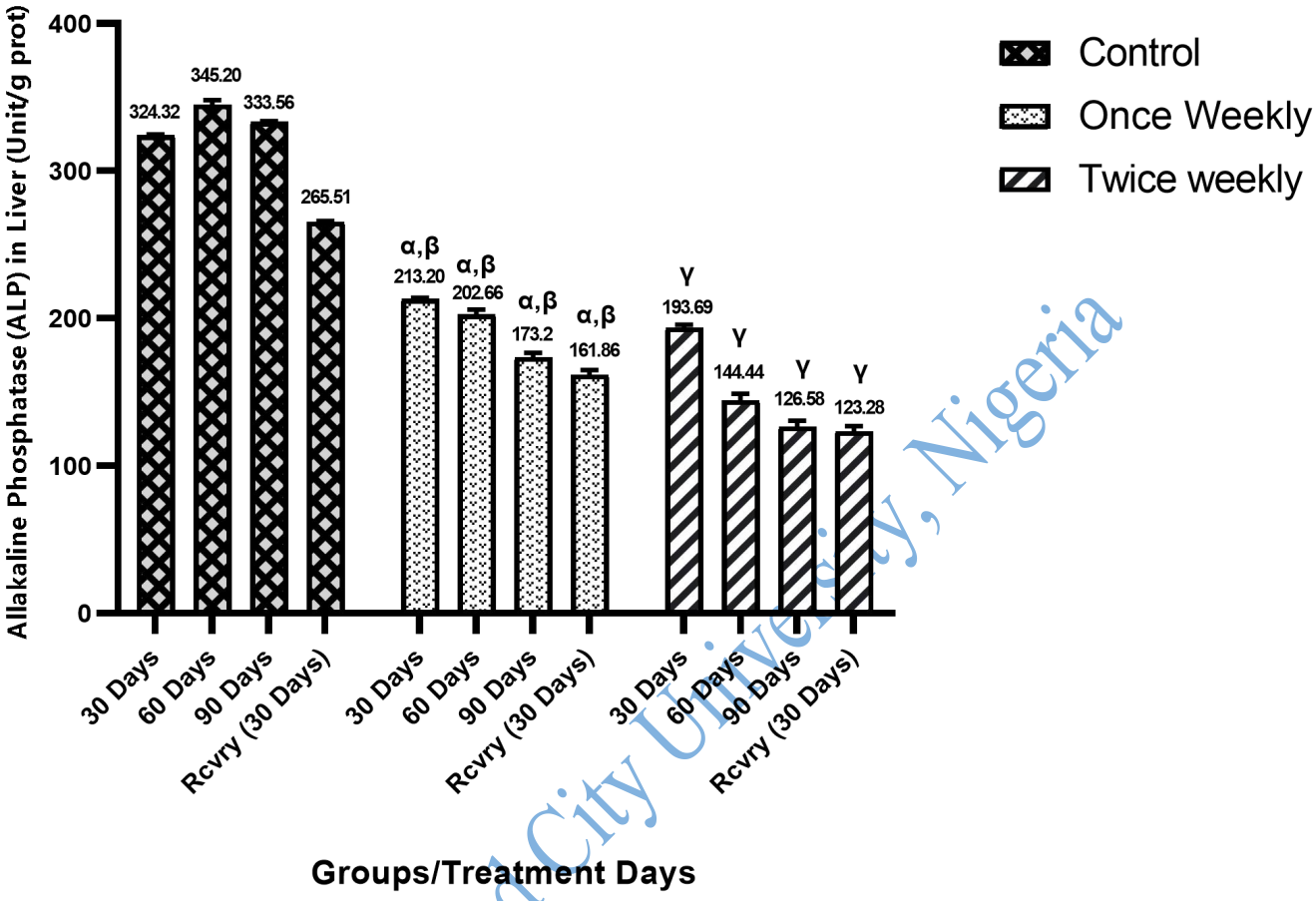

Figure 4.25b: Effect of Postinor 2 Intake on Alkaline Phosphatase (ALP) Activity in the Liver of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.5 Effect of Postinor 2 Intake on Total Protein

Compared to the control group, administration of Postinor-2 once weekly and twice weekly caused significant reduction in total protein concentration both in the liver and serum as shown in figure 4. 26a and b. This was seen in the time dependent decrease in the concentration of total protein in the order twice weekly < once weekly < control. The significantly reduced level of protein occurred throughout the treatment period and even in post treatment

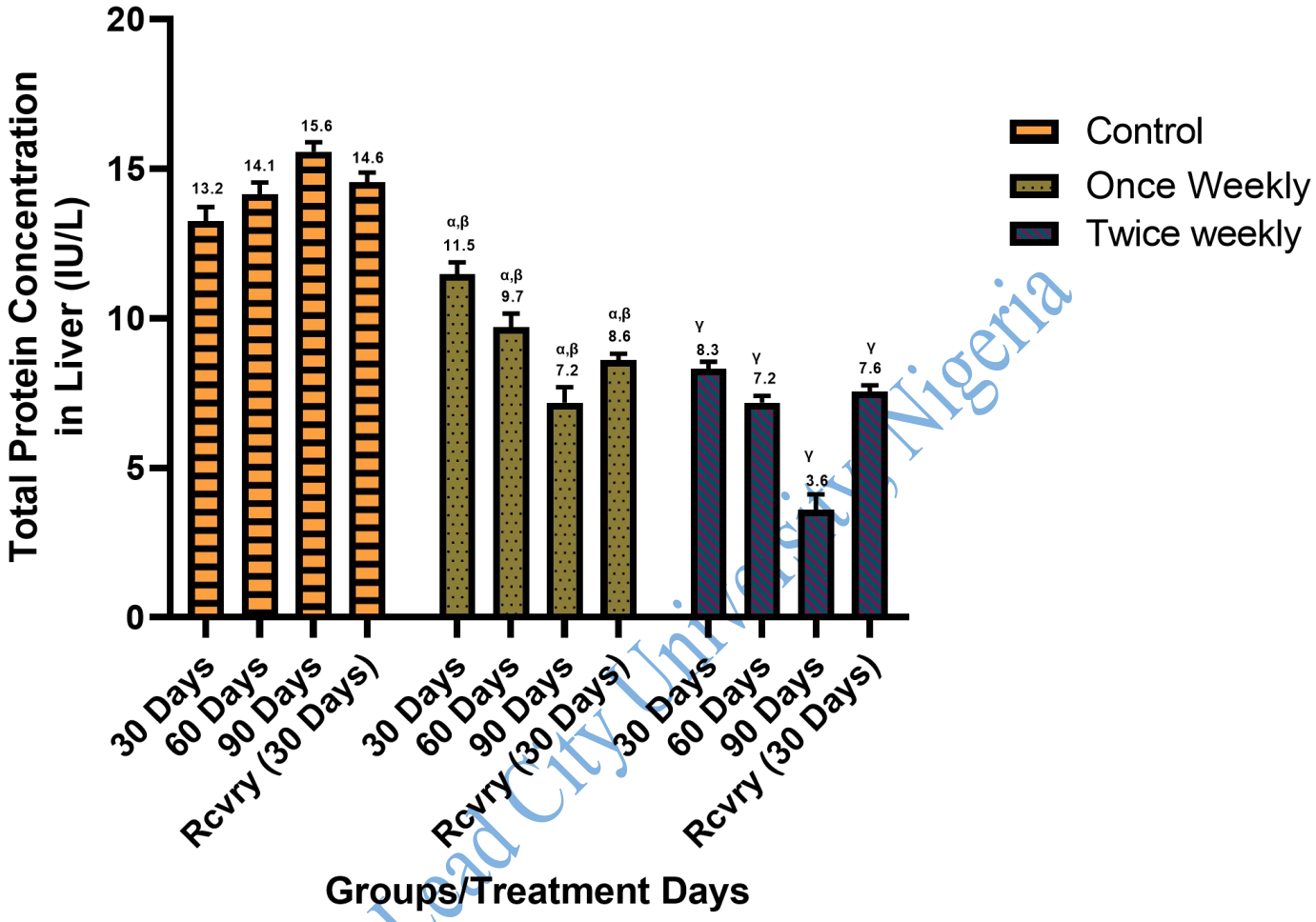

Figure 4.26a: Effect of Postinor 2 Intake on Total Protein Concentration in the Liver of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

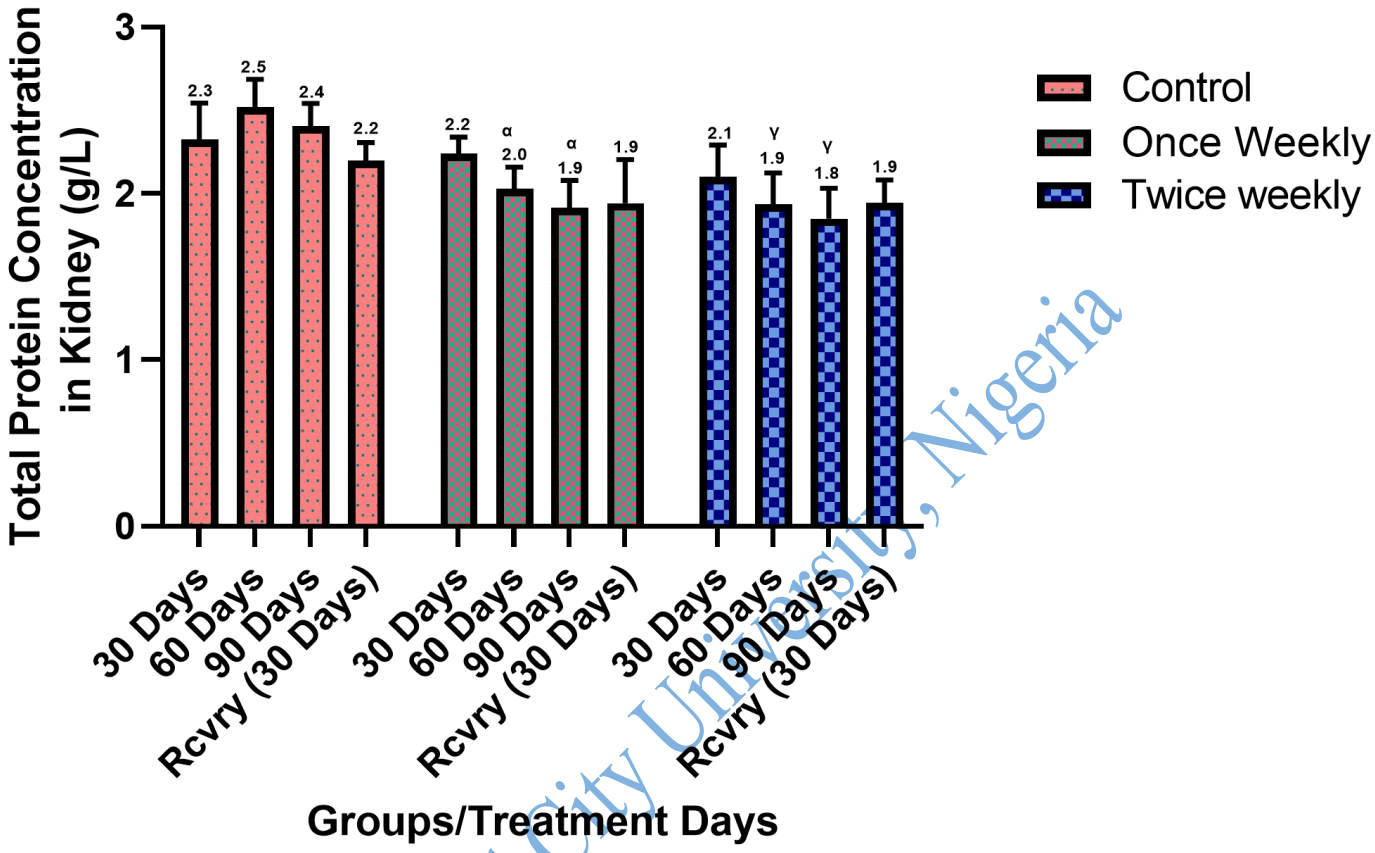

Figure 4.26b: Effect of Postinor 2 Intake on Total Protein Concentration in the Kidney of Wistar Rats

Values were expressed as mean ± standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.6 Effect of Postinor 2 Intake on γ-Glutamyl Transferase Concentration in Wistar Rats in the Serum and Tissue of Rats

Figures 4.27a and 4.27b shows the effect of postinor-2 intake on the activity of GGT in the serum of rats treated once and twice weekly.

In serum, twice weekly treatments of animals with Postinor-2 caused significant increase in the liver GGT activity, whereas once weekly treatment did not cause any change in GGT activity in the liver when compared to the control group ($p = 0.7279$) and this caused a significant difference between the treated groups in the first month ($p < 0.05$). This did not persist for long as a significant increase occurred in once weekly (0.7 ± 0.1) at the end of the second month which was the same as seen in twice weekly (0.7 ± 0.1) ($p = 0.8562$). This was significantly different from control ($p = 0.0019$ and 0.0089 respectively). This level of activity in once weekly was maintained over the next month but further increase in activity was observed in twice weekly (0.9 ± 0.1) which was significant from both control ($p < 0.05$) and once weekly ($p < 0.05$).

In the liver, twice weekly treatments of animals with Postinor-2 caused significant increase in the liver GGT activity, whereas once weekly treatment did not cause any change in GGT activity in the liver when compared to the control group ($p = 0.7279$) and this caused a significant difference between the treated groups in the first month ($p = 0.0398$). This did not persist for long as a significant increase occurred in once weekly (0.7 ± 0.1) at the end of the second month which was the same as seen in twice weekly (0.7 ± 0.1) ($p = 0.8562$). This was significantly different from control ($p = 0.0019$ and 0.0089 respectively). This level of activity in once weekly was maintained over the next month but further increase in activity was observed in twice

weekly (0.9 ± 0.1) which was significant from both control ($p < 0.0001$) and once weekly ($p < 0.0001$).

Posttreatment period of 30 days brought an improvement in the activity of GGT in the serum to a level where once weekly and twice weekly were no longer significantly different ($p = 0.9006$), though significantly higher than control ($p < 0.05$). Meanwhile in the liver, Recovery was observed most especially in once weekly as there was a 31 % decrease in the activity of GGT and 8 % decrease in twice weekly.

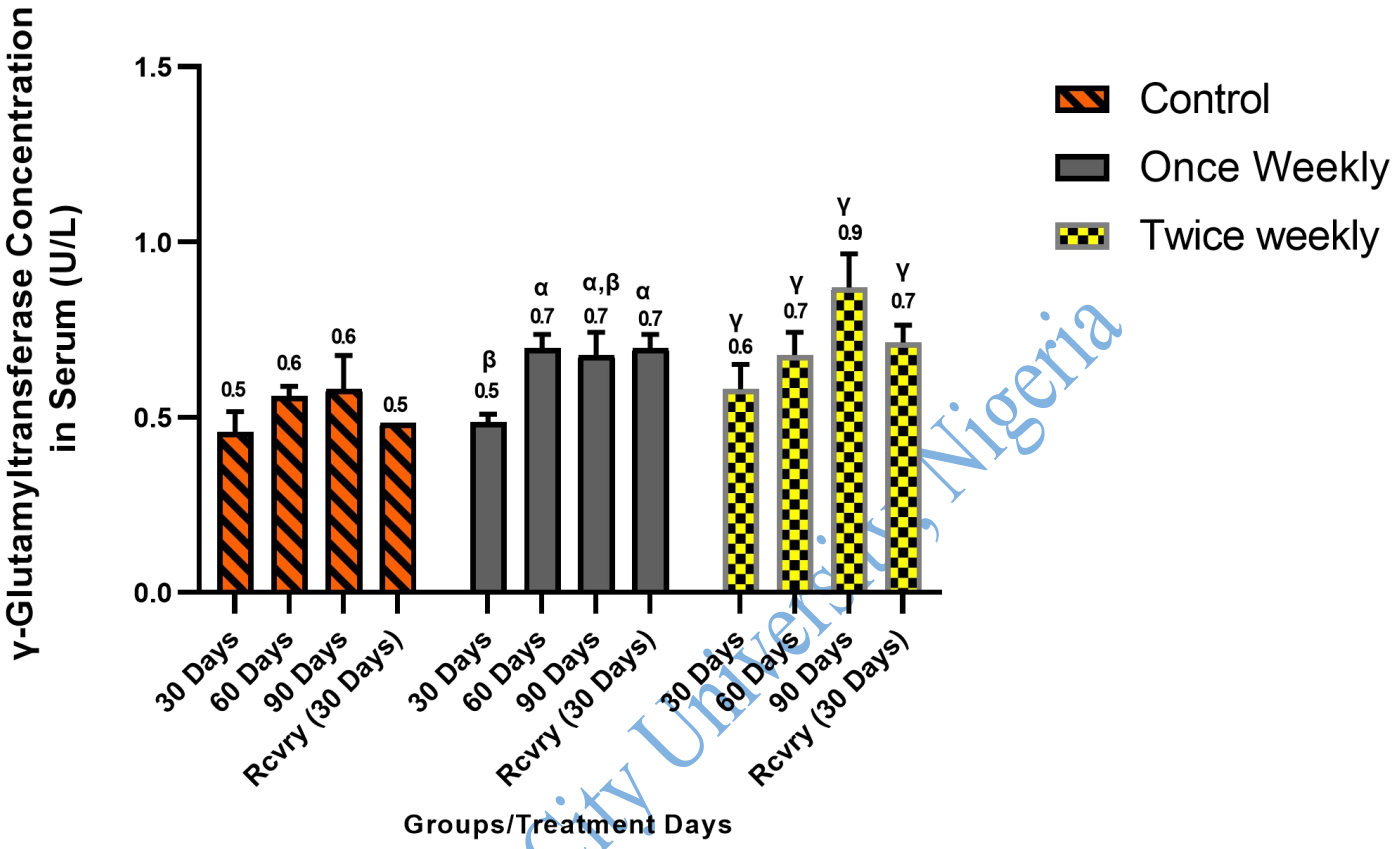

Figure 4.27a: Effect of Postinor 2 Intake on γ-Glutamyl Transferase Activity in Serum (U/L) of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.17.6.2 Effect of Postinor-2 on the activity of Gamma Glutamyl Transferase (GGT) in the Liver of Rats

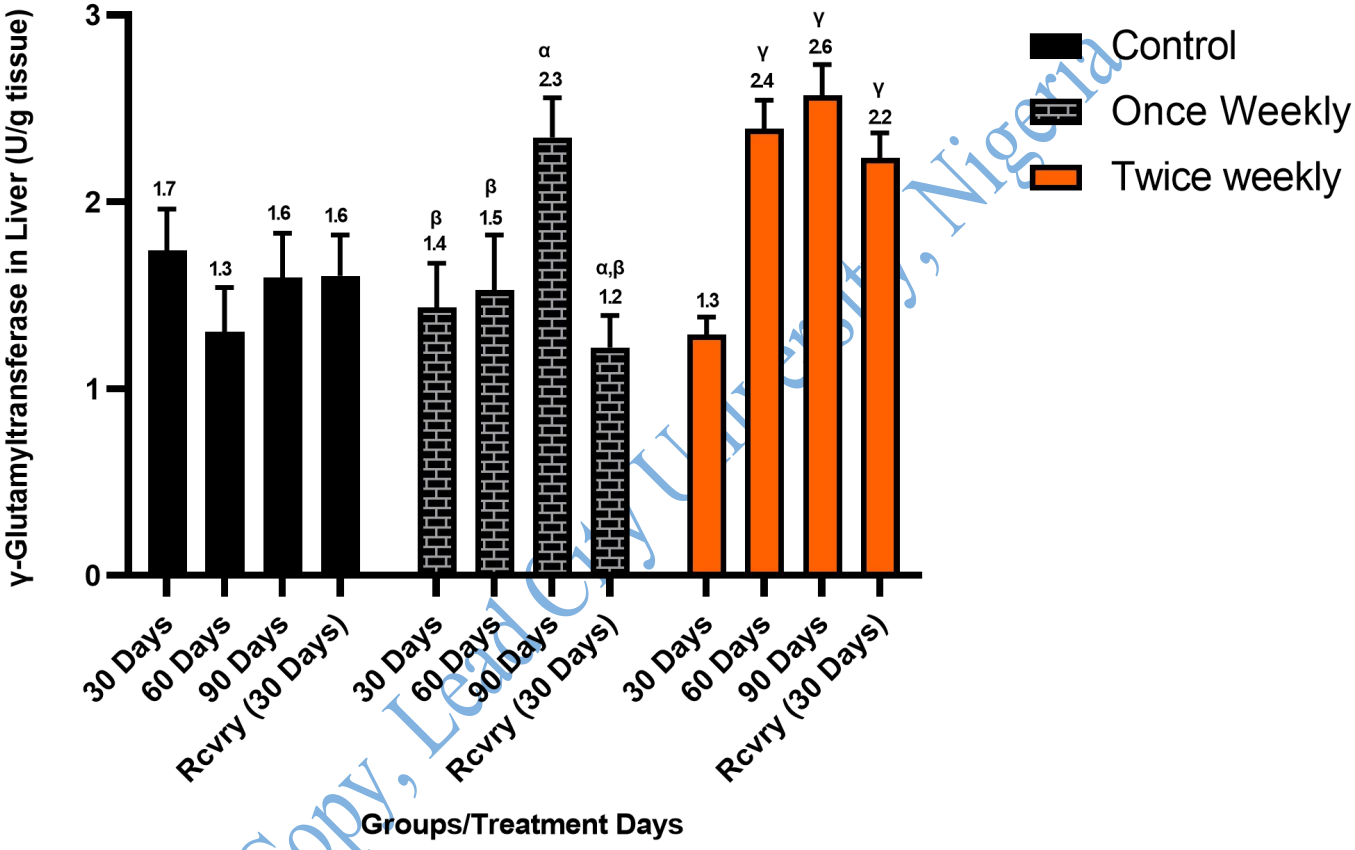

Figure 4.27b: Effect of Postinor 2 Intake on γ-Glutamyl Transferase Concentration in Liver (U/L) of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.18 Effect of Postinor 2 Intake on Kidney Function

4.18.1 Effect of Postinor 2 Intake on Cystatin C Concentration in the Serum of Wistar Rats

Figure 28a shows the standard curve of cystatin C which was used to extrapolate the concentration of cystatin C in the serum. Compared to the control group, administration of Postinor-2 once weekly and twice weekly caused significant increase in the concentration of cystatin C when compared with control in the second and third as shown in figure 4.28b. It was only significantly higher in twice weekly in the first month, while a slight increase was seen in once weekly ($p = 0.9999$). The increase was significant in twice weekly and between the treated groups ($p < 0.05$).

60 and 90 days of treatment presented a similar increase in activity in the once and twice weekly groups which was significant from the control ($p < 0.05$). At recovery, once weekly maintained cystatin c concentration while it was further increased in twice weekly and was significant from once weekly ($p = 0.0439$) and control ($p < 0.05$).

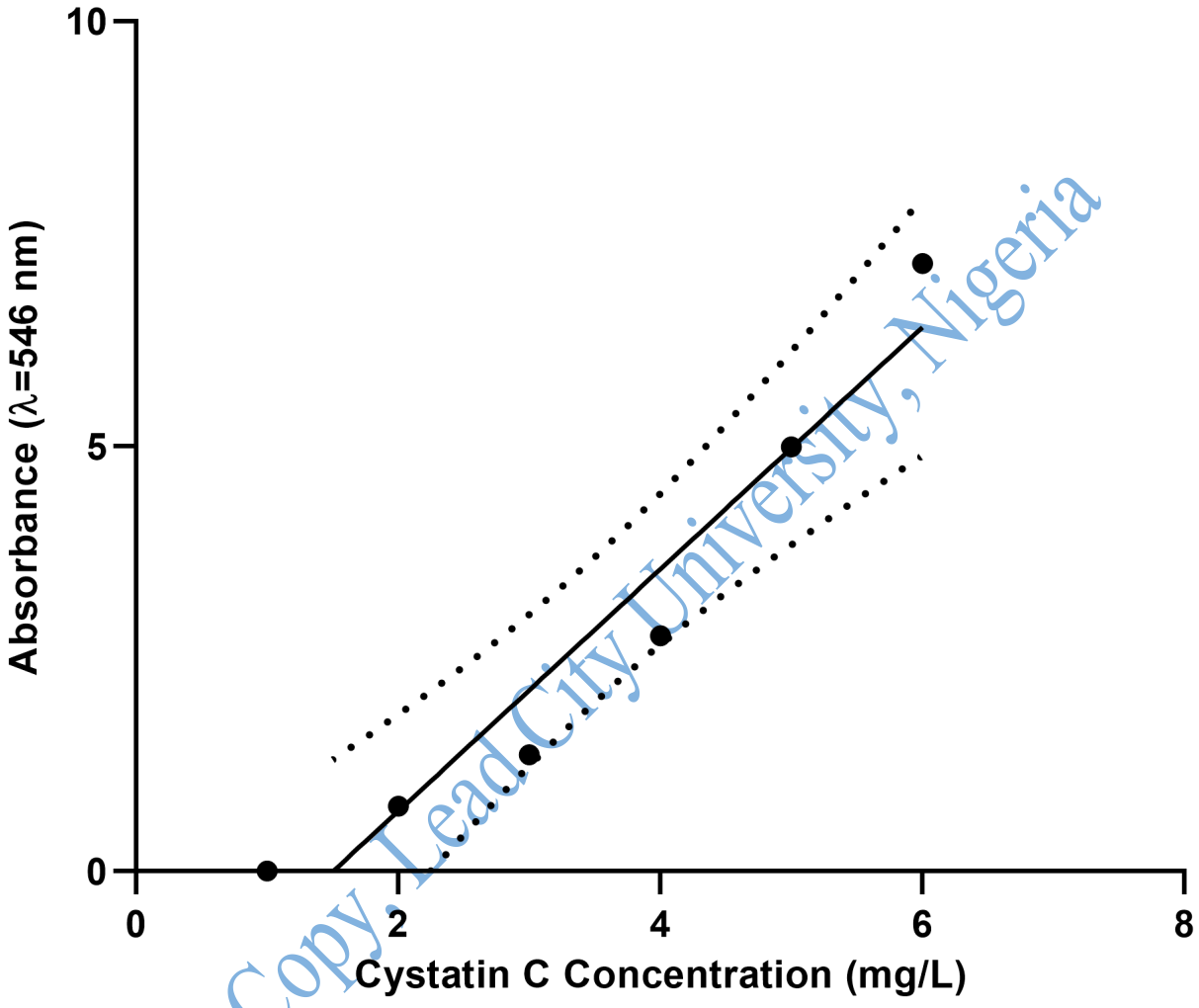

Figure 4.28a: Standard Curve for the Calibration of Cystatin C

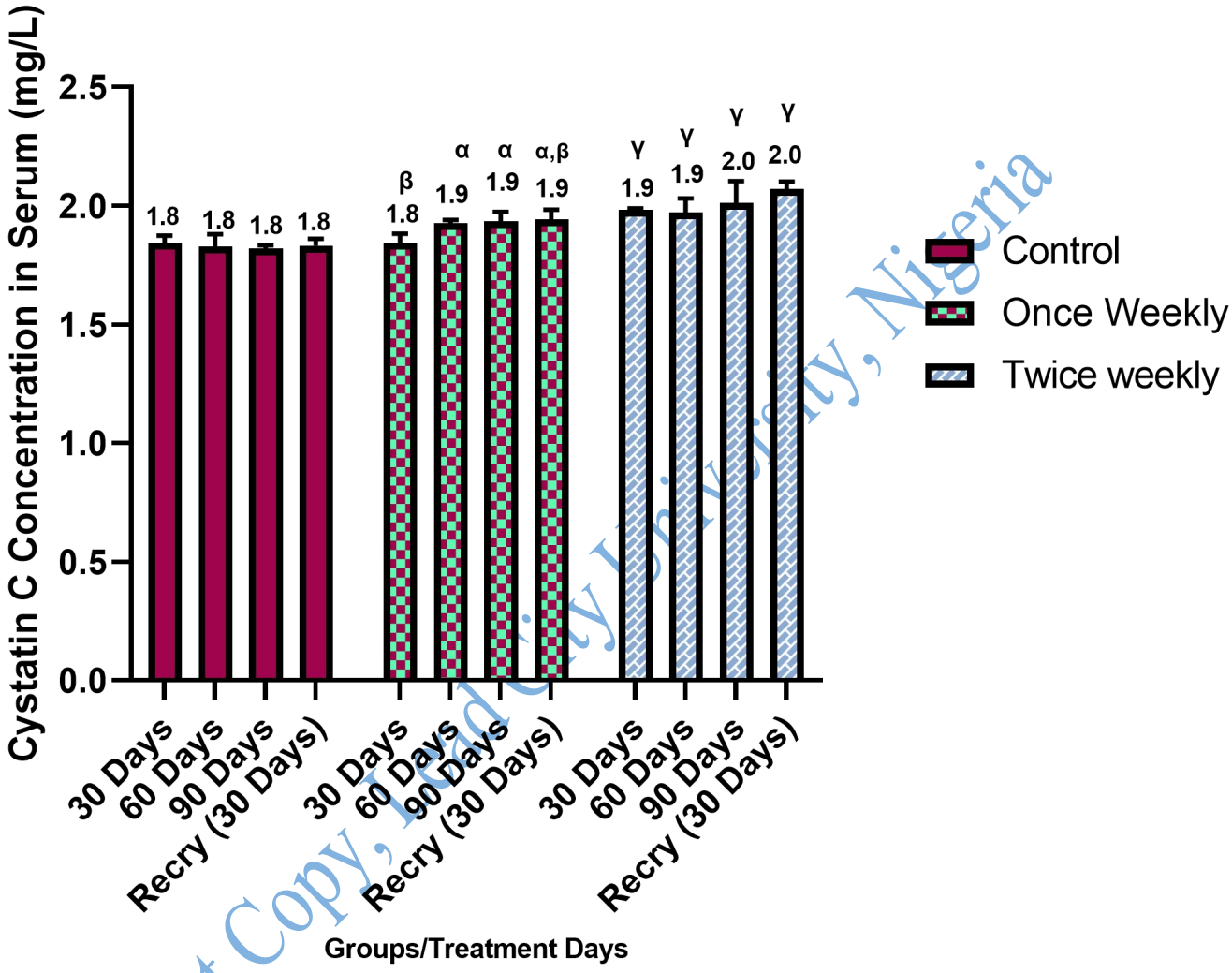

Figure 4.28b: Effect of Postinor 2 Intake on Cystatin C Concentration in the Serum of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical

significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **y'** showed significance when twice weekly was compared with control ($p < 0.05$).

4.18.2 Effect of Postinor 2 Intake on the Level of Creatinine (Cr) in the Kidney of Wistar Rats

The effect of Postinor 2 Intake on the concentration of creatinine in the kidney was shown in figure 4.29 to investigate the effect of Postinor 2 intake on the kidney. The treated groups had a reduced value that was significant when compared with the control. Creatinine level was constant and insignificant between once weekly and twice weekly treated groups at 30 and 60 days. At 90 days in twice weekly group, the value of Cr had reduced by 26 % in twice weekly and only 5.8 % in once weekly.

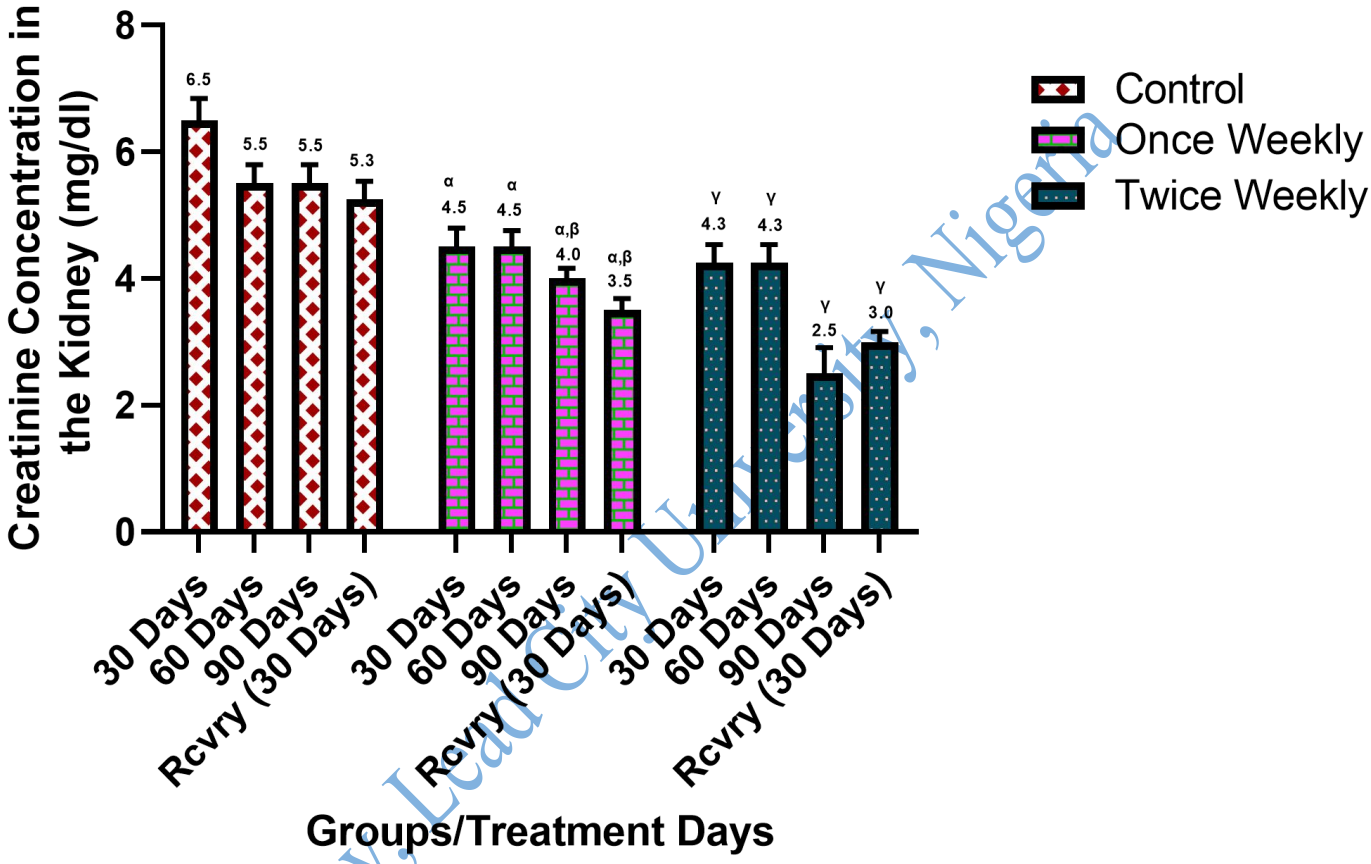

Figure 4.29: Effect of Postinor 2 Intake on the Concentration of Creatinine in the Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.18.3 Effect of Postinor 2 Intake on the Level of Urea in the Serum and Kidney of Wistar Rats

Urea concentration in the serum (figure 4.30a) and kidney (figure 4.30b) of the Wister rats to observe the effect of Postinor 2 intake on the integrity of the kidney. Though the concentration was fluctuating in the serum of control, it kept increasing significantly in the treated groups except in the first month where a non- significant increase in once weekly was observed ($p = 0.1730$).

30 days post treatment, the concentration of urea in once weekly was maintained (42.2 ± 2.8) while that of twice weekly still slightly increased (44.9 ± 0.7) which was significantly higher than control ($p < 0.05$).

In the kidney, the concentration of urea was increasing with treatment days and dropped by 23 % at recovery period.

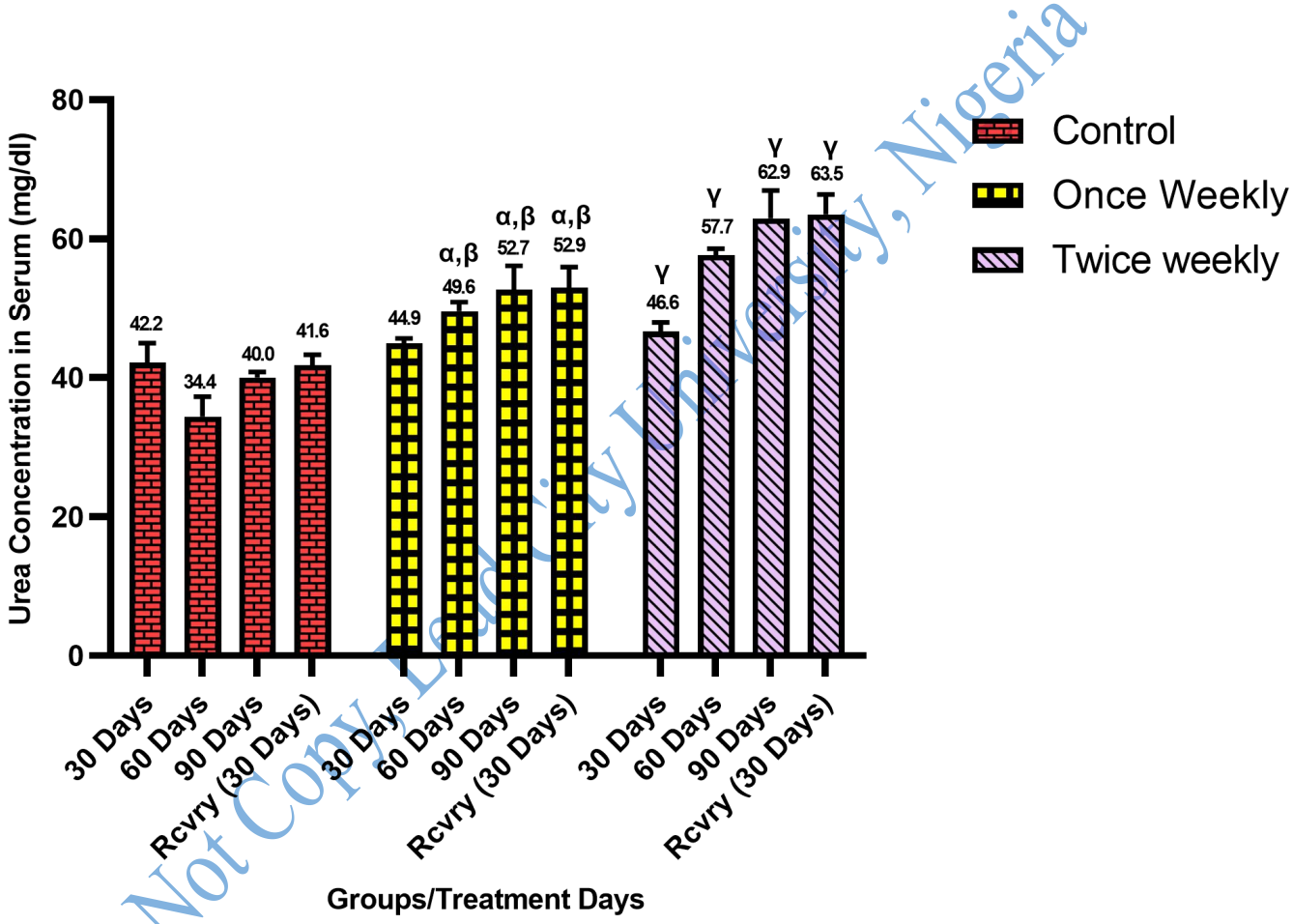

Figure 4.30a: Effect of Postinor 2 Intake on the Concentration of Urea in the Serum of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

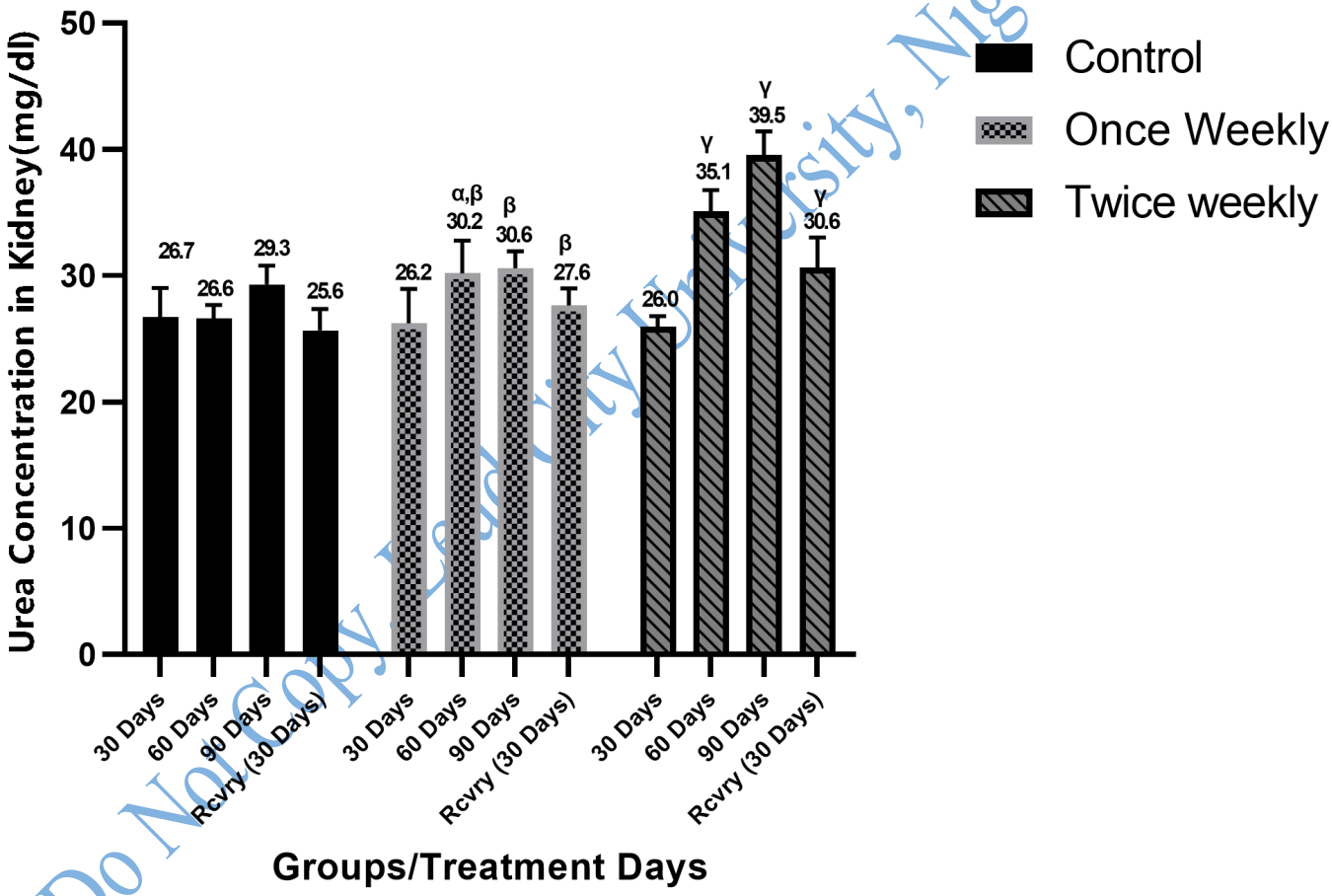

Figure 4.30b: Effect of Postinor 2 Intake on the Concentration of Urea in the Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.18.4 Determination of BUN: Creatinine Ratio

The ratio of BUN and creatinine in the kidney was shown in figure 4.31 to further evaluate the effect of Postinor 2 on the kidney. The treated groups presented a higher value that was significantly higher than in the control. At 90 days in twice weekly group, the value BUN:Cr ratio was 49 % higher than in the control group. There was reduced to about 35 % after post treatment

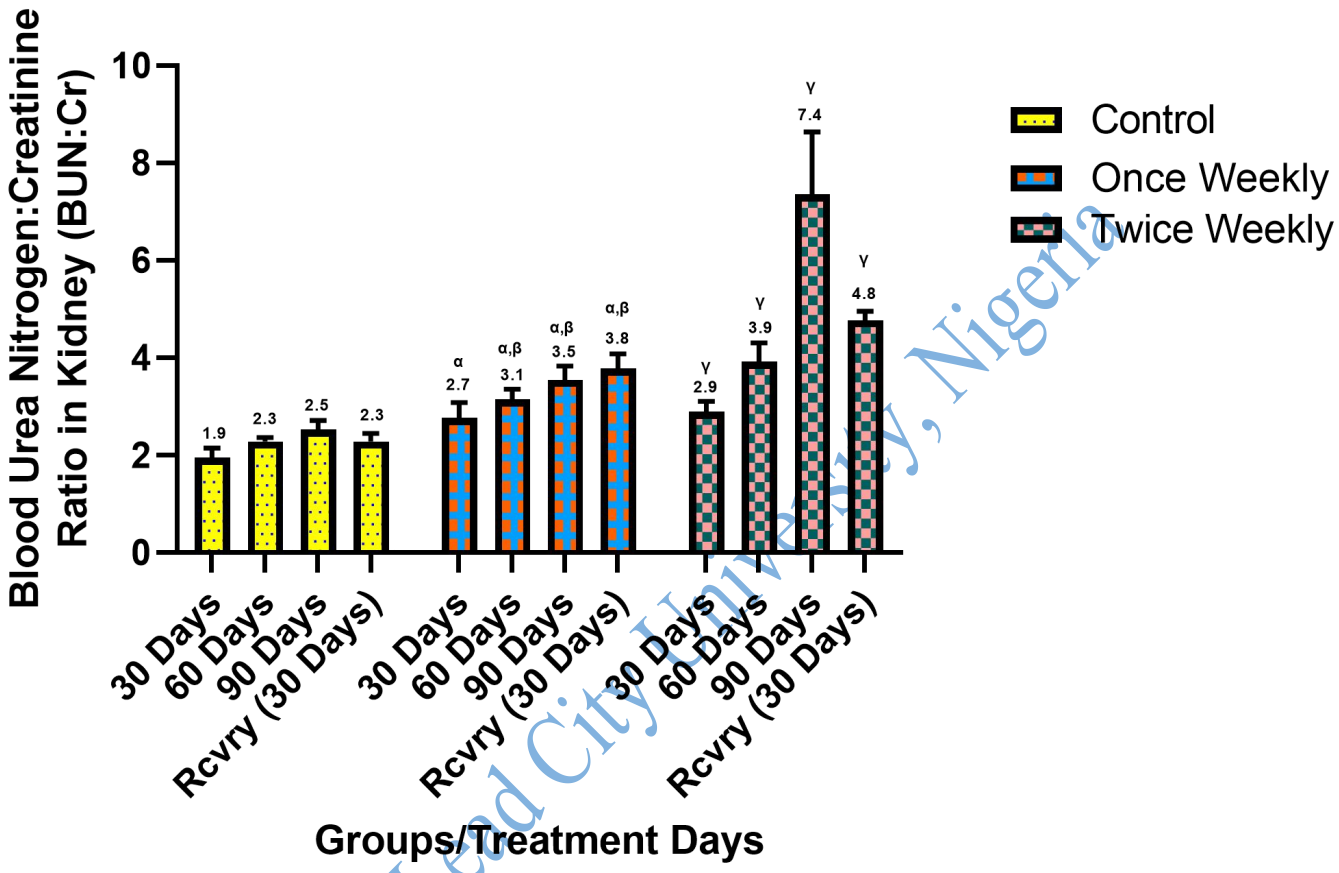

Figure 4.31: Effect of Postinor 2 Intake on the Level of Blood Urea Nitrogen: Creatinine Ratio (BUN: Cr) in the Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19 Assessment of Postinor 2 Intake on Oxidative stress Biomarkers

4.19.1 Effects of Treatment on Catalase Activity in Reproductive and Vital Organs of Wistar Rats

The effect of Postinor 2 intake on reproductive (Figures 4.32a and 4.32b) and vital organs (Figures 4.32c and 4.32d) that were harvested was reviewed and it was discovered that significant reductions occurred in both once weekly and twice weekly ($p < 0.05$). The effect of Postinor 2 intake was more pronounced on organs such as uterus, ovary and kidney than in liver. The reduction was consistent over the entire period of treatment and recovery period only brought about slight changes which are still lower than what was observed in control ($p > 0.05$).

The liver had a different experience with Postinor 2 treatment, it had no significant reduction in activity of catalase except in the third month of twice weekly treatment where significance was observed when compared with control ($p = 0.0098$).

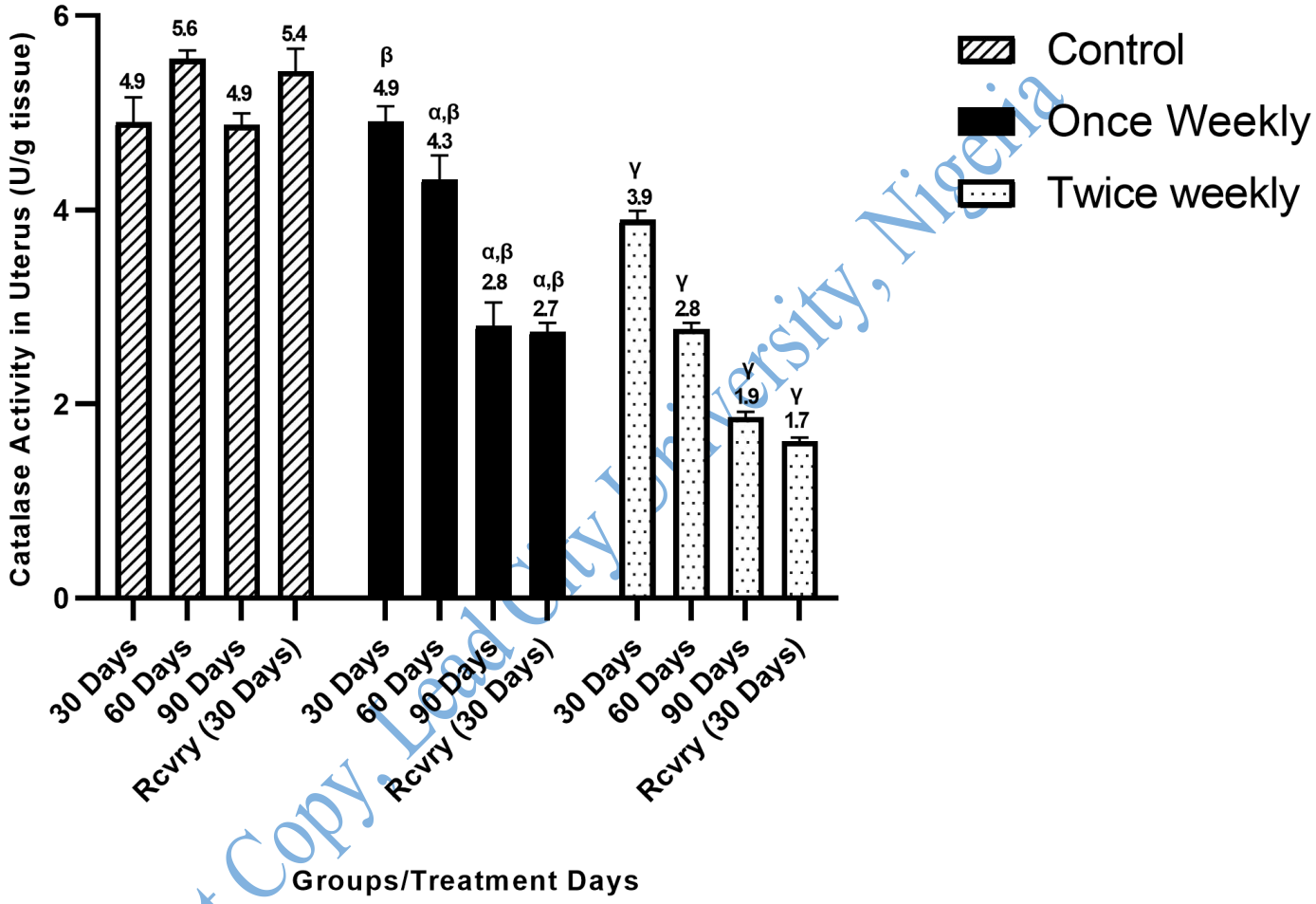

Figure 4.32a: Effect of Postinor 2 Intake on Catalase Activity in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

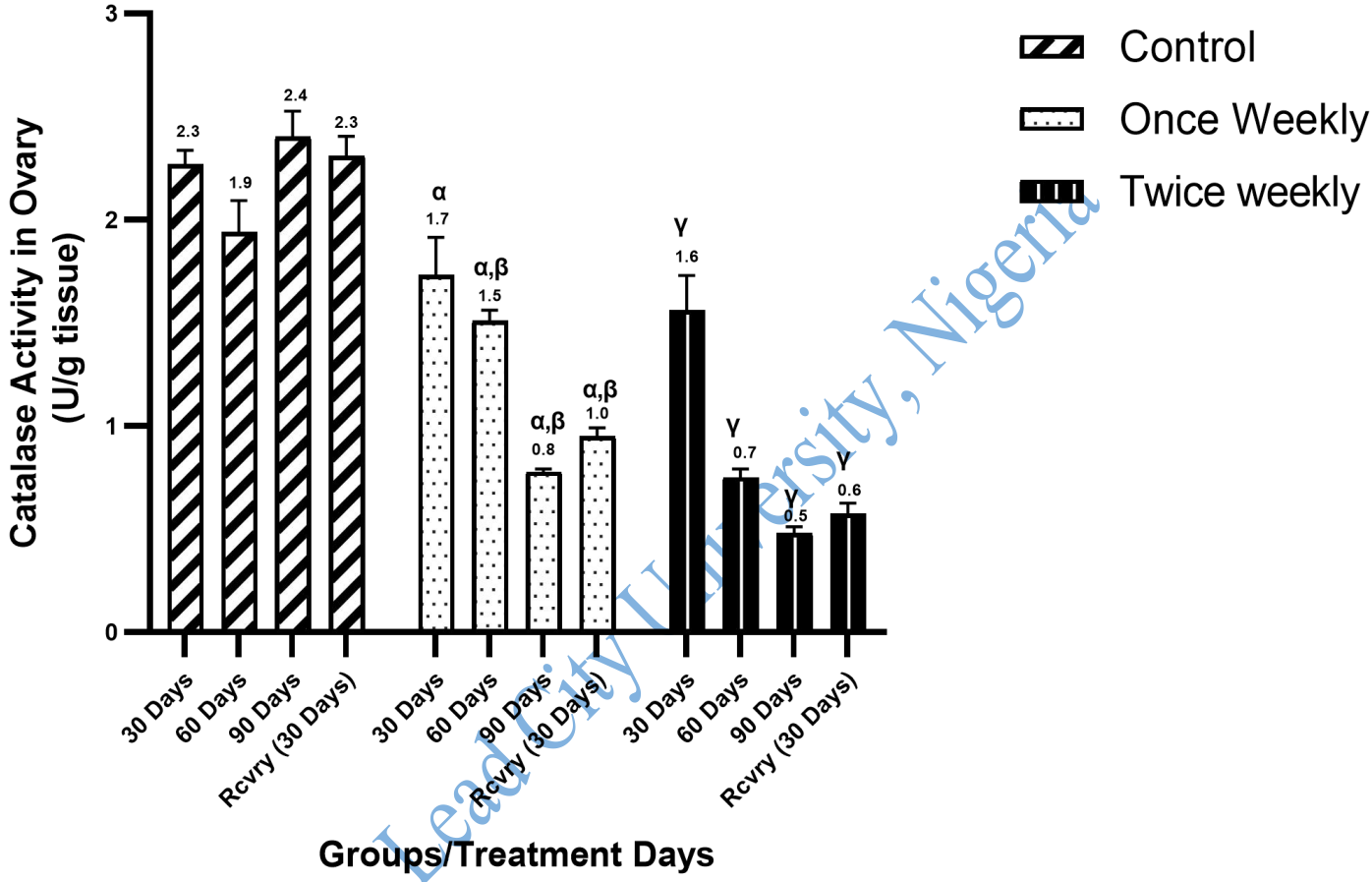

Figure 4.32b: Effect of Postinor 2 Intake on Catalase Activity in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

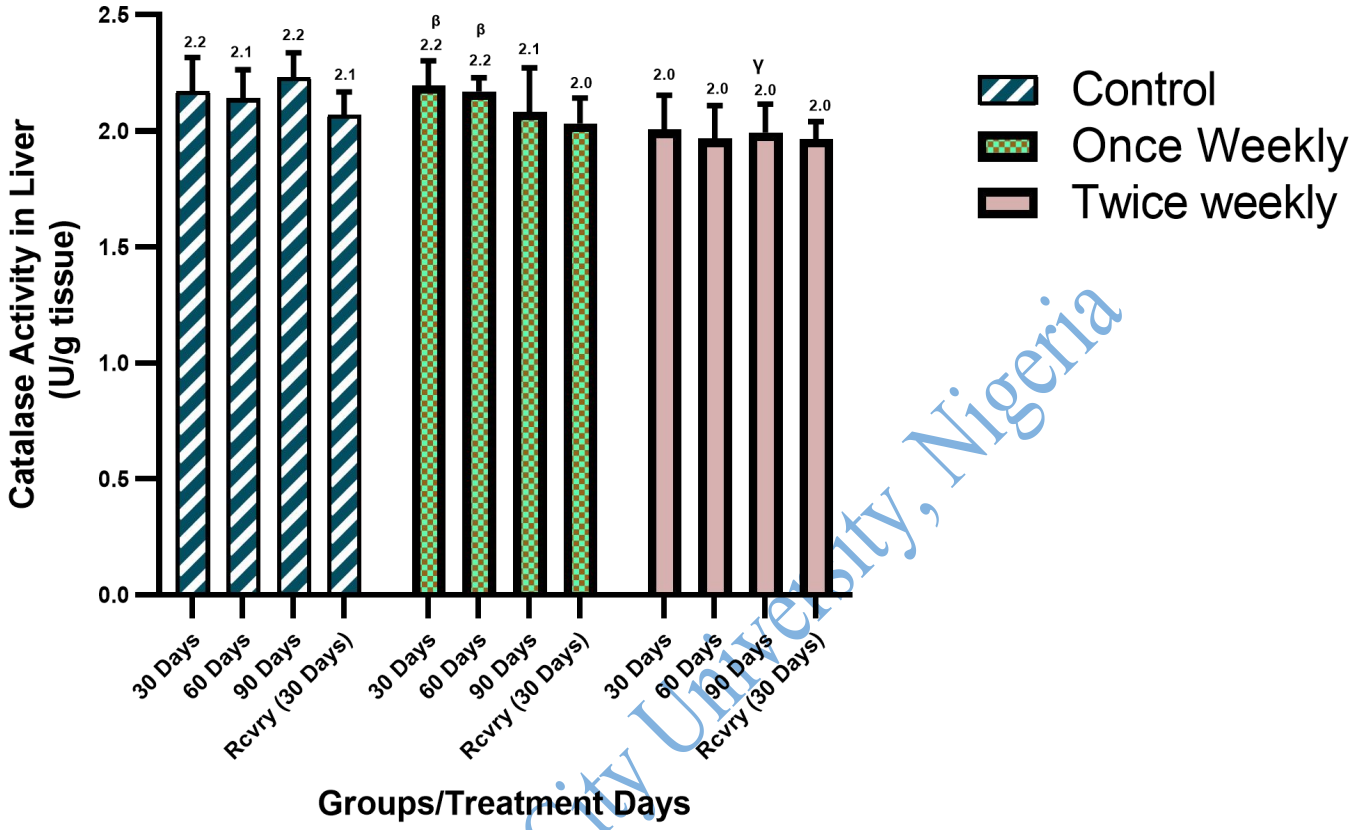

Figure 4.32c: Effect of Postinor 2 Intake on Catalase Activity in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

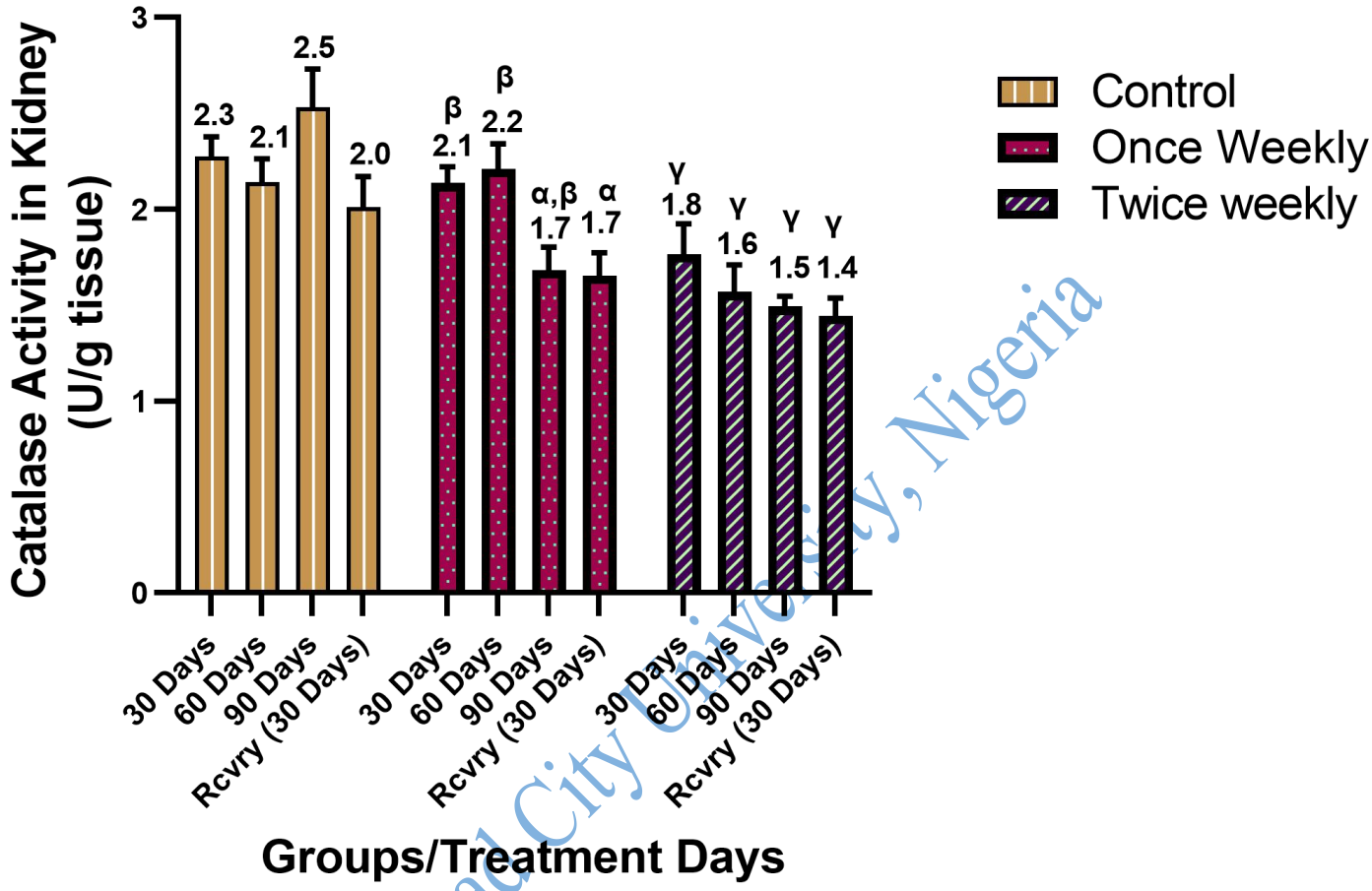

Figure 4.32d: Effect of Postinor 2 Intake on Catalase Activity in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19.2 Effects of Treatment on the Concentration of Reduced Glutathione (GSH) in Reproductive and Vital Organs of Wistar Rats

Figures 4.33a to 4.33d illustrate respectfully the effects of Postinor-2 intake on the level of Reduced Glutathione (GSH) in the uterus, ovary, liver and kidney of rats treated once and twice weekly for a period of 90 days and left for a recovery period of 30 days.

Compared to the control group, administration of Postinor-2 once weekly caused significant ($p < 0.05$) increase in the uterus GSH concentration in the first and second month of treatment. Whereas in the third month, there was a drastic fall in GSH concentration that was significant when compared with control in both once weekly and control ($p < 0.05$). The recovery phase returned the concentration of once weekly to a level that it was no longer significant different in once weekly compared to control ($p = 0.9371$) but was significantly lower in twice weekly ($p < 0.05$).

In ovary, treatment with Postinor 2 caused an elevation of GSH concentration in once weekly and twice weekly only in 30 days ($p < 0.05$). By 60 days, there was a reduction in concentration that was significant in once weekly but significant in twice weekly. The reduction continued in both groups at 90 days and was significant in both treated groups when compared with control ($p < 0.05$). A recovery occurred after been left untreated for 30 days causing an increase in the concentration of GSH in once weekly ($p = 0.2887$) but not in twice weekly which experienced only a 2.0 % decrease in concentration and significant against control ($p < 0.05$).

In the liver, the concentration of GSH was only significantly higher in once weekly ($p = 0.057$) and not in twice weekly ($p = 0.5114$) at 30 days. By the end of 90 days, there was a significant decrease in twice weekly ($p = 0.0026$). A comparison of both groups was also significant ($p =$

0.0352). The recovery period brought about not much difference in the treated groups ($p = 0.0014$) as there was further decrease in twice weekly ($p < 0.05$) but once weekly had gained a little increase in concentration though still significantly lower than control ($p < 0.05$).

Effects of Postinor 2 on the kidney was seen in the marked reduction in GSH concentration in once weekly and twice weekly ($p < 0.05$). This significantly reduced value was observed in all the treated months and also in the recovery period ($p < 0.05$). Although there was an increase in GSH concentration in the post treatment phase, it was not enough to match up with what was observed in control as the concentration of GSH in control was surprisingly increasing over the months.

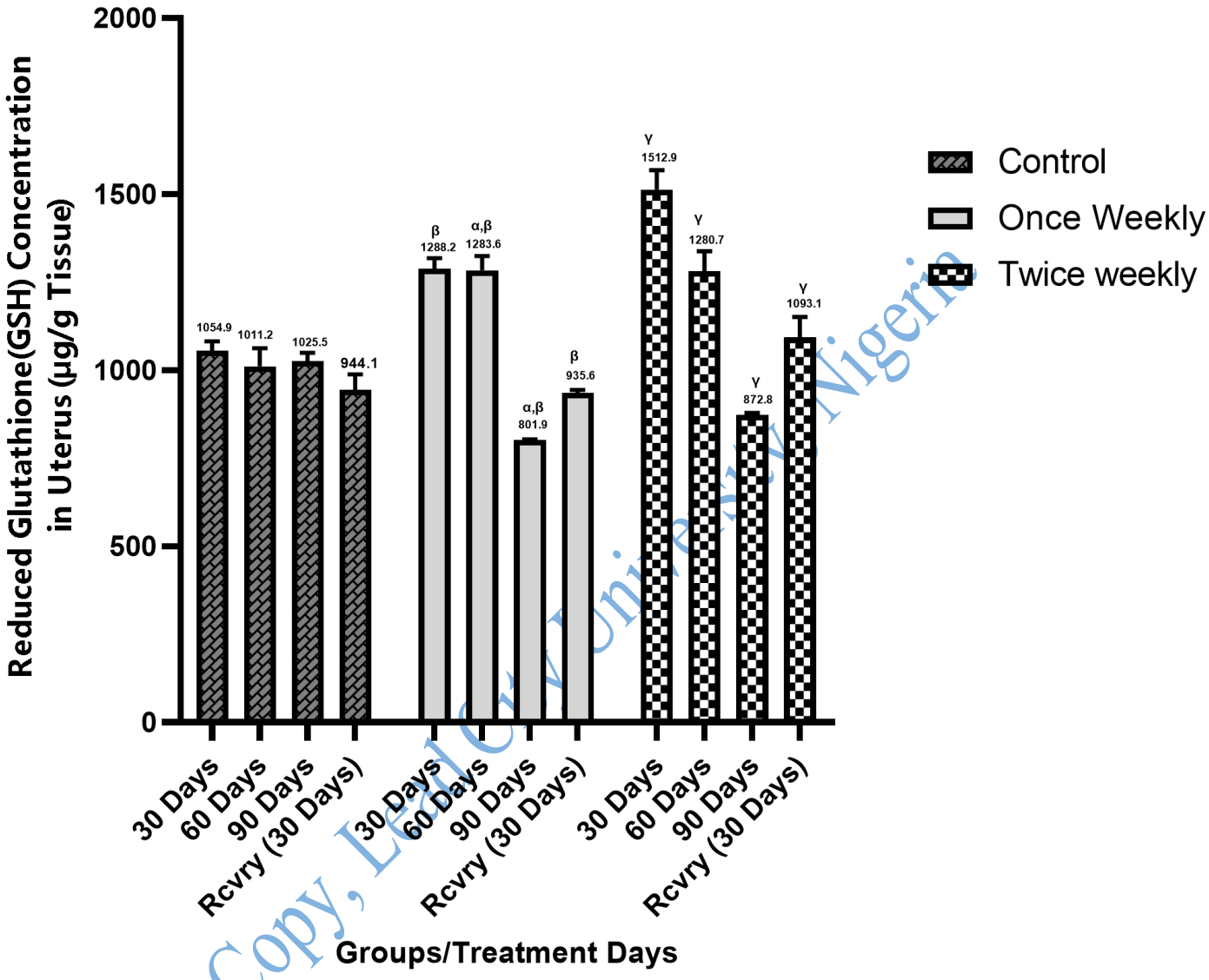

Figure 4.33a: Effect of Postinor 2 Intake on Reduced Glutathione (GSH) Concentration in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

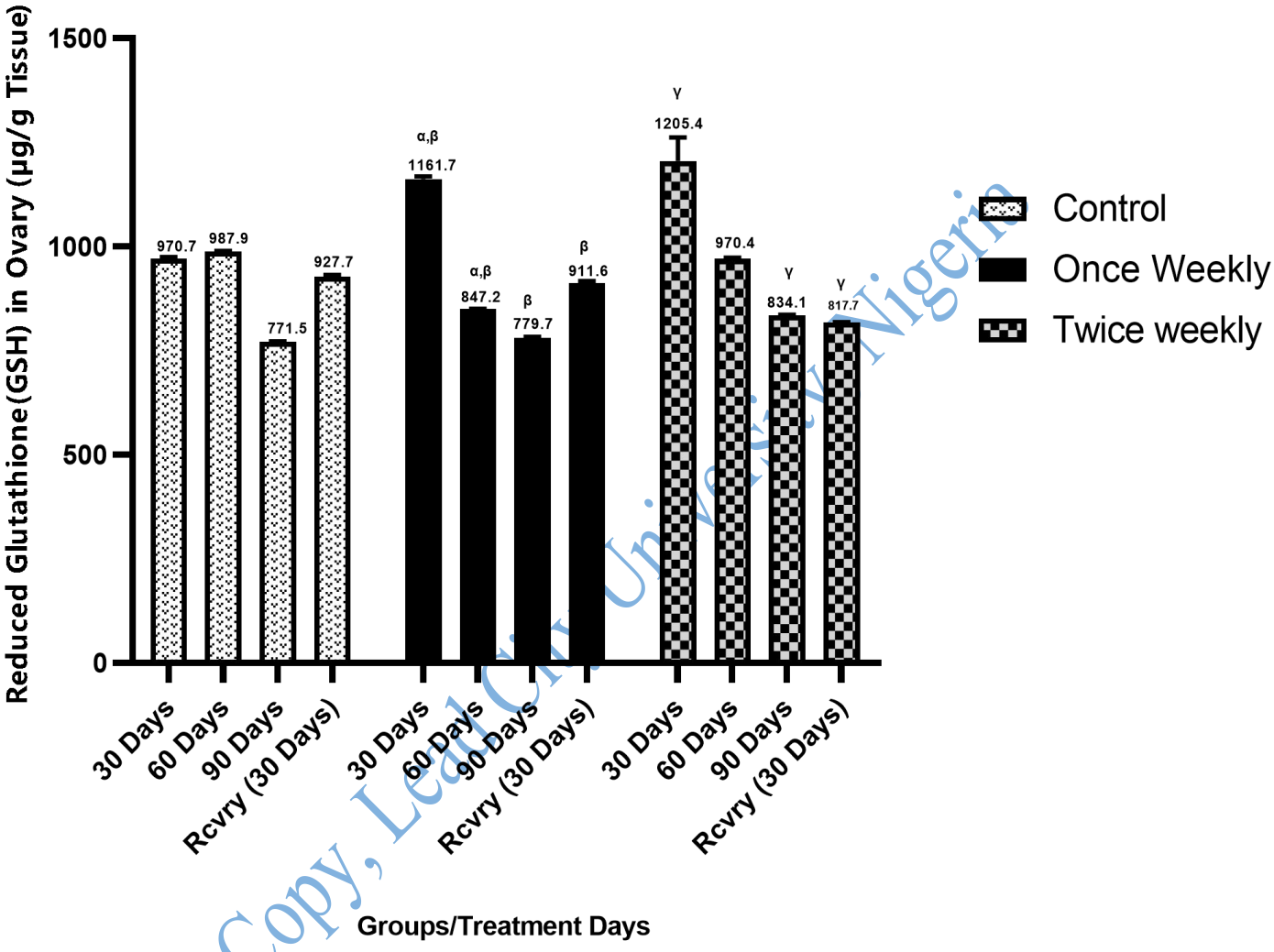

Figure 4.33b: Effect of Postinor 2 Intake on Reduced Glutathione (GSH) Concentration in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

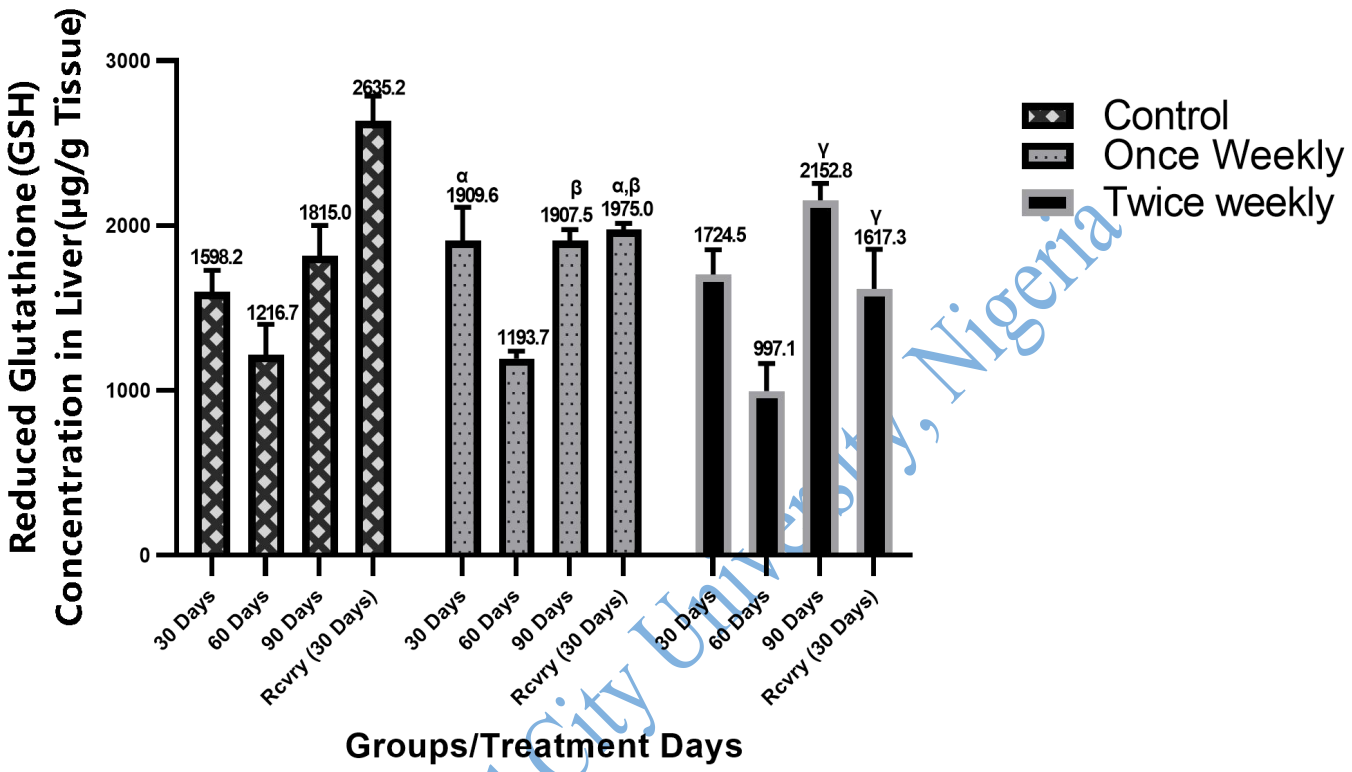

Figure 4.33c: Effect of Postinor 2 Intake on Reduced Glutathione (GSH) Concentration in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

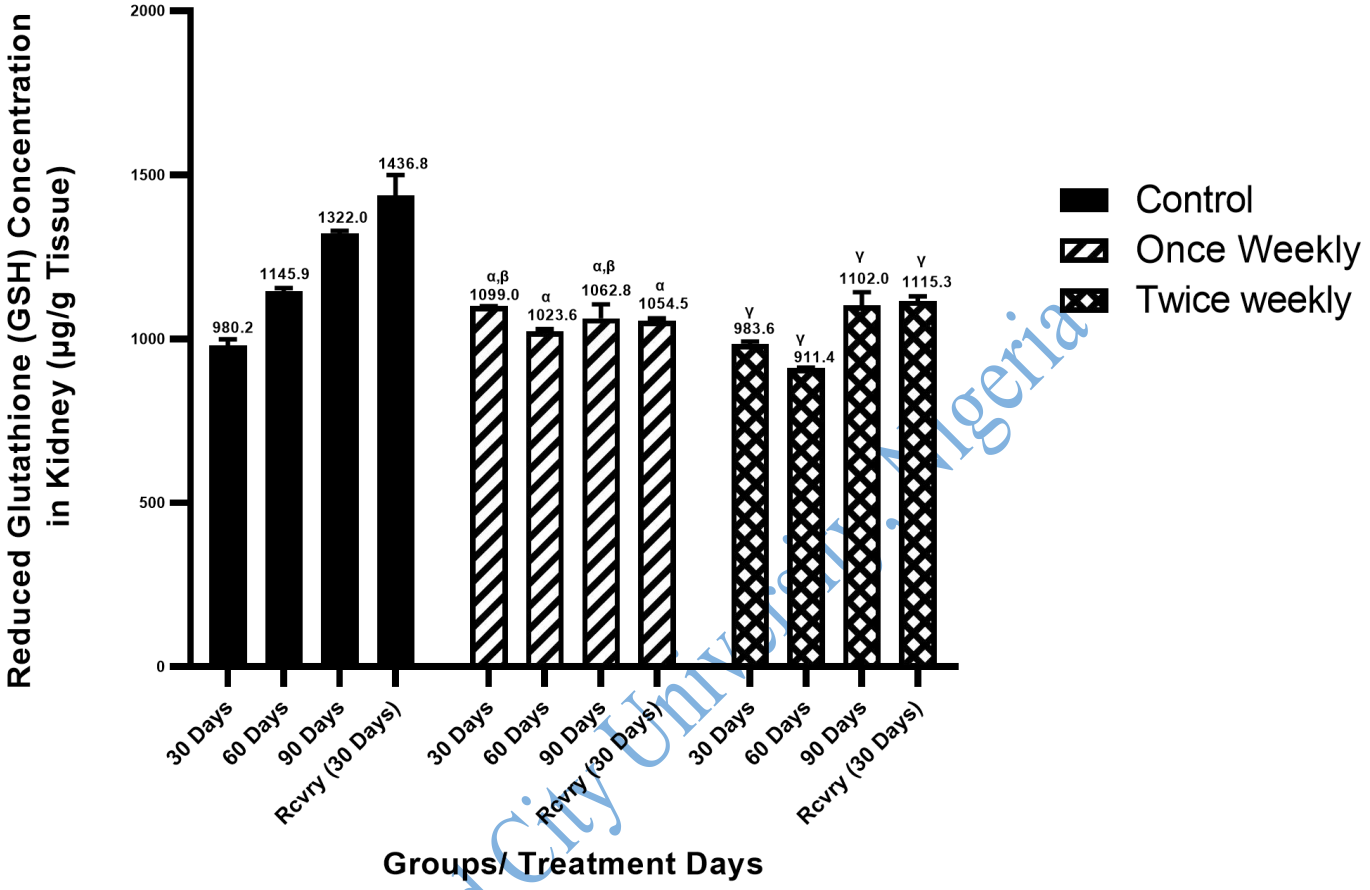

Figure 4.33d: Effect of Postinor 2 Intake on Reduced Glutathione (GSH) Concentration in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19.3 Effects of Postinor 2 Intake on the Concentration of Glutathione Peroxidase (GPx) in Reproductive and Vital Organs of Wistar Rats

The effects of Postinor-2 intake on the level of Glutathione peroxidase (GPx) in harvested organs namely; uterus (Figure 4.34a), ovary (Figure 4.34b), liver (Figure 4.34c) and kidney (Figure 4.34d) of rats treated once and twice weekly for a period of 90 days and left for a recovery period of 30 days. Compared to the control group, administration of Postinor-2 caused a significant decrease in the uterus GPx level once weekly and twice weekly ($p < 0.05$) in the first 30 days of treatment. The decrease continued in the second month ($p < 0.05$), third month ($p < 0.05$). A comparison of both treated groups showed no significance in all the months ($p > 0.05$).

In ovary, treatment with Postinor 2 did not cause any significant effect in the level of GPx in the treated groups in the first 30 days ($p > 0.05$). By 60 and 90 days, there was a reduction in concentration that was significant in once weekly and twice week ($p < 0.05$). A significant decrease in twice weekly relative to once weekly was observed only in the second month ($p = 0.0212$).

In the liver, there was no significant difference at 30 and 60 days in the treated groups until the end of 90 days, when significant decrease in twice weekly was observed ($p = 0.0026$). A comparison of both groups was also significant ($p = 0.0352$). The kidney showed significant reduction in GSH concentration in both treated groups that was time dependent ($p < 0.05$). Post treatment showed recovery in the uterus, only in once weekly of the ovary and liver while there was no improvement in the kidney GSH concentration.

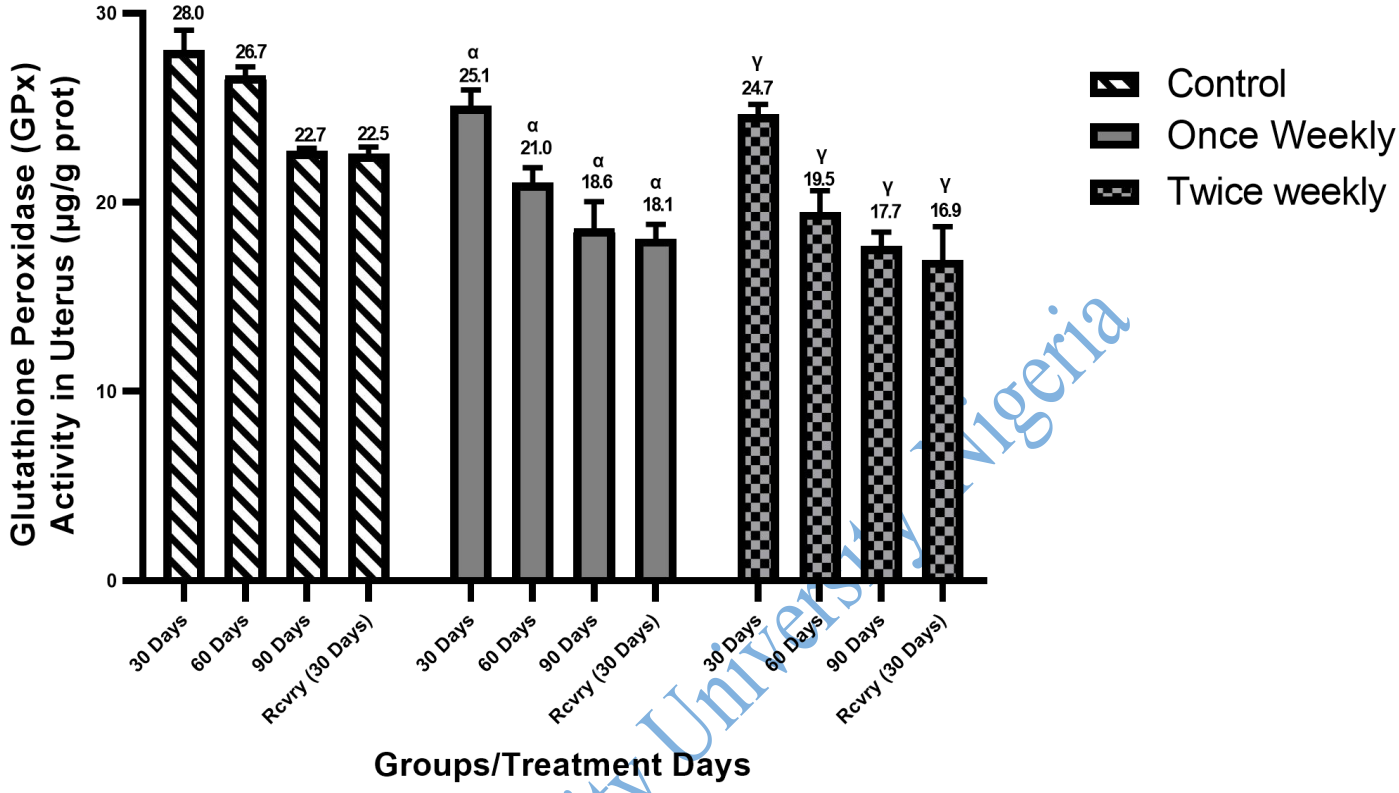

Figure 4.34a: Effect of Postinor 2 Intake on Glutathione Peroxidase (GPx) Activity in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

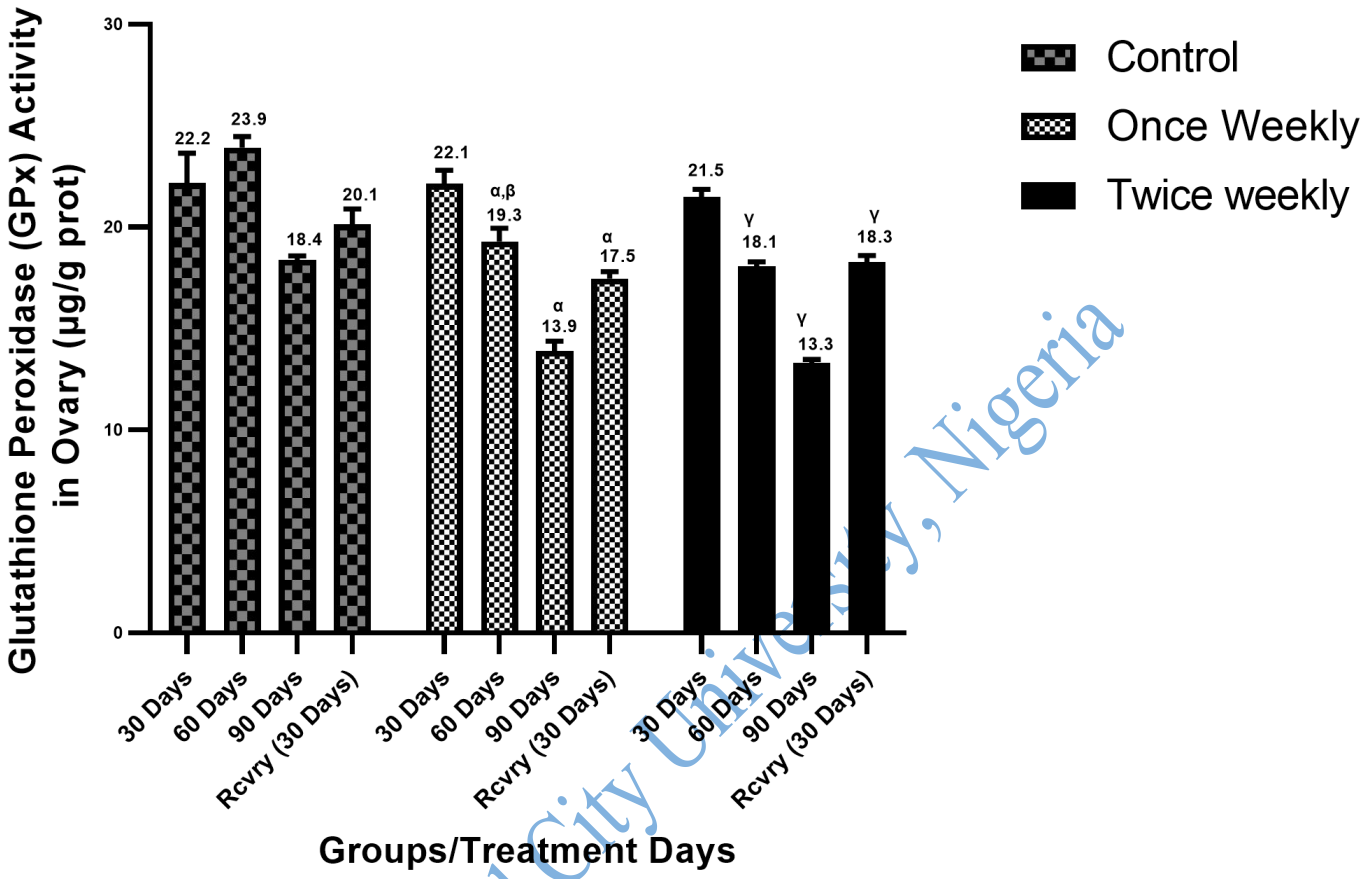

Figure 4.34b: Effect of Postinor 2 Intake on Glutathione Peroxidase (GPx) Activity in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

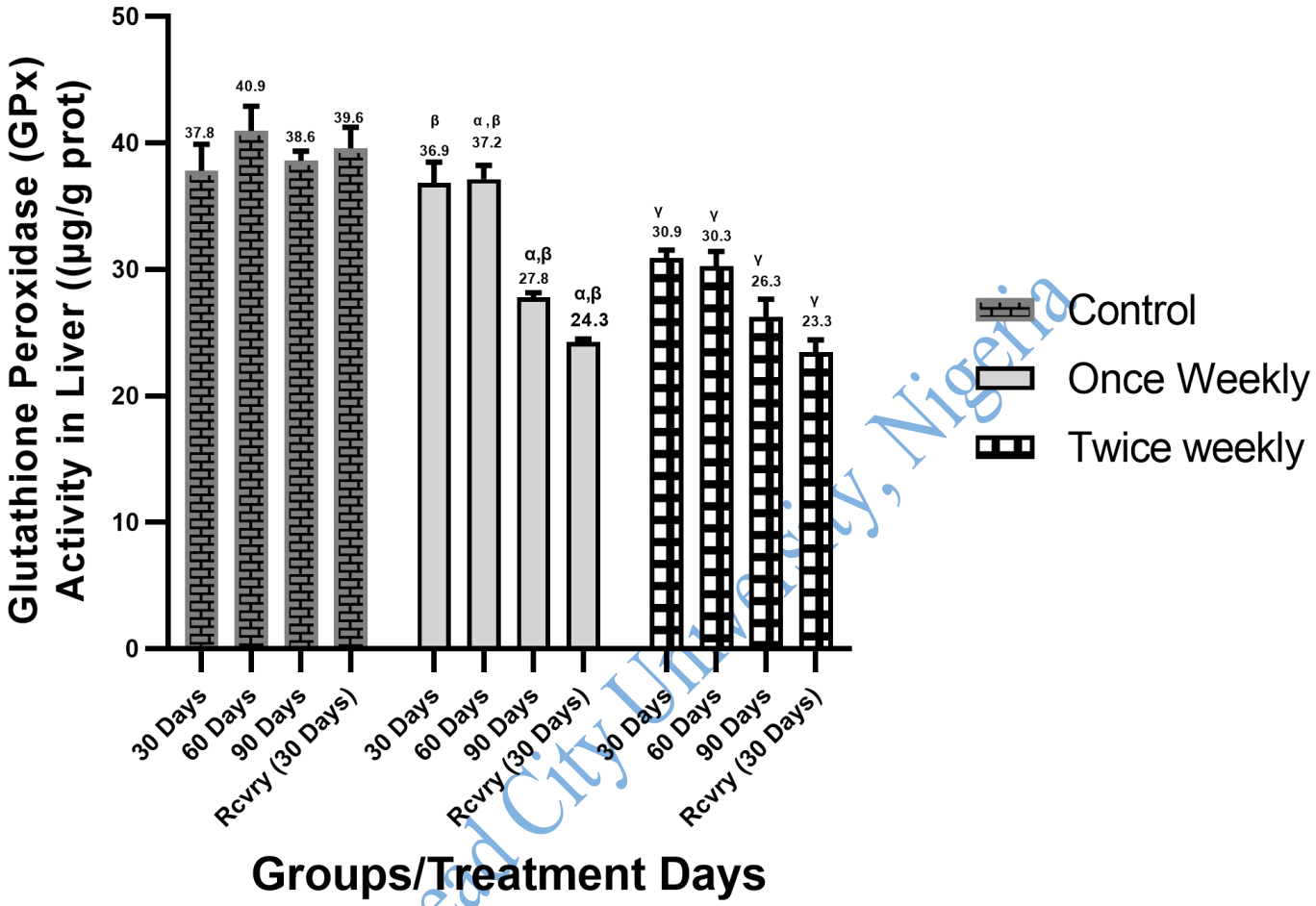

Figure 4.34c: Effect of Postinor 2 Intake on Glutathione Peroxidase (GPx) Activity in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

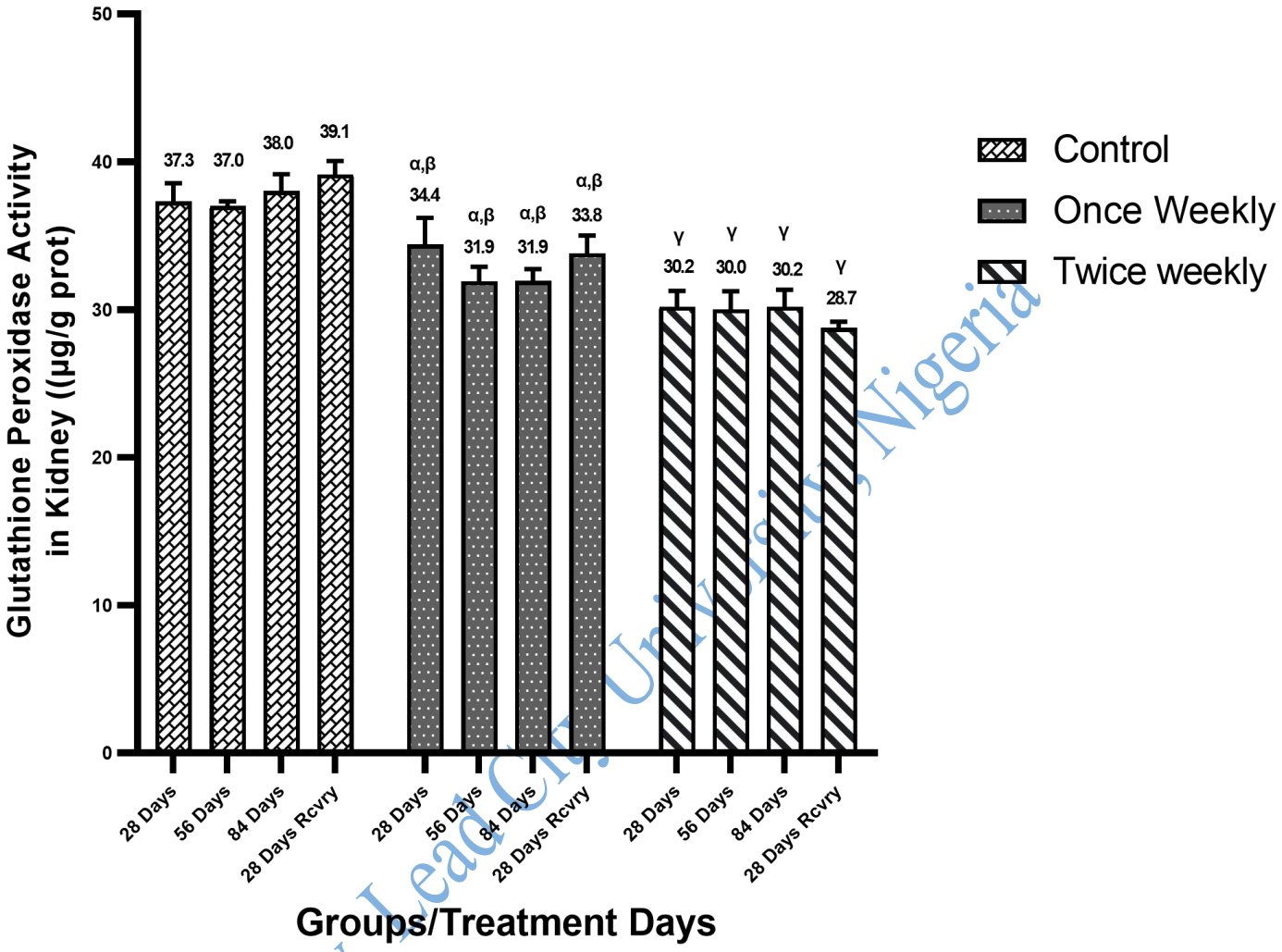

Figure 4.34d: Effect of Postinor 2 Intake on Glutathione Peroxidase (GPx) Activity in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19.4 Effects of Postinor 2 on the Concentration of Glutathione-S-Transferase in Reproductive and Vital Organs of Wistar Rats

The effect of Postinor 2 intake on the level of GST in the reproductive organs (Figures 4.35a and 4.35b) and vital organs (Figures 4.35c and 4.35d) that were harvested was reviewed and compared with control. At 30 days of treatment, the level of GST in the uterus of twice weekly was twice what was observed in the control while once weekly was about 9 % higher than control ($p < 0.05$). There was a further increase in GST at 60 days in once weekly while there was a 7 % decline in twice weekly, never the less they were both significantly higher than control. By 90 days, both groups had decrease in GST concentration which was still significantly higher than control ($p < 0.05$).

The ovary presented similar scenario in that there was also an elevated concentration of GST in twice weekly (43.9 ± 1.7) than in once weekly (28.1 ± 1.2) and both were significantly higher than control (23.1 ± 1.4) ($p < 0.05$). At 60 and 90 days, there was a decrease in the level of GST in both treated groups which was still higher than in control ($p < 0.05$).

In the liver, at 30 days, once weekly was not significantly higher than control but twice weekly was. At 60 and 90 days, both treated groups became significantly higher than control ($p = 0.0098$).

In the kidney, there was no significant difference among the treated groups and control in the first 2 months. By 90 days, only twice weekly was significantly higher ($p < 0.05$) than control.

The 30 days post treatment period caused a decrease in GST concentration in both treated groups in the ovary, liver and kidney whereas, only once weekly group of the Uterus show improvement while there was no improvement in twice weekly.

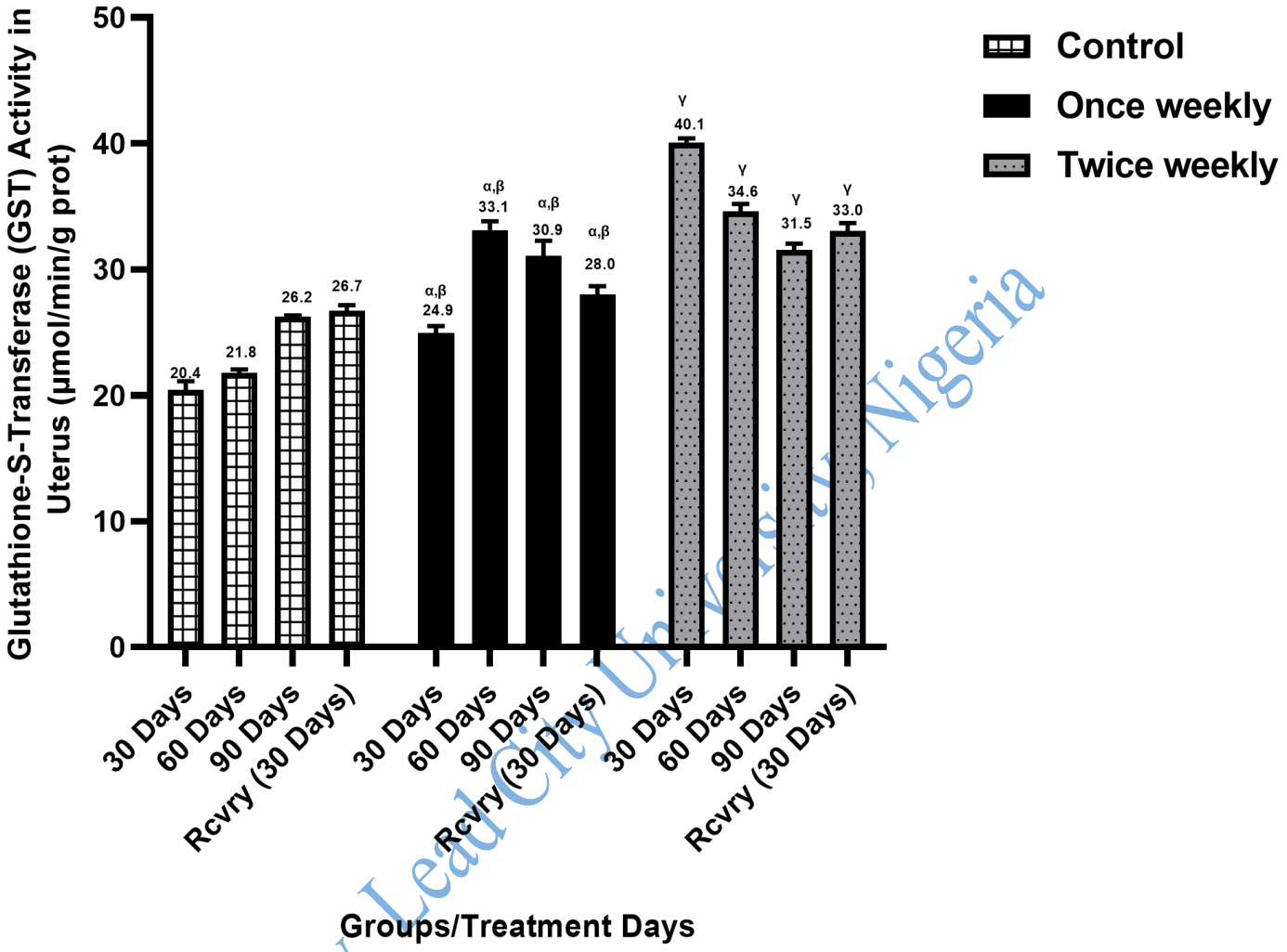

Figure 4.35a: Effect of Postinor 2 Intake on Glutathione-S-Transferase (GST) Activity in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

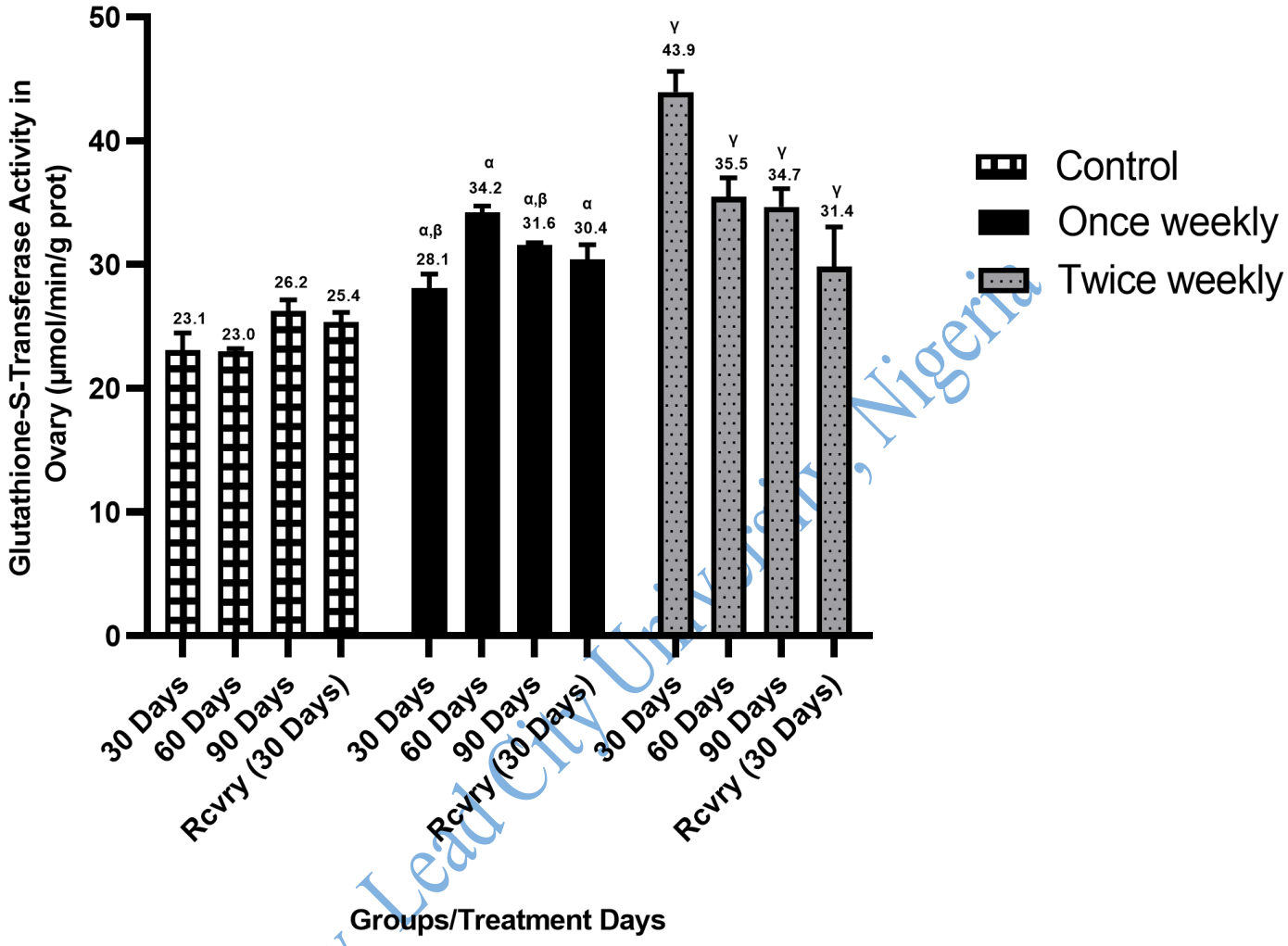

Figure 4.35b: Effect of Postinor 2 Intake on Glutathione-S-Transferase (GST) Activity in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

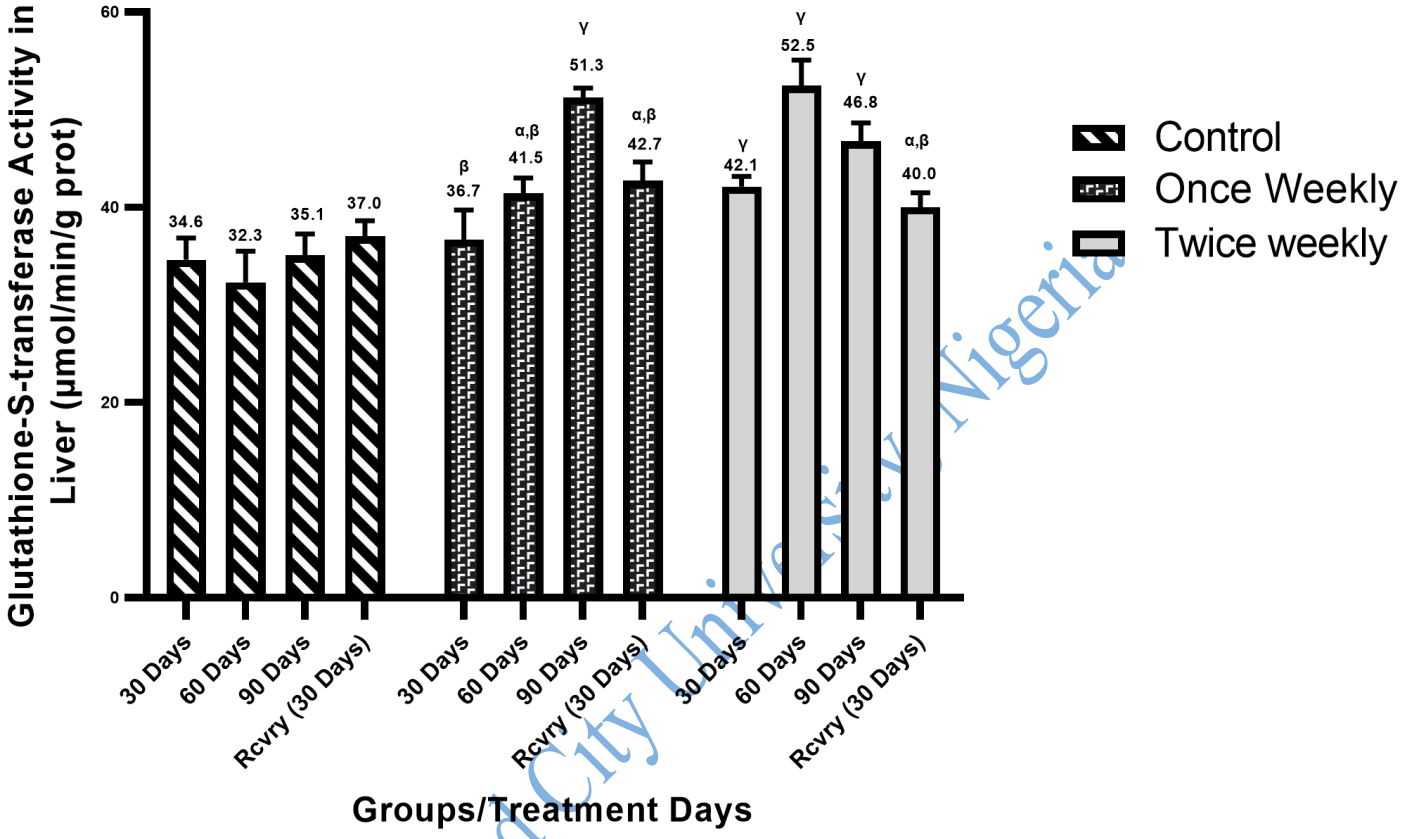

Figure 4.35c: Effect of Postinor 2 Intake on Glutathione-S-Transferase (GST) Activity in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

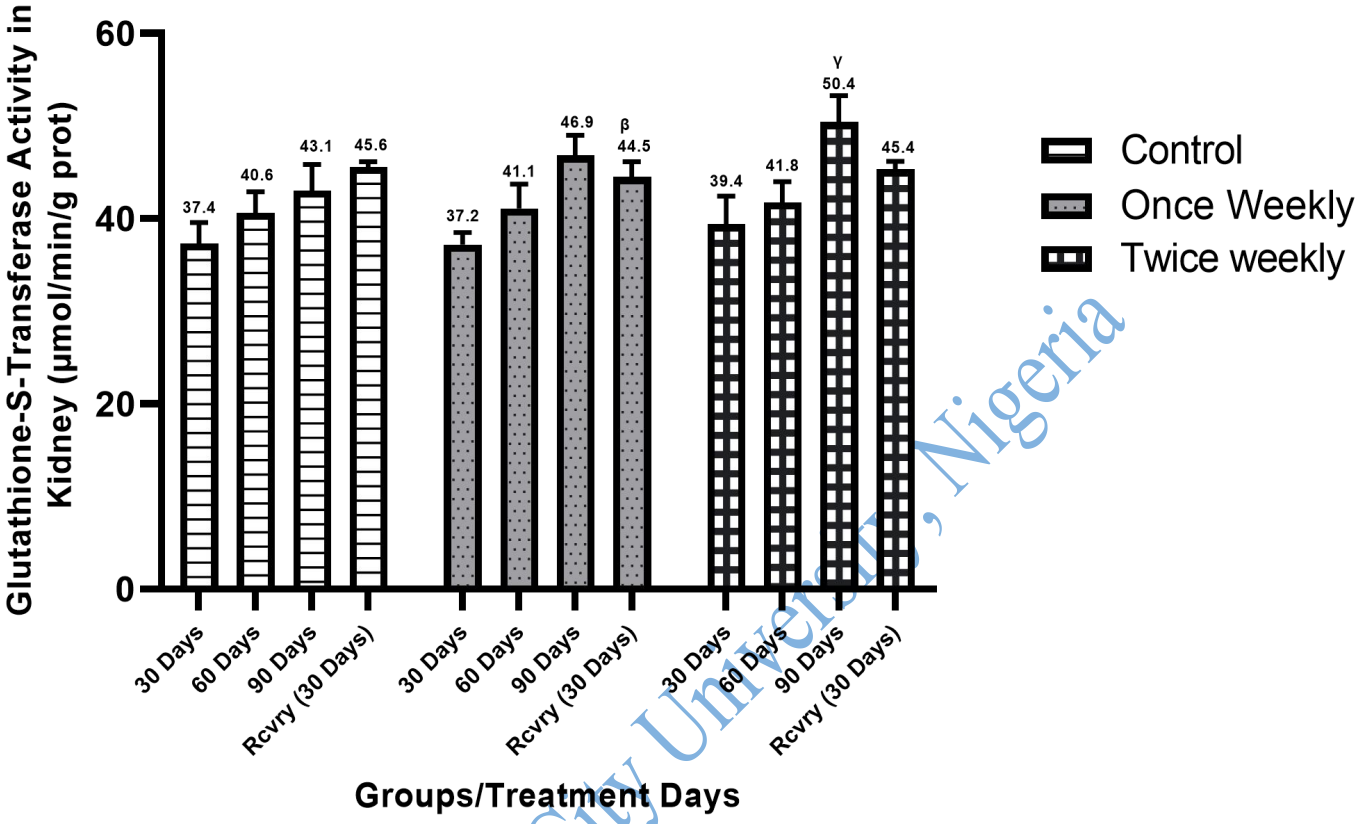

Figure 4.35d: Effect of Postinor 2 Intake on Glutathione-S-Transferase (GST) Activity in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19.5 Effects of Postinor 2 on the Activity of Superoxide Dismutase (SOD) in Reproductive and Vital Organs of Wistar Rats

The effect of Postinor 2 intake on the activity of SOD in the reproductive organs (Figures 4.36a and 4. 36b) and vital organs (Figures 4. 36c and 4. 36d) was compared with control.

In the uterus and ovary, there was a significant and progressive decrease in the activity of SOD with time and the worse decrease was recorded at 90 days. Similarly, decrease in SOD activity was also more pronounced in animals treated twice weekly when compared with once weekly ($p < 0.05$)

In the liver, at 30 and 60 days, activity of SOD in only twice weekly group was significantly lower than control. At 90 days, SOD activity in both treated groups were significantly lower than control. ($p < 0.05$). The decrease in SOD activity in twice weekly was also time dependent. In the kidney, there was no significant difference among the treated groups and control throughout the treatment periods ($p > 0.05$) except at 90 days where SOD level in twice weekly became significantly lower than control ($p = 0.0015$)

When treatment was withdrawn (recovery period), there was improvement only in the liver and kidney while none was observed in the uterus and ovary.

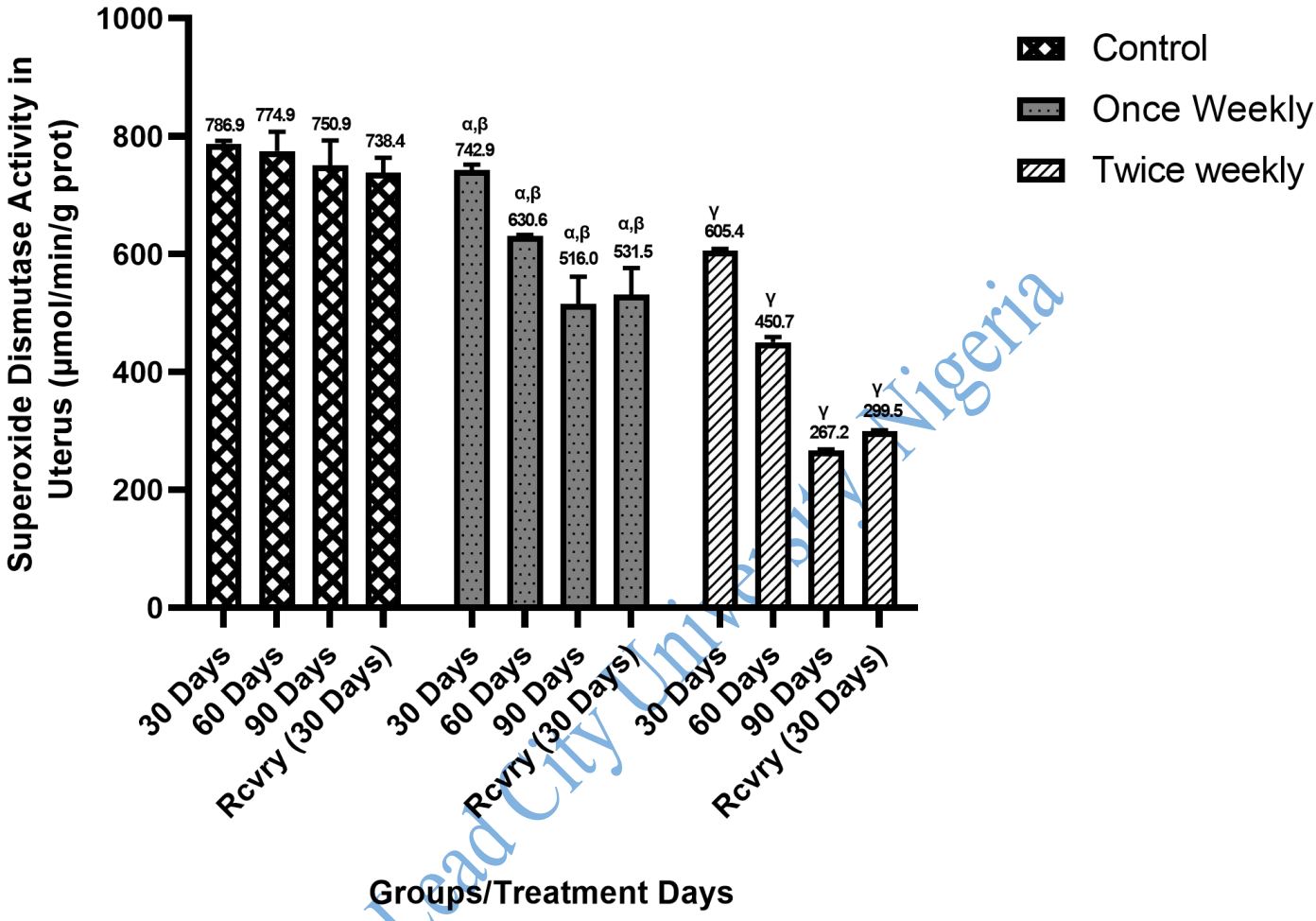

Figure 4.36a: Effect of Postinor 2 Intake on Superoxide Dismutase (SOD) Activity in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

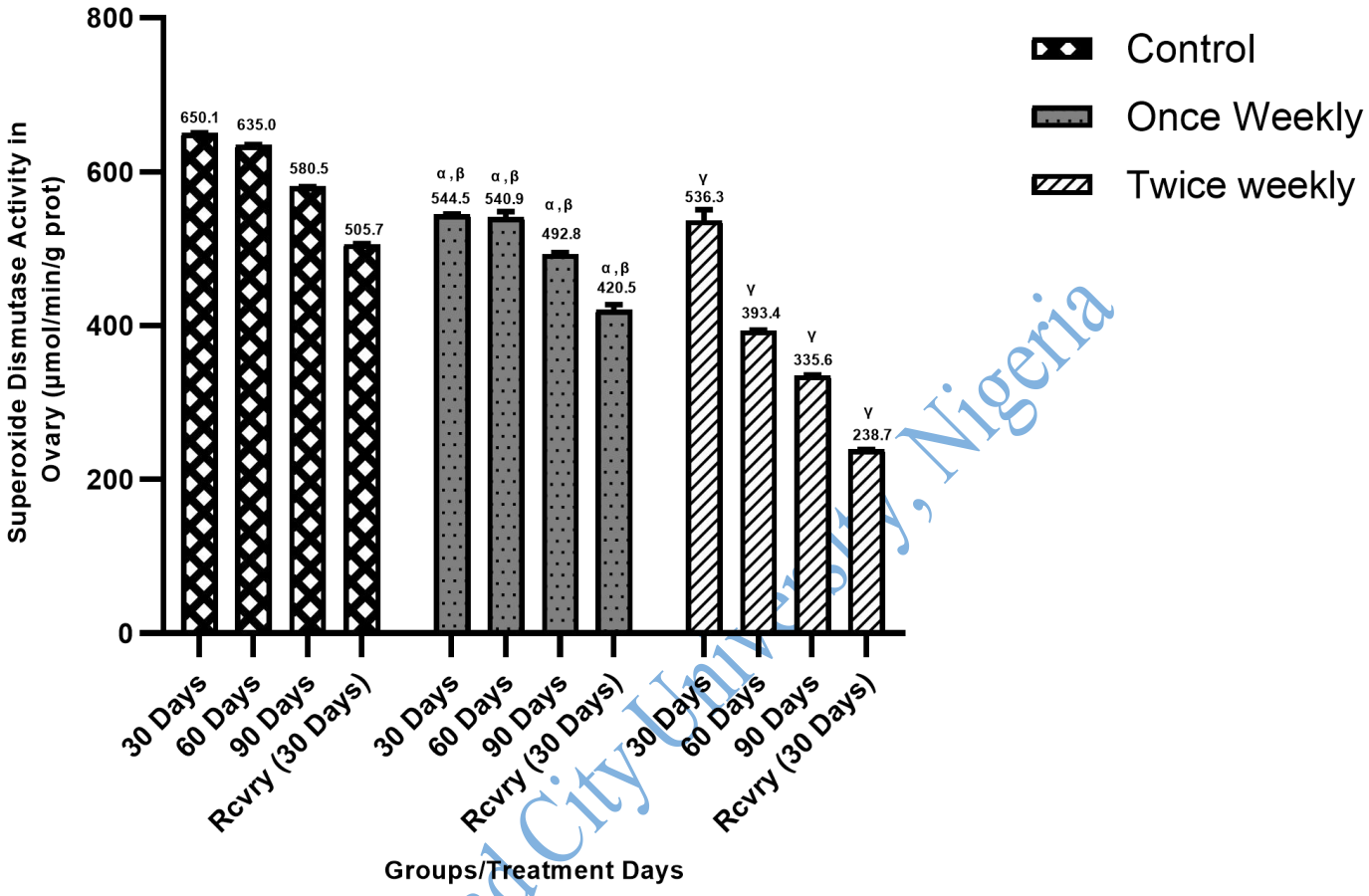

Figure 4.36b: Effect of Postinor 2 Intake on Superoxide Dismutase (SOD) Activity in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

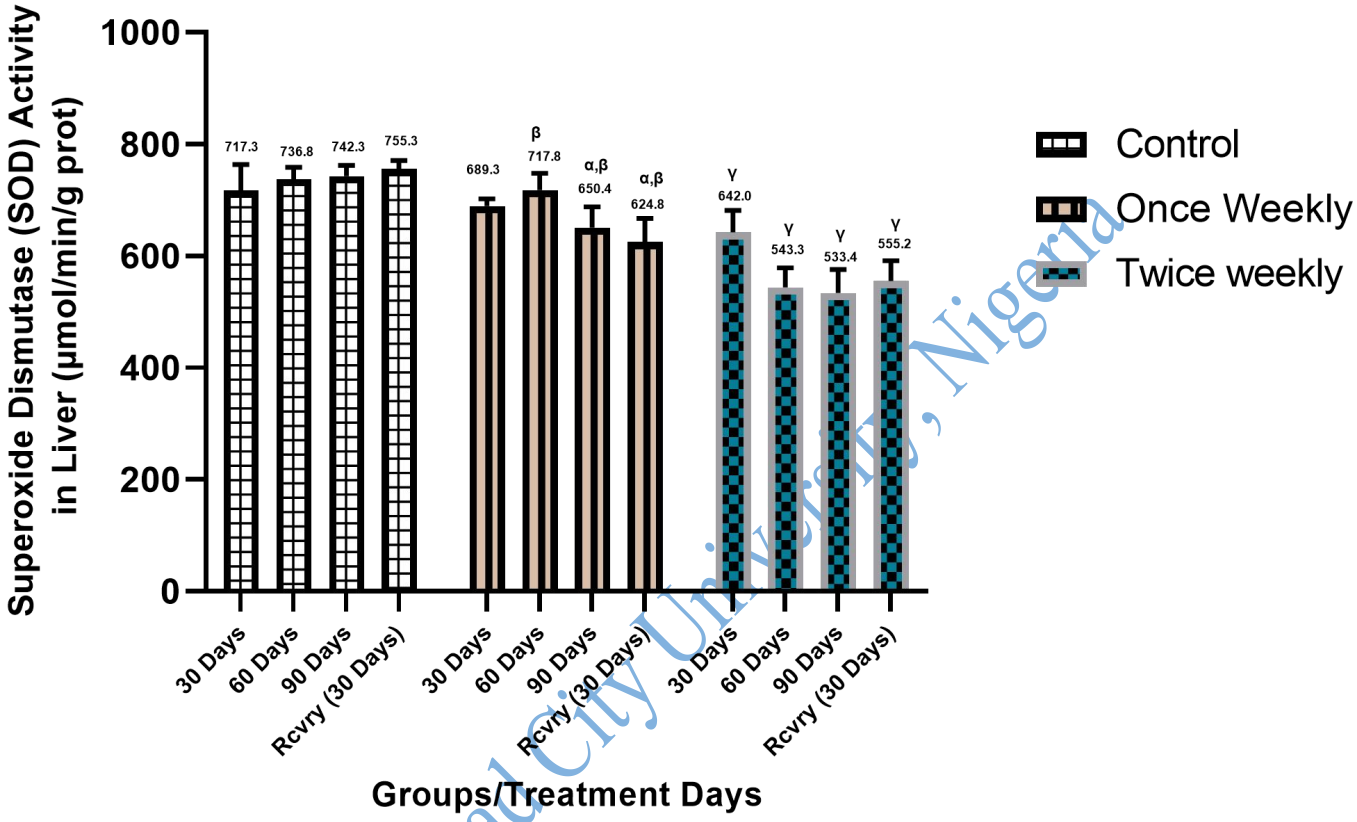

Figure 4.36c: Effect of Postinor 2 Intake on Superoxide Dismutase (SOD) Activity in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

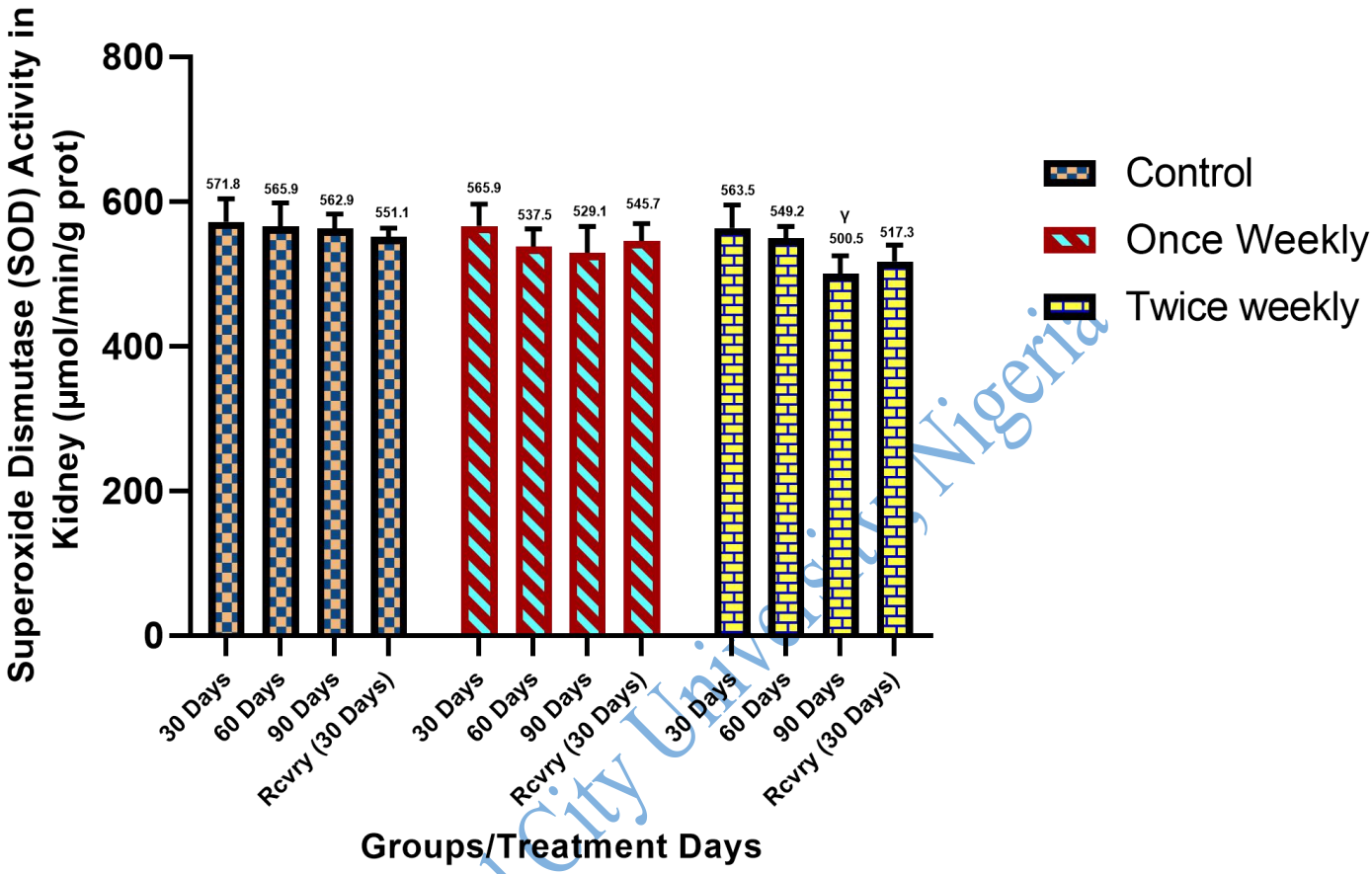

Figure 4.36d: Effect of Postinor 2 Intake on Superoxide Dismutase (SOD) Activity in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.19.6 Effects of Postinor 2 Intake on the Level of Malondialdehyde (MDA) in Reproductive and Vital Organs of Wistar Rats

The effects of Postinor-2 intake on the level of MDA in the uterus (Figure 4.37a), ovary (Figure 4.37b), liver (Figure 4.37c) and kidney (Figure 4.37d) of rats treated once and twice weekly for a period of 90 days and left for a recovery period of 30 days.

In the first 30 days of treatment, there was a 21 % increase in MDA level in the uterus of twice weekly which was triple what was observed in once weekly (7 %) when compared with the control ($p < 0.05$). In the second month, both once weekly and twice weekly had a reduction in concentration from 4.9 ± 0.1 and 6.7 ± 0.2 to 3.8 ± 0.1 and 5.7 ± 0.4 respectively, which was still significantly higher than control (3.7 ± 0.3). At 90 days, once weekly experienced an increased again in MDA level while twice weekly further reduced, yet MDA was higher in the treated groups than in control ($p > 0.05$).

In the ovary, treatment with Postinor 2 caused a significant increase in the level of MDA in the treated groups and this elevation was progressive throughout the treatment days in twice weekly group. Once weekly experienced a reduction at 60 days that was elevated again with time and these were significantly higher than in the control ($p < 0.05$).

In the liver, no significant difference was found between all groups in the first month. Also, once weekly and control were not significantly different in all the period of treatment and post treatment ($p > 0.05$). Meanwhile, twice weekly became elevated by 60 days and was significant compared to control and once weekly. This was also the case at 90 days and post treatment ($p < 0.05$)

Effects of Postinor 2 on the kidney showed no significant difference between once weekly and control in the first two months. Albeit, once weekly was significantly lower than twice weekly. The highest value of MDA was observed in twice weekly throughout the period of treatment ($p < 0.05$).

The Recovery period brought about a reduction in MDA level especially in the uterus, liver and kidney. The ovary only experienced recovery in the once weekly group while elevation in MDA concentration continued in twice weekly.

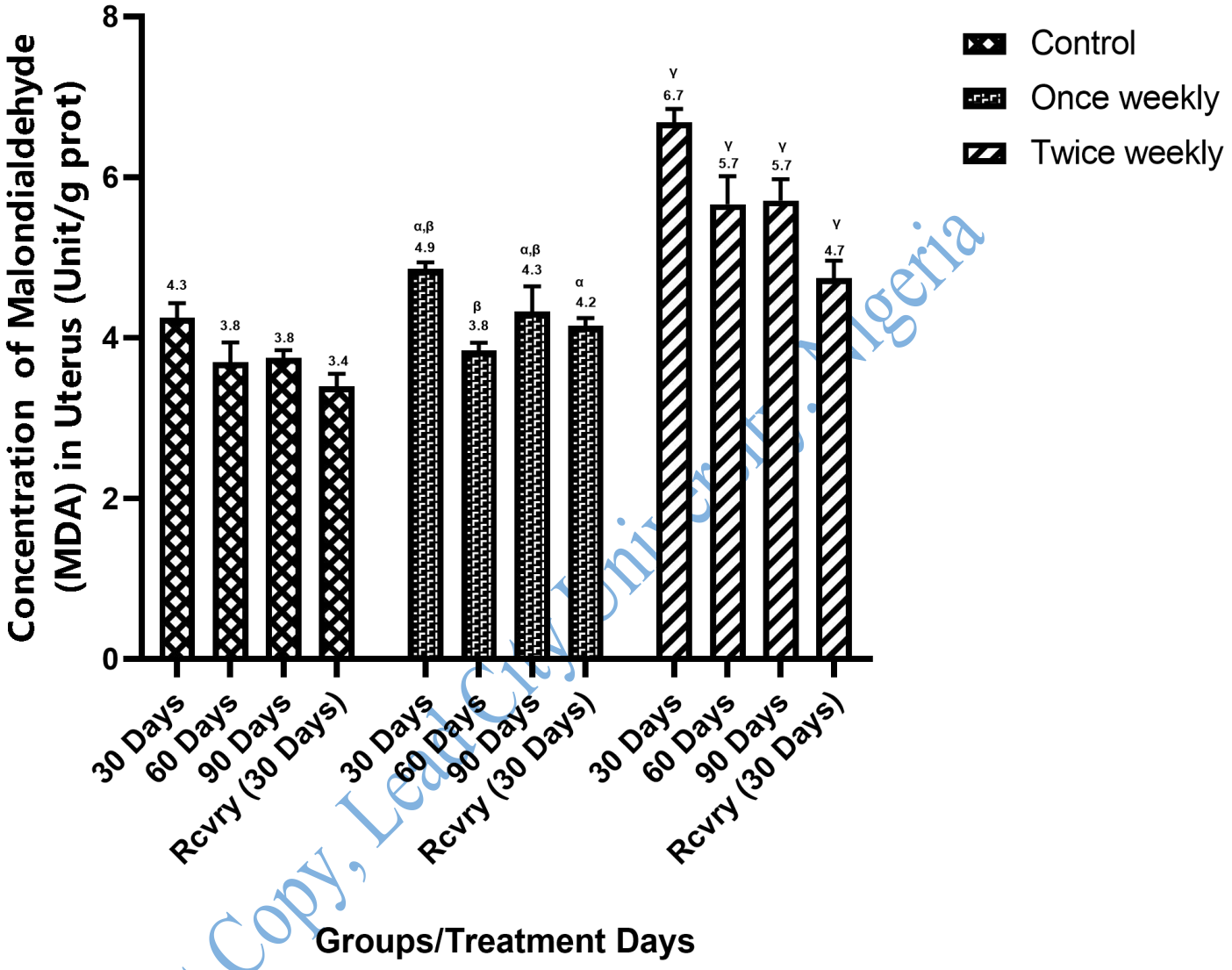

Figure 4.37a: Effect of Postinor 2 Intake on Malondialdehyde (MDA) Concentration in Uterus of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

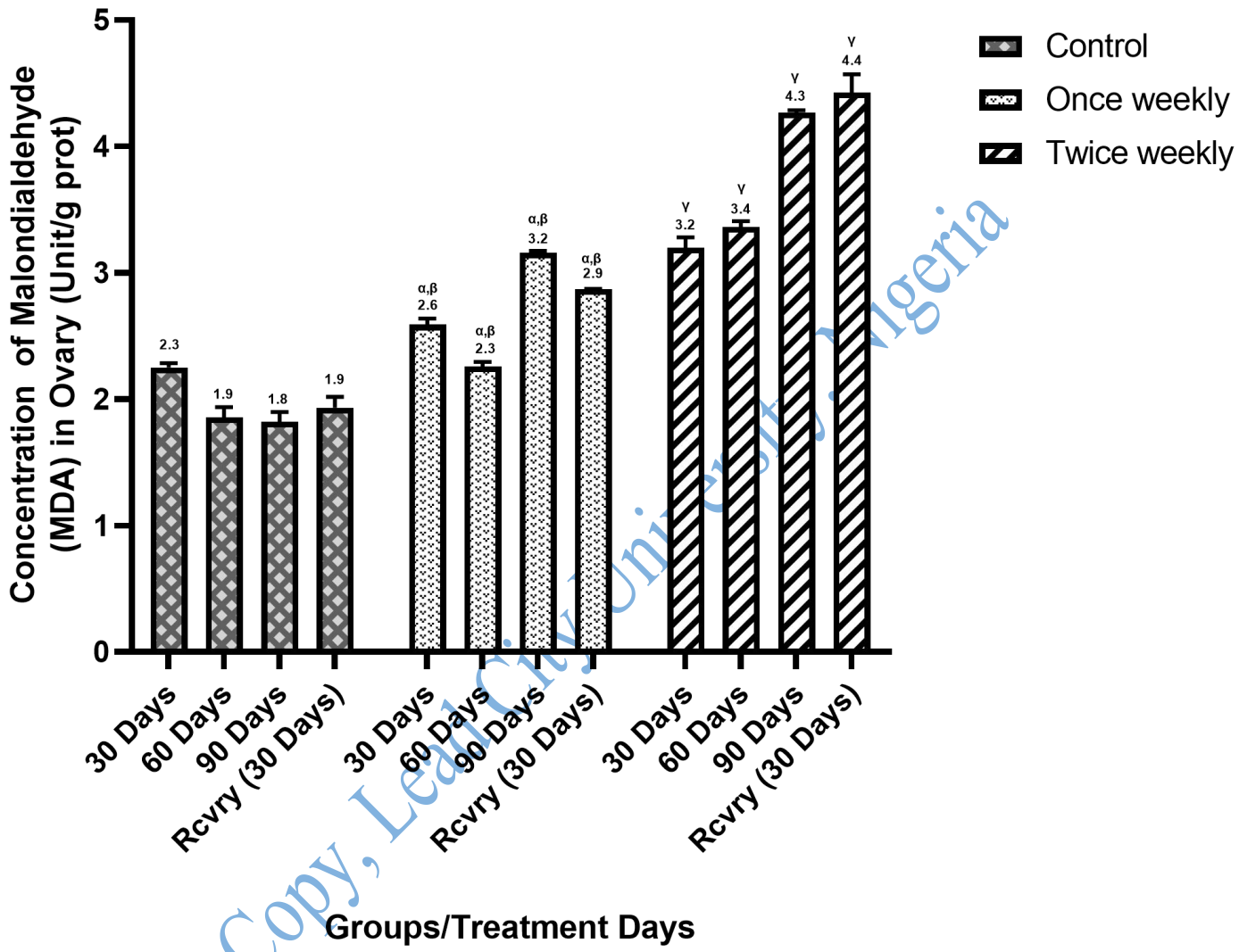

Figure 4.37b: Effect of Postinor 2 Intake on Malondialdehyde (MDA) Concentration in Ovary of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

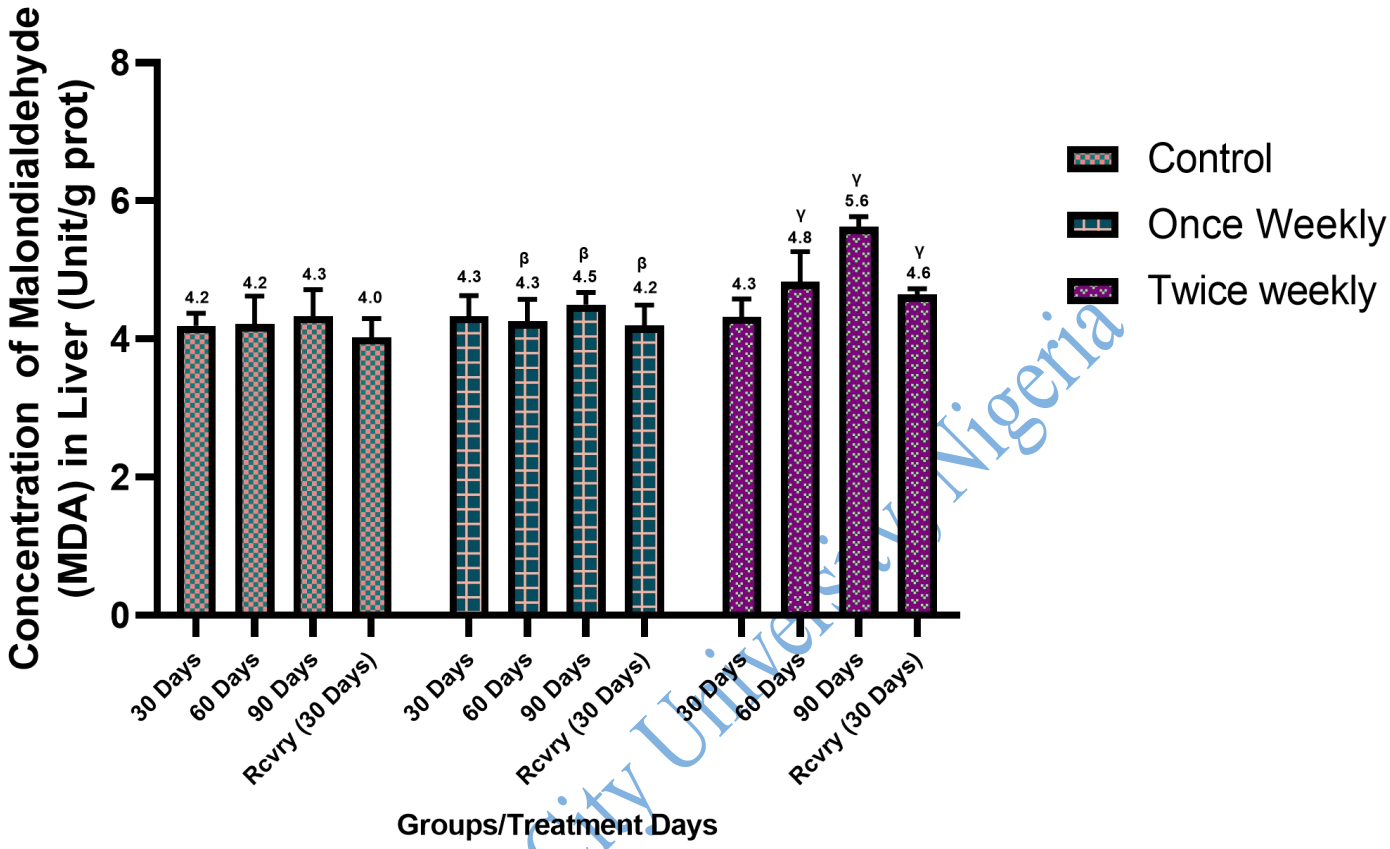

Figure 4.37c: Effect of Postinor 2 Intake on Malondialdehyde (MDA) Concentration in Liver of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

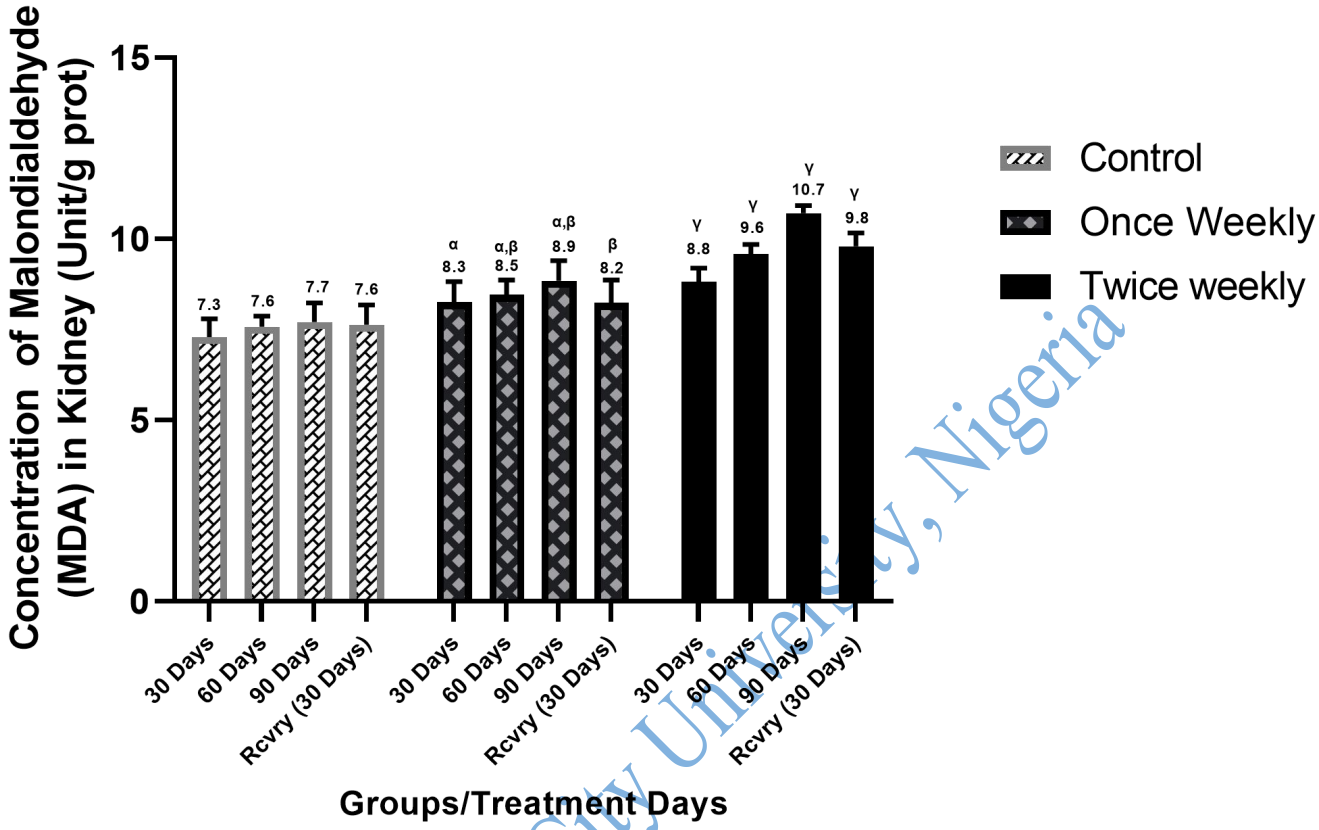

Figure 4.37d: Effect of Postinor 2 Intake on Malondialdehyde (MDA) Concentration in Kidney of Wistar Rats

Values were expressed as Mean ± Standard deviation (SD), n = 5. **α** showed a statistical significance when once weekly was compared with control ($p < 0.05$). **β** indicated a statistical significance when once weekly was compared with those treated twice weekly ($p < 0.05$). **γ** showed significance when twice weekly was compared with control ($p < 0.05$).

4.20 Discussion of Findings

Increased sexual activities among young adults has caused a high prevalence of unintended pregnancies and unsafe abortion. This situation has encouraged incessant and indiscriminate use of emergency contraceptives.

A cross sectional study conducted among the students of Lead City University, Ibadan using a semi-structured questionnaire gathered responses from 483 participants in total. This was done to investigate the knowledge and use of contraceptives, prevalent type of contraceptives, frequency of use, and common side effects experienced by users. The demographic characteristics showed the highest frequency of respondents were between the ages of 18 and 25 years 312 (64.59 %), followed by 85 (17.59 %) between 26 and 32 years old, 73 (15.11 %) were under the age of 18 years, and 9 (1.85 %) above the age of 32 years. This agrees with a study by Kgosiemang and Blitz [1] as well as Gbagbo and Nkrumah[2] where the highest frequency of ECP' users was below the age of 25 while Kalamer *et al* [3] found the highest frequency of ECP among women below 30. Out of the 85 under the age of 18, 26 ECPs (5.38 %) use contraception while 47 (9.73 %) did not. 208 (43.06 %) participants between 18 and 25 use contraception while 104 (21.53 %) did not. 31 (6.42 %) participants between the ages of 26 and 32 (11.18 %) use no contraceptive while 8 (1.66 %) participants above 32 years of age used contraception while 1 participant (0.21 %) did not.

The marital status of participants showed that the highest frequency, 422 (87.7 %), were single while 61 (12.63 %) were married. Most users of ECPs are unmarried sexually active students [1,3]. They concluded that students from urban areas, with higher level of education had better knowledge than their rural counterparts which may explain why the participants in this study have a good knowledge of contraceptive use. The high level of knowledge about contraception

observed in this study may be a result of the educational level of participants. This outcome is geographically influenced and many studies where low levels of knowledge and use of contraception were observed reported significant educational variations among participants [4,5,6,7].

The awareness and knowledge of contraception methods available showed (37.97 %) did not use any form of contraception because they have never had sex or by preference while 299 (62.03 %) students used one or more methods of contraception. This showed a good level of awareness and use of contraception among the students which is an indication of good knowledge of available contraceptive methods. This study agrees with a research study among Saudi women where adequate knowledge about ECPs was also observed [4]. Crawford *et al*, [5] observed a low level of contraceptive knowledge (16.7 %) among unmarried sexually active in Ilorin, southwestern Nigeria, which does not agree with this research.

The method of contraception with the highest frequency was pills only 196 (40.58 %), followed by 140 (28.99 %) who used condom alone, 82 (16.98 %) used a combination of condoms and pills, 24 (4.97 %) used injectible, 4 (0.83 %) used intrauterine devices, 11 (2.28 %) used herbs, concoction or other drugs which are designed originally as contraceptives such as ampicillin, salt and water mixture, alcohol or local herbs, 1 (0.21 %) used patches, and 2 (0.41 %) used implant. The specific type of pill among the participants who chose pills as their preferred method of contraception is 87 (43.07 %) used Postinor 2, 11 (5.44 %) used Afterpill, 78 (38.61 %) used Postpill, 10 (4.95 %) used Plan B, 12 (5.94 %) used combined oral contraceptives while 4 (1.94 %) used Morning After Pill. The frequency of pills taken by participants, 1 (0.69 %) used it daily, 97 (66.90 %) used it once in a while, 10 (6.90 %) used it once weekly, 26 (17.93 %) used it twice weekly, 10 (6.90 %) used it thrice weekly, while 1 (0.69 %) used it monthly. This disagrees with a study by Crawford *et al* [5] where they found the highest frequency of their

participants used male condoms (50.3 %) followed by ECP (16.7 %). A study by Kalamar *et al* [3] agrees with this study on the prevalent use of |ECPs among young women in Lagos and Ghana.

The fear associated with the use of contraceptives often comes from its negative side effects. Out of the 299 participants who used contraceptives, 49 (16.39 %) claimed they observed no side effects while 250 (83.61 %) said they did. The specific side effects observed by respondents from the use of contraceptives ranged from headache (8 %) to painful menstrual cycle (5 %), irregular menstruation (39 %), spotting (15 %), heavy menstrual flow (10 %), weight gain (20 %) and weight loss (3 %). The users of Pills have the highest distribution of side effects. A study by Elmaghraby *et al* [4] found that women who used ECPs experienced side effects but the prevalent side effect was mood swings. WHO statistics showed that the frequent use of ECP especially progestin-only contraceptives (POC) causes menstrual irregularities.

In the animal model aspect of the study, the observation of the Wistar rats before, during, and after treatments for physical and behavioral changes show that the control group looked healthier and more active throughout the experiment. The treated groups were usually sluggish immediately after treatment and took a while before resuming normal physical activities. The feeding pattern in the control group was more rapid and better than in the treated groups on the days of treatment but more rapid in the treated groups than in the control on other days. The death of rats in treated groups could be due to toxicity from the drug administered or from other diseases as a result of a compromised immune system.

Evaluation of the Effect of Postinor 2 on the Body Weights of Wistar Rats Before and After Treatments showed that th weight of control was significantly higher than the treated groups at 30 days. Surprisingly, by 60 days of treatment, once weekly was significantly lower than control

while twice weekly had become significantly higher. This was also the case at 90 days but by 30 days post-treatment and recovery, the body weight of the rats in the control had become significantly lower than both once weekly and twice weekly. This indicates that Postinor 2 caused a dose and time-dependent decrease in the body weights of the animals. This could be as a result of poor appetite after administration of the drugs. Moreover, the possibility that intake of Postinor-2 interfered with nutrient absorption and assimilation in the treated animals is worthy of note. Additionally, the decrease in weight may not be unconnected to the increased relative organ weights. This was not the observation of Shi *et al* [8], their study found no difference in the weights of animals treated with LNG and control.

The estrous cycle, which is the reproductive cycle in rodents, begins around the 26th day of life with the opening of the vagina. The estrus cycle is divided into four phases namely, proestrus, estrus, metetrus and diestrus. It is similar to the reproductive cycle in humans (the menstrual, ovarian, or uterine cycles) and lasts for 4 - 5 days. The cycle can be monitored using different processes that are widely acceptable. One of such is the visual assessment of the vaginal area. The four phases of the estrous cycle in rats are characterized by different features of the vaginal opening and were all observed across all groups. The first phase called the proestrus phase is characterized by a full, moist, and swollen vaginal opening with pink tissues and striations in both ventral and dorsal lips of the vulva. The estrous phase is the second phase and is characterized by similar features to the proestrus phase with more noticeable striations around the lips of the vulva but less swollen, less pink, and also less moist. Next is the metestrus phase where the vagina area is usually pale and dry and sometimes with a slough of whitish cellular debris. Lastly is the diestrus phase where the vaginal opening appears very wet, small, and closed with no tissue swelling.

Cytological assessment characterizes the estrus phase with the appearance and quantity of certain cells. The proestrus shows predominantly numerous nucleated cells; the estrus phase is marked by the presence of large numbers of cornified epithelial cells, metestrus is characterized by scattered squamous epithelial cells and several neutrophils; and lastly diestrus characterized predominantly by leucocytes. The estrus cycle in rats is essential for the elucidation of the mechanisms of reproduction and also to study the effects of advancements in fertility treatments and contraceptives since it is similar to the humans' menstrual cycle. It can also provide information that is crucial in pharmacological and toxicological studies since the cycle can be influenced by drug metabolism, toxicity, and efficacy [9]. This study explored the impact and effect of LNG (Postinor 2) intake on the estrous cycle of Wistar rats. The visual and cytological evaluation of the estrous cycle of the Wistar rats showed that Postinor 2 affected the treated groups most especially the twice-weekly treated group by prolonging the time in the metestrus phase and shortened the period of the estrus and diestrus phases. The metestrus phase is the period characterized with a decline of the corpus luteum functions in the absence of pregnancy when the reproductive organs have subsided in activities. Afterall, the drug is supposed to ensure the absence of pregnancy. The proestrus phase was first observed in the group treated twice weekly long before it was seen in the other groups. This means that Postinor 2 hastened the onset of sexual maturity in the treated rats. A study by Gemzell-Danielsson and Marions, [10] postulated that LNG can inhibit ovulation, which is the release of eggs from the ovaries during the estrus cycle. This effect is similar to its contraceptive action in humans. Lovick and Zangrossi [11] observed LNG may alter the estrus behavior of rats, including changes in the duration and frequency of estrus phases. Pennock-Speck and Lethbridge, [12], concluded that LNG treatment may affect the length and regularity of the estrus cycle in rats, potentially leading to irregular

cycles or prolonged periods of anestrus (non-reproductive phase). Allaway *et al.* [13] observed that implantation of progestin-only contraception altered the estrus cycle of rats. These previous reports corroborate the observation made in this study that LNG have the potential to disrupt the menstral cycle and may subsequently lead to some reproductive complexities or difficulties with time.

The effects of LNG (Postinor 2) on the selected organs were observed by determining the relative organ weight. Change (increase or decrease) in organ weight is accepted as a sensitive indicator of chemically-induced organ damage. The Utero-Somatic Index (USI) and Gonado-Somantic Index (GSI) are used in evaluating the reproductive potency and hence fertility in individuals [14]. The relative uterus weight also known as the Utero-Somantic Index (USI), was significantly higher in once weekly than in control while the twice weekly had the lowest weight at 30 days. However, at 60 days, twice weekly caught up with once weekly and were both significantly higher than control. At 90 days, twice weekly took a giant leap that surpassed once weekly though once weekly also increased but the increase was not significantly different from the increase in control. 30-day post-treatment recovery period showed a significant decrease in both once weekly and twice weekly than control. LNG-induced increase in organ weight was time-dependent, however, the LNG-induced increase in the relative weight of uterus was reversed, following the withdrawal or discontinuation of the drug for a period of 30 days.

The pattern in USI observed was similar to relative ovary weight, also known as Gonado-Somatic Index (GSI), and showed that Postinor 2 treatment increased the size of the reproductive organs which was corroborated by fat deposits observed especially in twice weekly group when the organs were physically observed after sacrifice. In 30 days, GSI was highest once weekly followed by twice weekly with both significantly higher than control. By 60 days, twice weekly

became the highest followed by once weekly and both were again higher than the control. This happened again at 90 days but the values dropped in treated groups to become significantly lower than control after the 30-day recovery period. This signifies that Postinor 2 caused an increase in both USI and GSI, an increase that was reversed following drug withdrawal. This was the case in the study by Palki *et al.*[15] 2018 where one-off treatment with LNG-treated rats had a significantly low USI and GSI as observed in this study at 30 days. A decreased GSI is usually indicative of a reduction in the rate of folliculogenesis or an increased rate of atresia in follicles. Reduction in folliculogenesis leads to decreased fertility. Further period of treatment brought about an increase in USI which suggests a structural anomaly. Physical observation of the uterus and liver especially in twice weekly groups showed accumulation of fat tissues which was corroborated by histological assessment which showed visible lesions in different sections.

Relative liver weight, also known as liver-to-body weight ratio, is used in medical and scientific research to assess the size and condition of the liver in relation to the overall body weight of an organism. In this study, postinor 2 caused a significant increase in relative liver weight only in the group treated twice weekly which was not reversed after 30-day-post-treatment recovery period. This increase could be as a result of decrease in body weight in proportion of the organ weight since there was a decrease I relative liver weight in once weekly group where the body weight was higher than twice weekly group. Also, the reduction observed in once weekly could be as a result of reduction in organ weight, for instance, the liver is reduced in proportion to the overall body weight, and this can have various health implications. Some chronic liver diseases, such as cirrhosis, can cause liver atrophy, which shrinks the liver tissue and to become scarred. This can result in a reduced relative liver weight. Liver diseases can have serious health implications, including impaired liver function, jaundice, and portal hypertension. Certain

chronic illnesses and conditions, such as cancer, autoimmune disorders, or chronic infections, can also affect liver size and function. A reduced relative liver weight may be a consequence of these underlying health issues as does age, especially with other underlying conditions, certain medications, and surgical procedures.

A measurement used to evaluate the size and health of the kidneys in relation to an organism's total body weight is known as relative kidney weight or kidney-to-body weight ratio. It is important to keep in mind that kidney health is a complicated issue influenced by numerous factors, a change in relative kidney weight can suggest a variety of health disorders and may have health repercussions. As the kidneys attempt to make up for their impaired function, an increase in relative kidney weight can happen in the early stages of renal disease. However, due to the strain imposed on the organs, this can eventually result in kidney hypertrophy (enlargement). On the other hand, a drop in relative kidney weight could mean renal atrophy, which is connected to reduced kidney function or chronic kidney disease [16].

In this study, Postinor 2 intake caused a significant decrease in the relative kidney weights of rats treated once weekly and an increase in those treated twice weekly. This could also be influenced by the body weights. The 30-day post -treatment recovery period returned the kidney-to-body weight ratio to the initial values at 30 days in both once-weekly and twice-weekly groups. This may also show that the kidneys had impaired function with the treatment of Postinor 2 which is dose-dependent. A study by Akinwunmi *et al* [17], found that the kidney and relative kidney weight in LNG treated group were similar to the control group which is contradictory to what was found in this study

An essential component of pathology practice, clinical diagnosis, as well as scientific and medical research, is the histopathological examination of organs. To determine their structure, composition, and any anomalies, tissues, cells, and organs are examined microscopically. It offers insightful information on the identification, management, and comprehension of a wide range of illnesses and ailments, ultimately enhancing patient care and promoting medical research [18]. This research work carried out histopathological examination of all organs in the treated groups and compared them with control. Once weekly group showed an infiltration of the mucosa as well as degenerated and hyperplastic uterine glands whereas severe endometrial ulcerations were observed in animals treated with Postinor 2 twice weekly. In the ovary, there was no visible sign of histological aberrations in the ovary of both treated groups. The liver showed that treatment of rats with Postinor 2, once weekly resulted in cogestion as well as mild focal periportal and disseminated infiltration by inflammatory cells while treatment twice weekly caused marked disseminated congestion, mild disseminated periportal infiltration by inflammatory cells and lymphoid aggregate in zone 2 and the focal area of hepatic nodules. In the kidney, the once weekly showed extensive tubular necrosis, mild disseminated tubular necrosis, glomerular messangialisation, and focal periglomerular while the twice weekly treated group showed marked to severe disseminated tubular necrosis, glomerular messagialisation, and necrosis. A study by Akinwunmi *et al.* [17] also observed tubular epithelial degeneration and necrosis was observed in levonorgestrel-treated rats.

The hormone, progesterone, which is largely produced by female ovaries, is crucial for the menstrual cycle, pregnancy, and general reproductive health. After ovulation, progesterone levels rapidly increase. The timing of fertility treatments and the ability to assess fertility depend on the ability to confirm the occurrence of ovulation by the measurement of progesterone levels [19]. Low

levels of progesterone during the luteal phase of the menstrual cycle may be an indication of luteal phase deficit, which can cause infertility or recurrent losses [20]. Pregnancy must be maintained during the early stages thanks to progesterone. In cases of impending miscarriage or to determine the viability of pregnancies, particularly in assisted reproductive technology (ART) operations, monitoring progesterone levels is essential. Progesterone levels can help diagnose and differentiate various menstrual disorders, such as anovulation, amenorrhea, and abnormal uterine bleeding. Combining progesterone measurement with other hormone testing can shed light on ovarian health and function, helping to diagnose diseases including polycystic ovary syndrome (PCOS) and ovarian malignancies. Determining progesterone is essential in numerous studies looking at hormone regulation, reproductive endocrinology, and fertility, which helps us understand reproductive health [21, 22, 23.] This research study observed a significant increase in the concentration of progesterone only in once-weekly and twice-weekly treated groups at 60 days of treatment when compared with the control. The progesterone-like effect of postinor 2 may be responsible for maintaining a balance in the progesterone level of the body. This result disagrees with Xiaohui *et al.* [24] who found that LNG treatment of Mongolian Gerbils resulted in the decrease concentrations of progesterone.

A class of steroid hormones known as estrogens are crucial for the establishment and maintenance of female sexual characteristics as well as the control of the female reproductive system. It plays a vital role in regulating the menstrual cycle by promoting the growth and development of the uterine lining (endometrium) during the follicular phase [25]. Estrogen levels considerably increase throughout pregnancy to aid fetal development and get the body ready for labor. It encourages blood flow to the placenta, supports uterine lining health, and promotes mammary gland development. It may have an impact on mood and emotional health. Mood and

mental health can be impacted by changes in estrogen levels, such as those that take place throughout the menstrual cycle or menopause. The vaginal and urinary tract tissues' health, which might impact sexual function and comfort, are also maintained by estrogen [26].

This study observed that Postinor 2 has an anti-estrogenic activity which hindered the production of estrogen by the pituitary glands. This was shown by the dose- and time-dependent reduction in estrogen concentration both in the once-weekly and twice-weekly treated groups. The anti-estrogenic activity of LNG may be why it is prescribed in hormone therapy to treat menorrhagia, endometrial hyperplasia and endometrioses. Estradiol is the key hormone associated with the growth and persistence of endometriotic tissues as well as the pain and inflammation associated with them Chantalat *et al*.[27] Reduction in E2 concentration may also be responsible for mood swings and depression associated with the use of LNG ECs as E2 can influence mood and emotional well-being. Shi *et al* [8] observed no significant difference in E2 level on Day 30 of LNG treatment but also observed that 6 of 7 females in the control group were pregnant (85.7 %) with signs of normal pregnancy whereas only one female in the LNG treated group experienced a failed pregnancy with signs of resorbed fetuses. Xiaohui *et al,* [24] observed an increase in the concentration of E2 with LNG treatment which does not agree with this study. Estrogen reduction is responsible for increase in the concentration of LH and FSH as a result of negative feedback mechanism. Increase concentration of E2 is responsible for the thickening of the endometrial walls [28]. A reduction in estrogen concentration may explain how LNG causes the thinning of the endometrial walls to prevent implantation and hence pregnancy. Additionally, E2 is responsible for maintaining the concentration and viscosity of the cervical mucus. A reduction in E2 concentration is also responsible for the change in the cervical mucus associated with LNG intake to prevent or slow down the swimming of the sperm cells up the female reproductive tract.

The anterior pituitary gland produces the gonadotropin hormone called luteinizing hormone (LH), which is responsible for the regulation of the menstrual cycle, ovulation, and fertility, as well as the identification and treatment of diseases including Poly cystic ovarian syndrome (PCOS) and hypogonadism. It is an important factor in reproductive endocrinology and hormone therapy [29]. This study observed a significantly higher concentration of LH in the serum of twice weekly than control and once weekly. There was no difference in the values of once weekly and control until 90 days and recovery. This shows that Postinor 2 intake influenced the level of LH at twice weekly which is also dose and time-dependent. Nappi *et al*, [28] explained that a reduced level of E2 is responsible for increasing the concentration of LH and FSH due to negative feedback effects, especially during the late follicular phase.

The regulation of reproductive functions, particularly the female menstrual cycle and fertility, depends also on the hormone, follicle-stimulating hormone (FSH). From its involvement in the menstrual cycle and fertility to its uses in assisted reproduction, hormone therapy, and the identification of reproductive illnesses, it plays a variety of roles in numerous facets of reproductive health. For both male and female reproductive health, fertility preservation, and monitoring of FSH levels [30]. The follicle-stimulating hormone was lower in once weekly and increased in twice weekly than the control group and was time-dependent. The recovery period brought about a marked decrease in FSH concentration twice weekly. LNG treatment on day 30 by Shi *et al.* [8] found lower FSH concentration than the control group which agrees with this study.

Progestagen-Associated Endometrial Protein (PAEP), also known as glycodelin or placental protein 14 (PP14), is a glycoprotein primarily produced in the endometrial tissue of the female

reproductive system. PAEP is under the influence of the hormone progesterone, and its expression varies throughout the menstrual cycle, peaking during the luteal phase (post-ovulation) when progesterone levels are high. PAEP is involved in several important reproductive processes and plays a role in suppressing the maternal immune response to a developing embryo during early pregnancy, preventing the immune system from recognizing the embryo as a foreign invader. It is believed that PAEP has a role in preparing the uterine lining for embryo implantation, and its expression is frequently linked to fertility. Pregnancy issues like repeated miscarriages, preeclampsia, and premature birth have all been linked to abnormal PAEP levels. Its dysfunction may throw off the equilibrium between the fetal and maternal immune systems. For a number of gynecological and reproductive illnesses, such as endometriosis and infertility, PAEP has been studied as a potential biomarker. The development and progression of cancer may be affected by the dysregulation of PAEP expression [31]. There was a reduction in the level of PAEP in the serum of the treated rats continuously over the treatment period and this reduction was more in twice weekly than once weekly.

Leukemia inhibitory factor (LIF) is a cytokine, a subclass of signaling protein that is crucial for many bodily physiological functions. Its name comes from the fact that it was initially found to be able to stop the proliferation of leukemia cells. Since then, a variety of processes, including embryonic development, immunological response, and tissue homeostasis, have been discovered to be included in its range of functions. It performs an essential role in embryo implantation and early pregnancy by maintaining pluripotency in embryonic stem cells, an important aspect of its function [32]. In this study, except at 30 days of treatment once weekly, LIF concentration was significantly lower than the control in both treated groups. A study by Matsuo *et al.* [33] reported

that LIF was significantly reduced in LNG-treated rats which agrees with the findings in this research.

Prolactin (PRL) is a versatile hormone that is produced not only in the anterior pituitary but also directly synthesized by the endometrium under stimulatory action of progesterone. The functions of PRL include the promotion of uterine smooth muscle cell growth and proliferation. It also primarily controls breastfeeding and reproduction in mammals. In conjuction with its receptor (PRL-Rs), they play a role in the activation of signaling pathways involved in uterine cancers and so serve as discriminative biomarker of uterine cancer Its secretion and functions are strictly regulated by a number of factors, and problems with this regulation can result in illnesses that have an impact on fertility and general health. Prolactin is frequently linked to severe endocrine disorders like ovulation suppression, which leads to infertility by centrally modulating Kisspeptin secretion or peripherally affecting progesterone or FSH level [24,34]. Its pathogenetic role in malignant, premalignant and benign uterine diseases has been of increasing research focus in recent years. This study found that Postinor 2 intake caused a considerable decrease in the level of prolactin in the serum of Wistar rats in once weekly and more significantly in twice weekly. The significant decrease in twice weekly was throughout the treatment period and also in 30 days post-treatment. The shift in readings following the treatment period might be a sign that PRL concentration restoration could occur with additional time without therapy. Low levels of PRL leads to increase secretion of FSH. This disagrees with a study by Xiaohui *et al,* [24] which observed a stimulatory effect of LNG on PRL concentration in serum.

The liver produces a sex hormone transporter called sex hormone-binding globulin (SHBG), which binds to circulating sex steroids especially estrogen with a high affinity to control the concentration of physiologically active sex hormones in the blood, their access to tissues and

thus their bioavailability. In light of this, SHBG can be used to gauge the extent of hyperandrogenism and evaluate treatment efficacy [35]. This current research observed that Postinor 2 caused a dose-dependent decrease in the concentration of SHBG which was more pronounced in twice weekly. The decrease in once weekly only became significant at 90 days in once weekly. It was observed that decrease level of SHBG is associated with decreased availability of estrogen which is associated with ovarian pathology, anovulation, liver disease, PCOS, cardiovascular disease and hormone imbalances [36,37]. Reduction in SHBG causes an increase in free levonorgestrel index (FLI) which is a strong indicator of poor luteal function, implantation failure and low pregnancy rates. This agrees with a study by Alvarez *et al.*[38] who observed a reduction in SHBG with LNG intake and subsequently leads to ovarian suppression.

A family of transcription factors known as nuclear factor-kappa B (NF-kB) is essential in controlling a variety of biological processes, including inflammation, immunological response, cell proliferation, and apoptosis. NF-kB is involved in the transcriptional regulation of a wide variety of genes and is crucial for the management of immunological and inflammatory responses. It is involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, radiation, oxidized LDL, and bacteria/viral antigens. Incorrect regulation of KF-kB has been linked to cancer, inflammation, autoimmune diseases, infection, septic shock, or improper immune development [39]. A study by Hall and Klein, [40] observed the modulatory effect of progesterone-based contraceptives such as Postinor 2 on immune responses throughout the body, particularly at mucosal sites. This current research found that Postinor 2 caused an increase in the concentration of NFkB in Wistar rats. This was proven by the continuous increase in its concentration as treatment progressed especially in twice-weekly treated group in all the assessed organs.

TNF-α is a strong proinflammatory cytokine that affects the immune system and many different tissues in a variety of ways. A number of diseases, such as autoimmune disorders, chronic inflammatory conditions, viral diseases, and some malignancies, are thought to have their etiology influenced by dysregulated TNF-α production and signaling. TNF-α is a target for therapeutic intervention due to its essential function in inflammatory and immunological responses [41]. The level of increase in TNF-α in the uterus compared to the ovary, which experienced more elevation only in twice weekly group showed that the impact of Postinor 2 is greater in the uterus.

The liver is a vital organ responsible for numerous metabolic, detoxification, and regulatory functions in the body. The effects of Postinor 2 intake on liver function biomarkers were also assessed in this study. Liver function biomarkers such as AST, ALT, ALP, GGT, and Total protein are essential tools for assessing liver health, diagnosing liver disorders, and tracking disease progression and treatment efficacy [42]. The biomarkers determined in this study were done both in the serum and liver and the results showed that Postinor 2 intake affects liver function.

AST plays a key role in the metabolism of amino acids, maintenance of NAD+/NADH ratio in cells, Krebs cycle activity, synthesis of purine/pyrimidine bases, urea and protein synthesis and gluconeogenesis. AST activity is widely distributed across human tissues with the highest activity found in heart, liver, skeletal muscle, kidney and brain. An elevated AST activity may reflect tissue damage (plasma membrane disruption or apoptosis), plasma membrane bleb formation, increased tissue expression and macroenzymes (complexes of AST with plasma proteins), increased cardiovascular risk related to nonalcoholic fatty liver disease (NAFLD), cardiometabolic risk factors (metabolic syndrome, abdominal obesity, insulin resistance and diabetes), chronic alcoholism and structural heart diseases [43]. In this research, AST concentration

in the serum was elevated and the increase occurred progressively with treatment days while there was a decrease in AST concentration in the liver.

Alanine Aminotransferase (ALT) found primarily in the liver plays a vital role in amino acids metabolism, represents the most used liver damage biomarker worldwide. ALT and ALP levels are determined to assess liver health, liver diseases including hepatitis, cirrhosis, fatty liver disease, and alcoholic liver disease, medication or toxin-induced liver damage, and monitoring liver disease progression [44]. ALT concentrations measured in this study became significantly elevated in the serum at 90 days and the increase was not reversed at post-treatment while there was a decrease in ALT concentration in the liver.

Alkaline Phosphatase (ALP) is a broader marker that can originate from several tissues in the body, including the liver, bone, intestines, and placenta (in pregnant women). Its increase in the blood is associated with biliary tract disease though not specific to liver diseases [45]. ALP concentration determined in this study was also increasing in the serum while decreasing in the liver with treatment days. The reduction was significant against control and more pronounced on 90 days of treatment.

Total protein measures the total amount of protein in the body and it provides a combined measurement of both albumin and globulin in the blood, reflecting the balance between these two protein groups. Proteins are essential molecules in the body that serve various functions, including maintaining the structure of cells and tissues, regulating fluid balance, and functioning as enzymes, antibodies, and hormones [46]. Total protein level was reduced in this study up to about 70 % in the liver at 90 days of twice-weekly treatment. The dose and time-dependent

decrease was significant when compared with control and this may be a strong indication for chronic inflammation, liver disease, kidney disease, malnutrition, or other health conditions.

Gamma-glutamyl transferase (GGT) can also be determined to provide valuable information about liver and biliary system health. They are elevated in different diseases affecting the liver, pancreas, and cardiovascular [47]. The concentration of GGT determined in this study was elevated significantly in the treated group when compared with control. Although the increase was not significant in once weekly until 60 days it promptly reduced during the post-treatment period. Twice weekly experienced an increase that was significant from the first month up until the treatment period.

The pattern of liver function biomarkers in this research is indicative of a compromised liver's integrity and function. This was corroborated by the increase in relative liver weight and histological aberrations to the liver. It may suggest that cellular metabolism of Postinor-2 in the animals may have resulted in oxidative stress-induced tissue atrophy. Metabolism of LNG occurs majorly in the liver to sulfates and glucuronides. Although glucuronides are considered non toxic, there is morphine-6-glucuronides which are toxic and since the pathway is not yet fully understood, toxic metabolites may arise from LNG metabolism. Since the severity of the effects of Postinor 2 on the liver occurs with duration of use, this puts long-term users at a high risk of liver damage. The liver function results from this research agree with the one done by Chane *et al*, [48] where they found elevated concentrations of AST, ALT, ALP, and GGT in participants on oral contraceptives than in control. It should be noted that the most adverse effects of contraceptive use are metabolic impairment and cardiovascular complications. This study has presented a metabolic impairment in relation to hepatic function evident in liver test alteration

which is related to duration of usage. This disagrees with Connoly and Zuckerman, [49] who suggested that progestins are safe in patients with liver diseases.

There is also the report that several prescribed and over-the-counter drugs have been implicated in Drug-induced liver infection (DILI). This is the most common justification for taking many drugs off the market and caution is advised to be considered in abundance, with close monitoring when taking ECPs especially with other drugs and in situations of other underlying risks of liver diseases [50]. The DrugBank online, hosted by Wishart *et al* [51], listed several drugs such as Alteplase, Ancrod, Acenocoumarol, Abciximab, Alteplase, Anistreplase, Antithrombin alfa among several others that are capable of increasing the risk or severity of adverse effects of LNG when taken in combination. Several drugs were also listed that can either increase or decrease the metabolism, bioavailability, and therapeutic effect of LNG. The point about this is, several users of ECPs are unaware of the health risks associated with the use of levonorgestrel in combination with other drugs, especially to the liver [52].

De Ritis ratio also known as AST/ ALT ratio is used as a prognostic biomarker for various malignancies such as chronic liver diseases, renal cell carcinoma, pancreatic cancer and breast cancer. Patients whose value of De Ritis ratio is higher than one is at risk of liver damage. The importance of the test lies in its easy accessibility and low cost since AST and ALT are one of the most demanded serum biomarkers in daily liver function clinical investigation [53]. Women with liver dysfunction or prior transplants should be advised by reproductive health counselors for safer contraception methods other than hormonal contraceptives in family planning [54]. It might also be important to note that animals' cellular metabolism of Postinor-2 led to tissue shrinkage brought on by oxidative stress. This theory is in line with the findings of Sridharan *et al.* [55] who suggested that several excipients in postinor-2, including silica colloidal anhydrous,

cause harmful effects on the liver such as inflammatory diseases and necrosis. Also, Adigun *et al.* [56] observed that damage to the cells and tissues of the liver is associated with excessive intake of Postinor 2.

The health and function of the kidneys, which are crucial for preserving the general homeostasis of the body, are evaluated using renal function biomarkers. The regulation of cardiovascular parameters like blood pressure and endothelial factors depends critically on renal function. The kidneys control blood pressure and produce vital hormones in addition to filtering wastes, extra fluids, and electrolytes from the blood. For the detection of kidney disorders, evaluating renal health, and managing a variety of medical conditions, monitoring kidney function is crucial [57]. Biomarkers of renal function such as Cystatin C, creatinine, urea, and BUN: Cr were determined in this study to investigate the effects of Postinor 2 intake on renal metabolic function in female Wistar rats.

Cystatin C is an important piece in the puzzle of assessing kidney function and monitoring conditions such as chronic kidney disease (CKD), acute kidney injury (AKI), and estimating glomerular filtration rate (GFR), which is a key indicator of kidney function. A member of the cystatin superfamily of cysteine protease inhibitors, cystatin C is a low-molecular-weight protein. Given its widespread distribution in various organs and tissues and low molecular weight, cystatin C is also utilized as a measure of glomerular filtration rate. Cystatin C is less prone to biological interference and more sensitive to early impairment in renal function compared to other renal biomarkers, such as serum creatinine. Additionally, there is growing proof that cystatin C is linked to a variety of immunological responses to exogenous and endogenous antigens and that the regulation of its encoding gene during inflammation and infection is mediated by a number of cytokines [58,59]. In this study, the concentration of cystatin c

was significantly higher in the serum of Postinor 2 treated rats than in the control. The significant increase in the concentration of cystatin C was more pronounced in Twice-weekly than once weekly when compared with the control. Kidney function and cystatin C levels are inversely correlated, meaning that as cystatin C levels in the blood tend to rise as renal function declines.

During regular metabolism, the muscles produce creatinine as a waste product which is eliminated by the kidneys. Because the kidneys typically filter and eliminate creatinine from the blood, elevated creatinine levels may indicate decreased kidney function [60]. This study observed a reduction in the concentration of creatinine in the kidney of both treated groups which is up to over 50 % reduction in twice weekly at 90 days. The reduction in once weekly treated group was not as bad as in twice weekly but nevertheless significant when compared with control.

One of the most important markers for evaluating kidney function and general metabolic health in clinical settings is urea. Increased urea levels can be a sign of a number of illnesses, including kidney disease, dehydration, heart failure, and bleeding in the gastrointestinal tract [61]. This study observed the concentration of urea was elevated in the serum and kidney of rats treated with Postinor 2 than the control. Akinwunmi *et al*, [16] found an elevated concentration of urea and creatinine when they treated rats with levonorgestrel which agrees with what was found in this study. Brookes and Power, [62] observed that an elevated urea and creatinine levels in the blood is associated with reduced or compromised function of the kidney since both are filtered by the glomerulus. About 40 - 50 % of urea is reabsorbed in the glomerulus unlike creatinine, a compromised attempt of the kidney to perform this function may explain why urea level is elevated in both serum and kidney.

The BUN (Blood Urea Nitrogen) to creatinine ratio is a test of great importance in clinical settings to compare the levels of two substances, blood urea nitrogen (BUN) and creatinine, in the blood. It is often used as part of a comprehensive metabolic panel (CMP) or basic metabolic panel (BMP) to assess kidney function and to help diagnose certain medical conditions. It is commonly acknowledged that renal tubular lesions result in a decrease in the blood-uremic nitrogen to creatinine ratio (BUN: CR) which has a normal range between 5 – 20 mg/ mL [63]. The BUN:Cr ratio was higher in the treated groups than the control but still within the normal range. The higher value calls for attention because an increase in urea out of proportion compared to creatinine in the serum causes an elevated and reflects a critical condition. Urea and Cr concentrations in the serum usually increase with an inverse proportionality to renal function [62]. The use of high-dose progestins, even though not contraindicated in chronic kidney disease (CKD) should be avoided as much as possible, the paradoxical issue is that a significant number of women on ECPs do not realize they are affected by CKD even in the early stages [64]. Attini *et al,* [64] argued that, even though pregnancy is possible in all CKD- stages, progestin contraceptives should not be an option. This argument may be due to the fact that progestins can exacerbate kidney disease and impair the outcome of treatment. The study by Micheal *et al.* [65] disagrees with this study with a claim that estrogen-progestin treatment did not affect the renal concentration of AST and ALT but a reduced renal ALP concentration reported agrees with this study. Rojas *et al*, [66], explained that drugs that disrupt the hypothalamic-pituitary-gonadal axis play a critical role in contributing to decreasing kidney function and development of uremia.

Oxidative stress is a biological phenomenon that occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. Reactive oxygen species, which include molecules like superoxide radicals,

hydrogen peroxide, and hydroxyl radicals, are highly reactive and can cause damage to cellular components such as DNA, proteins, and lipids. This damage is often referred to as oxidative damage. Oxidative stress is a normal part of the body's metabolic processes, and ROS serve important roles in various physiological functions, including immune response and cell signaling. However, when there is excessive production of ROS or a deficiency in the body's antioxidant defense mechanisms, oxidative stress can become harmful and lead to a variety of health problems. Oxidative stress has a wide range of effects, some of which can be deleterious to cellular and tissue function [67]. The front liners in the antioxidant defense system of the body are the SOD, CAT and which work together in the entire mechanism against free radicals Engwa *et al*, [68]. SOD causes the dismutation of superoxide anion radicals (O2-) into hydrogen peroxide (H_2O_2), CAT breaks down hydrogen peroxide (H_2O_2) to water and molecular oxygen while GPx reduces hydrogen peroxide (H_2O_2) to water, and lipid peroxides to their corresponding alcohols using glutathione as a substrate [69].

In this investigation, the potential of frequent intake of Postinor-2 to cause oxidative damage in the organs of rats was assessed using female Wistar rats as experimental animals. There was a significant reduction in the concentrations of CAT, SOD and GPx. The reduction in CAT activity was more severe in the ovary and uterus with about a 75 % decrease from the control value. The reduction in the liver and kidney was significant in twice weekly while once weekly was only significantly lower at 90 days. The reduction of GPx in the ovary and liver became significant from 60 days while in the uterus and kidney, a significant reduction was from the first month. SOD reduction was more pronounced in the uterus with up to 70 % difference when compared with control while it was about 37 % decrease in the ovary compared to control. Wusu *et al*, [70] observed the downregulation of CAT and SOD genes in the liver, brain, and kidney of rats

treated with ethinylestradiol and levonorgestrel which aligns with what was observed in this research work. Mannai *et al*, [71] observed an enhancement of the antioxidant defense against oxidative stress in female Clams treated with LNG alone through the induction of CAT and GST

It is possible that the metabolism of Postinor-2 in the tissues may have produced too many free radicals, which could have caused oxidative damage, as evidenced by the suppression of SOD and CAT activities in the ovary and uterus of rats and GPx activity in the ovary alone after weekly intake of Postinor-2. This finding is in line with observations from other research that showed exposure to medicines or toxicants whose metabolism is linked to the production of reactive species or free radicals reduced the activities of SOD, CAT, and GPx [72,73,74].

GSH is undoubtedly the most significant intracellular antioxidant molecule crucial for the detoxification of xenobiotics [75]. It is implied that Postinor-2 metabolism in the ovary may have resulted in the production of excess reactive species, which have the potential to cause oxidative stress and tissue damage, as a result of the significant decrease in GSH concentration in the ovary of rats treated with Postinor-2. This study supports a previous one by Venter and colleagues [11], which suggested that the intake of oral contraceptives may increase the generation of free radicals that can lower the quantity of GSH inside cells.

Glutathione-S-transferase (GST) is needed as one of the enzymes in the GSH redox cycle, which guarantees that oxidized GSH molecules are transformed back to reduced GSH. By reducing electrophilic chemicals to reduced glutathione (GSH), the antioxidant enzyme GST performs a crucial function in detoxification [76]. The significant increase in GST activity observed in the uterus, ovary, liver, and kidney of rats given Postinor-2 both once and twice a week in this study suggests that Postinor-2 induced the production of free radicals that oxidized GSH, which led to

an increase in GST activity as an animal's response. It is worthy of note that the increase in GST activities was significant in the uterus and ovary right from the first month of treatment while the liver and kidney became significant at 60 days and 90 days respectively. This shows that Postinor 2 has a greater effect on the reproductive organs where they carry out their function. This finding resembles one made in a prior study by Gonzalez *et al*, [77] which found that mice exposed to toxins that could cause oxidative stress experienced a large increase in GST activity.

Lipid peroxides, which are indices of membrane lipid peroxidation, are formed when polyunsaturated fatty acids, which are found in cell membranes, are subjected to peroxidative processes. A biomarker of oxidative stress called lipid peroxidation is frequently characterized by high levels of thiobarbituric acid reactive species (TBARS) or malondialdehyde (MDA) [78]. In this investigation, Postinor-2 administration once and twice weekly increased MDA concentration in all the examined organs most especially in the reproductive organs. It appears that the frequency of exposure of the animals to Postinor-2 determines the severity of the increase in MDA concentration in all the tissues, with the twice-weekly treated animals exhibiting somewhat greater MDA levels. The rise in MDA levels suggests that Postinor-2 consumption may have triggered the generation of hydroxyl or peroxyl radicals linked to the membrane lipid peroxidation observed in the organs. This finding is in line with the findings of Akinwunmi *et al,* [16] who observed an increase in MDA concentration with LNG treatment in rats. Other studies by Park *et al*, [79] and Cengiz *et al*, [80] also proved that exposure to substances whose metabolisms are characterized by the buildup of free radicals, such as pharmaceuticals, can raise MDA concentration.

The observed modifications in the antioxidant molecules examined in this study strongly suggest that continuous Postinor-2 intake has the potential to cause oxidative damage to the reproductive

organs, uterus, and ovaries as well as the metabolic organs liver, and kidney. In fact, its toxic effects may be as a result of oxidative stress which could have been as a result of its metabolism in the tissues.

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Chapter Five

Conclusion

5.1 Summary of Findings

In a bid to avoid unintended pregnancies and unsafe abortions, the use of Postinor-2 has increased drastically more than ever before. Probably due to its easy accessibility as an emergency contraceptive, there has been an increased rate of abuse of the drug among young female adults. This practice is arguably a serious cause for concern in light of the potential damage that it may cause to the female reproductive health. The fear of abortion which is one of

the leading causes of death among young females have led many into the use of contraception without the knowledge of possible toxicity associated with its use to various organs of the body. The introduction of emergency contraceptives has led to a reduction in the contribution of unsafe abortion to maternal mortality from 13 to 8 % worldwide. This is a good success nevertheless, the thrill of the success should not make researchers especially toxicologists neglect the fact that everything is a potential poison, it is the dose that matters. The young adults who simply wants results do not have this knowledge and take drug with discretion not forgetting that this ECPs is available over the counter.

This research work validated the knowledge and usage of contraceptives among the students and that some of them have side effects from its intake. The highest frequency of users is within the age group 18 – 25 years old and that Postinor 2 is the most patronized type of contraceptive pills. The side effects from the intake of ECPs include headache, spotting, mood swing, weight loss, irregular and delayed menstrual cycle among others. The treatment of Wistar rats with Postinor 2 hastened the onset of sexual maturity and the estrous cycle became irregular with prolonged metestrus and diestrus phases which are characterized by elevated circulating progesterone levels. The average weight of Wistar rats and relative organ weights was reduced by Postinor 2. Intake of Postinor 2 caused significantly elevated concentration of FSH especially in twice weekly group. A higher concentration of FSH than LH is believed to be a sign of diminished ovarian reserve and also a cause of reduction in egg quality. With higher FSH level than normal it becomes difficult to achieve pregnancy. Since FSH suppresses follicular development, there is prolonged luteal phase by the presence of corpus albican which is the Metestrus and diestrus phase in rats.

Postinor 2 also reduced the level of Progesterone associated endometrial protein (PAEP), leukemia Inhibitory factor (LIF), Prolactin and serum hydrogen-binding globulin. These proteins regulate fertilization and implantation and the results seen in this research may be an indication of poor fertility and pregnancy outcome in incessant users of the drug. There is severe aberration to the histological features of the reproductive organs and the liver especially in twice weekly group. Circulating levels of liver markers showed a degree of liver dysfunction among treated rats. Renal function biomarkers also indicated an increased level of stress on the kidney caused by intake of P2. There are also high concentration of NF-kB and TNF-α, which is suggestive of stress, inflammation or infection as well as oxidative stress determined in the organs of the body.

5.2 Conclusion

In conclusion, this research project discovered that:

i. There is high awareness of contraceptive methods among the students of Lead City University, Ibadan
ii. The prevalent method of contraception among them is the emergency contraceptive pills (ECPs)
iii. A high percentage of ECP users observed one or more side effects from the use of the pills
iv. Postinor 2 is commonly used once in a while to prevent unwanted pregnancy and unsafe abortions among students
v. Postinor 2 intake hastened the onset of sexual maturity in Wistar rats treated twice weekly

vi. Postinor 2 intake lengthened the metestrus phase and shortened the diestrus phase in Wistar rats

vii. Postinor 2 intake caused physical and behavioral changes in Wistar rats which were shown by reduced physical activity, agility, and feeding

viii. Postinor 2 intake caused noticeable weight loss, hair loss, and even death in some Wistar rats

ix. Postinor 2 caused an aberration to the structural make-up of the uterus and ovary as seen in the histopathological examination

x. Postinor 2 decreased the concentration of vital endometrial proteins involved in successful implantation and fertility namely PAEP, LIF, PRL, and SHBG in Wistar rats

xi. Intake of Postinor 2 compromised the integrity of the liver as evident in the elevated levels of hepatocyte function markers as well as the histopathological examination

xii. Oxidative stress was triggered by the intake of Postinor 2 in organs such as the uterus and ovary as well as in the kidney

xiii. Postinor 2 intake caused noticeable stress to the kidney as evident in the elevated concentration of renal function markers

xiv. Postinor 2 caused increased production of early mediators of inflamed tissue such as TNF-α and NF-kB in Wistar rats

xv. There is also a modulatory effect on the concentration of LH and FSH as a result of Postinor 2 intake

Hence, LNG treatment caused time- and dose-dependent hormonal modulation and organ toxicity. The uterus and liver were particularly affected, and animals exposed to LNG twice a

week apparently suffered more assaults. The ability of the animals to recover following drug withdrawal was more evident in the once-weekly treated group, particularly with respect to hormonal, liver, and kidney aberrations

5.3 Recommendations

Based on this research findings, the following recommendations were made:

i. The use of ECPs is efficient in preventing unwanted pregnancy and unsafe abortion but must be taken with great caution because of the toxic effects on reproductive and vital organs

ii. Postinor 2 and other ECPs should be used as prescribed by its manufacturer, for emergency reasons only, and not as regular and/or long-term methods of contraception

iii. Other long-lasting methods of contraception with lower doses of LNG or other products should be considered in case of continuous use

iv. Periodic evaluation of the metabolic profile of long-term oral contraception users should be done to evaluate general wellness

v. Individuals with liver disease, kidney disease, organ transplant or any other serious diseases should embrace tubal ligation in cases of complete families or barrier methods, intrauterine devices as other safer alternative contraceptive methods

5.4 Contribution to Knowledge

This study has:

i. showed a high prevalence, knowledge and use of ECPs especially Postinor 2 among the students of Lead City University, Ibadan

ii. the students who use it most are between the ages of 18 and 25 and they observe certain side effects such as headache, weight loss, spotting, and irregular menstrual cycle among others

iii. intake of Postinor 2 affects the estrous cycle of rats

iv. incessant use of Postinor 2 is toxic to the reproductive organs and vital organs

v. Postinor 2 intake over a long period of time may increase the risk of liver damage

vi. the downregulation of the endometrial proteins shows the possible correlation between incessant Postinor 2 intake and recurrent implantation failure, spontaneous abortion as a result of implantation failure

vii. there are hormonal imbalances associated with the use of Postinor 2 which may be the reason for mood swings, depression and infrequent menstrual cycle as complained by users

5.5 Suggested Areas for Further Research

i. The contribution and association of Postinor 2 intake to fibroid development

ii. the implications of ECPs in hormonal imbalances and mental health

iii. Potential toxic effects associated with the incessant intake of ethinylestradiol in Wistar rats

iv. Assessment of established and emerging biomarkers of cardiovascular diseases and cancer among Postinor 2 treated Wistar rats and control

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Appendix

Questionnaire

Department of Chemical Sciences

Faculty Of Applied and Natural Sciences

Lead City University, Ibadan

Good day. This questionnaire is being administered to evaluate the perception, knowledge and awareness of the female students of Lead City University of emergency contraceptives (EC) in preventing pregnancy. Please respond honestly to the questions below by filling out or ticking

the appropriate box in each case, and be assured that the confidentiality of your response is guaranteed.

Thank you.

Catherine B. ADENIJI.

DATE _____

SECTION A (Demographic Characteristics)

Age (years): Less than 18 [ ] 18 – 25 [ ] 26 - 32 [ ]

Marital status: Single [ ] Married [ ]

Academic level: 100L [ ] 200L [ ] 300L [ ] 400L [ ] 500L [ ] Postgraduate [ ]

SECTION B

Age at first sex _____

How often do you have sex: > 3 times in a week [ ] 3 -5 times a week [ ] More than 5 times a week [ ] Once a while [ ]

Do you use any form of contraceptive? Yes [ ] No [ ]

If yes, what type of contraceptives do you use? Pills [ ] Injectables [ ] Herbs [ ] Concoctions [ ] Condoms [ ]

If pill, tick the specific contraceptive you use. Postinor 2 [ ] Post pill [ ] After Pill [ ] Morning after pill [ ] Combined birth control pills [ ] Plan B [ ] Others, please specify _____

How often do you use your contraceptive? Once in a week [ ] Twice in a week [ ] Thrice in a week [ ] others, please specify _____

How did you learn about the contraceptive? _____

What other method of contraception do you know apart from the one you use? _____

Where do you get your contraceptives from? _____

Did you experience any changes in your menstrual cycle since you started using it? Yes [ ] No [ ].

If yes, please specify _____

Did you experience any changes in your body since you started using it? Yes [ ] No [ ]

If yes, please specify _____

Has it been effective? Yes [ ] No [ ]

Have you ever recommended it to other friends? Yes [ ] No [ ]

Appendix II

Preparation of Drug

60 kg BW animal = 110 mg of Postinor 2

1kg BW animal = 110/60 mg of Postinor 2

110/60 mg/kg BW = 1.83 mg/kg BW

1.83 mg/kg BW (Postinor 2 dosage used in this study)

This dose (1.83 mg/kg BW**)** was used to estimate the actual amount of Postinor 2 appropriate for the body weight of the animals used in this study.

The average body weight of the experimental animals was estimated to be 140.8 g. The required amount of Postinor-2 for each animal was estimated as follows:

1 kg (1000 g) BW = 1.83 mg of Postinor 2

1 g BW = 1.83/1000 of Postinor 2

= 0.000183 of Postinor 2

140.8 g BW = 0.000183 x 140.8 of Postinor 2

= 0.25 mg of Postinor 2

Therefore 140.8 g BW animal required 0.25 mg of Postinor 2

0.2 mL of normal saline was used as vehicle for the drug.

Hence, 0.25mg of Postinor 2 was dissolved in a 0.2mL of freshly prepared normal saline, and administered to each animal at 12 hours interval. To represent a dose of **1.83 mg/kg BW.**

Biodata

A. Personal Data

Full Name: ADENIJI, Catherine Bosede

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Date and Place of Birth: May, 10, 1987, Akure

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B. Educational Background: Institutions Attended with Dates and Qualifications

2014 – 2017	University of Ibadan, Ibadan	M.Sc Chemical Pathology
2007 – 2011	Ekiti State University, Ado Ekiti	B. Sc. Biochemistry
1997 – 2004	Akure Sec. Commercial School	SSCE
1990 – 1997	St. Joseph's RCM Pry School, Akure	Primary School Certificate

Work Experience with Dates:

i. Assistant Lecturer, Lead City University, Ibadan. March 2020 – Till date

ii. Administrative Executive, Buff-i Global Consult Limited May, 2014 – Nov 2019

iii. Teacher (Biology and Chemistry), Ultimate Brain College, Umukalu 2012 – 2013

(National Youth Service Corps, February 2012 – January 2013)

Membership of Academic Professional Bodies:

- Member, Society of Toxicology
- Member, Society of Environmental Toxicology and Chemistry (SETAC)
- Affiliate member, Chartered Institute of Environmental Health (CIEH)
- Founding member, Earth Club: Environmental Conservation and Protection Organization, Lead City University, Ibadan.

Research Completed and Ongoing with Dates

i. Adeniji Catherine Bosede (2016). Oxidative Stress Biomarkers in Automobile Mechanics in Ibadan, Oyo State. Submitted to the Chemical Pathology Department, University of Ibadan, February, 2016. Pp 98 (MSc.)
ii. Arewa Catherine Bosede (2012). Quantitative Determination of Total Phenolic Content of various Extracts of *Enantia chlorata.* Submitted to Biochemistry Department, Ekiti State University, Ado Ekiti, March, 2012. Pp 57 (BSc.)

Papers Accepted for Publications:

i. Catherine Adeniji, Micheal Akinosun, Osasenaga Ighodaro, 2022. Assessment of some Oxidative Stress Biomarkers in Automobile Mechanics in Selected Local Government Areas in Ibadan, Oyo state, Nigeria, International Journal of Recent Research in Interdisciplinary Sciences, 9 (2): 72 – 80. Doi: https://doi.org/10.5281.zenodo.6637499

ii. A. O. Oyeyemi and C. B. Adeniji, 2022, Mineral Composition and Marker Enzymes Status of Children on Daily Multivitamins Intake, International Journal of Advanced Biochemistry Research, 6 (2): 179 - 185. Doi: https://doi.org/10.33545/26174693.2022.v6.i2c.154

iii. Grace Oloukoi, Kehinde J. Olamiju, Catherine B. Adeniji, 2023, Water, Women and Development, Water Affairs in a changing World, 10: 275 – 302

iv. Oluwatosin A Saibu, Gagandeep Singh, Damilola A Omoboyowa, Oyejoke O Kayode, Sunday A Olugbodi, Abayomi Bamisaye, Catherine B Adeniji, Temitope M Ajayi, Yetunde I Akinpelu, Christianah A Ogunwole, Osasenaga M Ighodaro, Ann Christopher Francis, 2023, Discovery of Putative Natural Compounds Inhibitor of the Germinant Spore Receptor Cspc in Clostridiodes Difficile Infection: Gaining Insights via in Silico And Bioinformatics Approach, Informatics in Medicine Unlocked, 42, 101339, https://doi.org/10.1016/j.imu.2023.101339

Conference / Workshop Participation with Papers Presented

i. The Article Publishing Process: An Elsevier virtual Workshop, June 1, 2020

ii. The Book Publishing Process: An Elsevier virtual Workshop, June 3, 2020

iii. Building your Academic Career Workshop, Liprorich Consulting Limited, March 26th – 27th, 2021

iv. 1st Scientific Conference, Faculty of Applied Sciences, Kola Daisi University, Ibadan, 2 – 4 March, 2022 (**Paper presented: Assessment of Heavy Metals in Soil and Water Samples from Abattoirs Located in Ibadan, Oyo State, Nigeria**)

v. 2nd International Conference, Faculty of Natural Sciences, Ajayi Crowther University, Oyo, Oyo State. 4 – 6 April, 2022. (**Paper Presented: Oxidative Stress Biomarkers among Auto Mechanics in Selected Local Government Areas in Ibadan, Oyo state**)

vi. Faculty lecture, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, July 1, 2022

vii. 32nd Annual Conference, Society of Environmental Toxicology and Chemistry (SETAC) COPENHAGEN, Denmark, 15 – 19, 2022. (**Paper Presented:**

Assessment of Cement Production on Water Quality in Sagamu, Ogun State, Nigeria).

viii. SETAC workshop on Recommendations for Microplastic Toxicity Testing and Hazard Characterization. July 18, 2022

ix. Society of Toxicology (SOT), Ohio Valley Regional chapter, 2022 Summer Virtual Workshop on July, 18, 2022

x. 3rd International Conference, Faculty of Natural and Applied Sciences, Lead City University, 2 – 4, November, 2022 (**Paper Presented: Mineral Composition and Marker Enzymes Status of Children on Daily Multivitamins Intake)**

xi. Lead City University Teaching Staff Training on Capacity Building on Contemporary Management Skills, Development and Effectiveness of Lead City University organized by Fortune Royal Multiconcepts, 16 - 17 December, 2022.

xii. Lead City University Teaching Staff Training on Capacity Building on Contemporary Management Skills, Development and Effectiveness of Lead City University organized by Fortune Royal Multiconcepts, 20th – 21st July, 2023.

xiii. Faculty of Natural and Applied Sciences Lecture, Nature and her Resources: Seeing beyond Looking, July 27, 2023.

_____ _____

Signature **Date**

The University Compliance Certification

This is to certify that this thesis written by Catherine Bosede ADENIJI with Matriculation Number LCU/PG/002032 in the department of Chemical Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan is in full compliance with the approved University format and style

_____ _____

Signature **Date**