JOSTIR Publication 2025 | ISSN: 1118-3896

Special Issue December 2025 | 25-40

Journal of Science, Technology and Innovation Research
Volume 1 Special Issue | December 2025

Prolonged Intake of Levonorgestrel Oral Contraceptive Caused an Increase in Tumor Necrosis Factor Alpha, Nuclear Factor -Kappa B and Oxidative Stress Biomarkers in the Uterus and Ovary of Female Wistar Rats

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ABSTRACT

The incessant use of contraceptives among young adults is a cause for concern. This study assessed the levels of some proinflammatory and oxidative stress biomarkers associated with incessant intake of levonorgestrel (LNG) in the uterus and ovaries and the outcome of drug withdrawal using a rat model. Sixty female Wistar rats weighing 110-120 g were randomized into three groups (n = 20). Groups A and B were administered 1.83 mg/kg body weight (BW) of LNG orally once and twice weekly, respectively, while group C served as the control. Five animals from each group were sacrificed at 30, 60, and 90 days. The remaining were observed for 30 days post-treatment to assess possible recovery. The serum levels of tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NF-kβ) were determined using the ELISA technique. Oxidative stress biomarkers such as catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), reduced glutathione (GSH) and lipid peroxidation (LPO) in the uterus and ovaries were determined using standard procedures. Results showed an increase in TNF-α and NF-kB, especially in the group administered LNG twice-weekly. There was a significant reduction in CAT, GSH, SOD, and GPx in both organs. The GST and MDA were significantly elevated, especially in the uterus. Recovery was observed only in TNF-α, GSH, and GPx in the ovary of the once-weekly group. The uterus was more affected, and animals exposed to LNG twice-weekly suffered more assaults. The ability of the animals to recover following drug withdrawal was observed in the once-weekly treated group and in the ovary.

Keywords: Levonorgestrel; Inflammation; Oxidative stress; Contraceptives; uterus; ovary; reproductive abnormalities

Introduction

The total well-being, physically, emotionally, behaviourally, and in the social aspects of life, termed reproductive and sexual health is a global concern. The reproductive and sexual health of women is seriously challenged by several gynecological abnormalities worldwide. Crucial contributors to these are physiological processes that increase the markers of inflammation and oxidative stress (Agarwal *et al*., 2012). Oxidative stress, caused by an imbalance between the physiological generation of reactive oxygen species (ROS) and combating ability of the body through the production of antioxidants (Adeniji *et al*, 2022), has been noted to negatively impact female reproduction, and whatever can lead to increased generation of ROS and inflammation

doi.org/10.51459/jostir.2025.1.Special-Issue.0143

will contribute to reproductive abnormalities (Pyeon *et al*., 2021). This is because ROS damages the oocytes, causes a decline in oocyte quality, modulate estrogen receptors, leads to luteal regression, inhibits steroidogenesis in the corpus luteum, inhibits follicullogenesis, which are all associated with the incidence of reproductive anomalies (Phang *et al*., 2023).

The increased generation of markers of inflammation and oxidative damage has been greatly contributed to by hormonal modulation resulting from several factors, including synthetic hormones. One of such is hormonal emergency contraceptive pills (ECPs), which are increasingly used as a result of increased sexual activities among young adults. Although known for multiple advantages such as high effectiveness, accessibility, and non-invasiveness, ECPs are associated with several challenges, and these include a lack of information or misinformation, negative past experiences, mixed feelings about side effects, among others.

Several methods have been developed to prevent pregnancy including the use of devices (such as implants, injections, patches, vaginal rings, intrauterine devices) and oral pills (containing progestin, estradiol or a combination of both). Oral pills used in emergency situations up to about 72 hours after sexual intercourse to prevent pregnancy are called emergency contraceptives. Levonorgestrel (LNG) is a widely used synthetic progestin found in hormonal contraceptives, including emergency contraception, intrauterine devices (IUDs), and combined oral contraceptives (Crawford *et al*., 2021). While LNG is highly effective in preventing unintended pregnancies, reducing the risks associated with abortions, and decreasing maternal and child mortality, its prolonged use has raised concerns regarding potential adverse effects on reproductive health. Studies suggest that long-term exposure to synthetic hormones can disrupt normal physiological processes in reproductive tissues, leading to inflammatory responses, oxidative stress, and possible tissue dysfunction. Additionally, they have also been linked with clotting disorders, behavioural changes (Yan *et al.*, 2018), hormonal imbalances and this has been studied to promote the onset of cervical and breast cancer, and thrombosis (Shukla *et al.,* 2017). Despite the widespread use of LNG-based contraceptives, there remains a need to better comprehend their long-term impact on female reproductive organs, particularly the uterus and ovaries, which play vital roles in fertility and hormonal regulation.

One of the primary concerns associated with prolonged LNG intake is its potential to induce chronic inflammation in reproductive tissues. Synthetic hormones, including LNG, have been reported to alter redox homeostasis, leading to increased lipid peroxidation, DNA damage, and mitochondrial dysfunction in reproductive tissues (Yan *et al*., 2022). These can affect a variety of physiologic functions and pathologic processes involving the female reproductive tracts such as oocyte reserve, folliculogenesis, ovarian steroidogenesis, and ovulation (Aslan *et al*., 2025). The uterus and ovaries, being highly sensitive to hormonal fluctuations, may suffer from oxidative damage, which could compromise their normal function and structure. Given these concerns, it is critical to investigate whether prolonged LNG intake influences TNF-α, NF-κB, and oxidative stress biomarkers in the reproductive system. By analyzing the impact of LNG on inflammatory and oxidative stress markers in the uterus and ovary, this study aims to provide crucial insights into the potential risks associated with prolonged hormonal contraceptive use and the effects of drug withdrawal. Understanding these mechanisms could aid in developing strategies to mitigate adverse effects and ensure safer contraceptive options for women.

Materials and methods

Experimental Design, Sacrifice of Animals, Homogenization and Centrifugation

Sixty (60) female rats of the Wistar strain weighing between 110g and 150g were used for the study and

acclimatized for a period of 28 days in the departmental animal house under suiTable environmental conditions in plastic cages with wire nets for 28 days. They were purchased from the Physiology Department of the University of Ibadan, Nigeria. They were randomly assigned into three (3) groups of twenty animals each (n = 20). All the animals were allowed equal access to food, 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 (NIH, 2011). The treatments of the animals lasted for a period of ninety (90) days. The animals in Group A (Once Weekly) were orally administered 1.83 mg/Kg per body weight (BW) LNG one day in a week at a 12-hour interval (morning at 7 a.m. and evening at 6 p.m.). Group B (Twice Weekly) animals were orally administered LNG at a dose of 1.83 mg/Kg BW two days in a week, 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 30 days of treatment, five (5) animals from each group were sacrificed by cervical decapitation. The tissues of interest, namely the ovaries, and uterus, were harvested, homogenized (ice-cold Phosphate buffer, 0.1M, pH 7.4) using Teflon homogenizer to make 2 % homogenate and centrifuged at 10,000 g for 10 minutes in a Microlfield refrigerated centrifuge (Model: MF – TGL16) at 4 ^{0}C to obtain the post-mitochondrial fractions and stored for biochemical analysis. This procedure was repeated at 60, and 90 days respectively. The remaining five animals in each group were left for a 30-day post-treatment recovery period. Thereafter, they were sacrificed, tissues were processed and analyses were carried out on the harvested and processed tissues.

Determination of Proinflammatory Markers

The quantitative determination of Tumor Necrosis Factor-α **(**TNF-α) and Nuclear Factor Kappa B (NF-κB) in uterus, and ovaries were assayed using a Microplate Immunoenzymometric assay (ELISA) whose kits was supplied by Mornmed Medical Equipment Company Limited, United Kingdom.

Determination of Oxidative Stress Biomarkers

Catalase activity was determined in all tissue homogenates according to the method of Claiborne, (1985). Reduced glutathione level was estimated in all tissue homogenates using the method of Ellman (1959). The activity of Glutathione peroxidase (GPx) was measured according to the method of Rotruck *et al.,* (1973), which was based on the reaction between glutathione remaining after the action of GPx. Glutathione -S- transferase activity was determined according to the method of Habig *et al.,* (1974). 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. The activity of SOD was determined in all tissue homogenates by the method of Misra and Fridovich, (1972). The generation of thiobarbituric acid reactive substances (TBARS) contained in the tissue homogenates was measured in order to quantify the level of lipid peroxidation using the method of Varshney and Kale, (1990).

Statistical Analysis

Data obtained was expressed as mean ± standard deviation (SD), n = 5, using Graph Pad Prism version 9. 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 ($p < 0.05$). **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 ($p < 0.05$).

Results

Effect of LNG Intake on the Concentration of Nuclear Factor -Kappa B (NF-kB) in the Reproductive Organs of Wistar Rats

The concentration of NF-kB was measured in the uterus (Figure 1a) and ovary (Figure 1b) of the Wistar rats treated with LNG was compared with control.

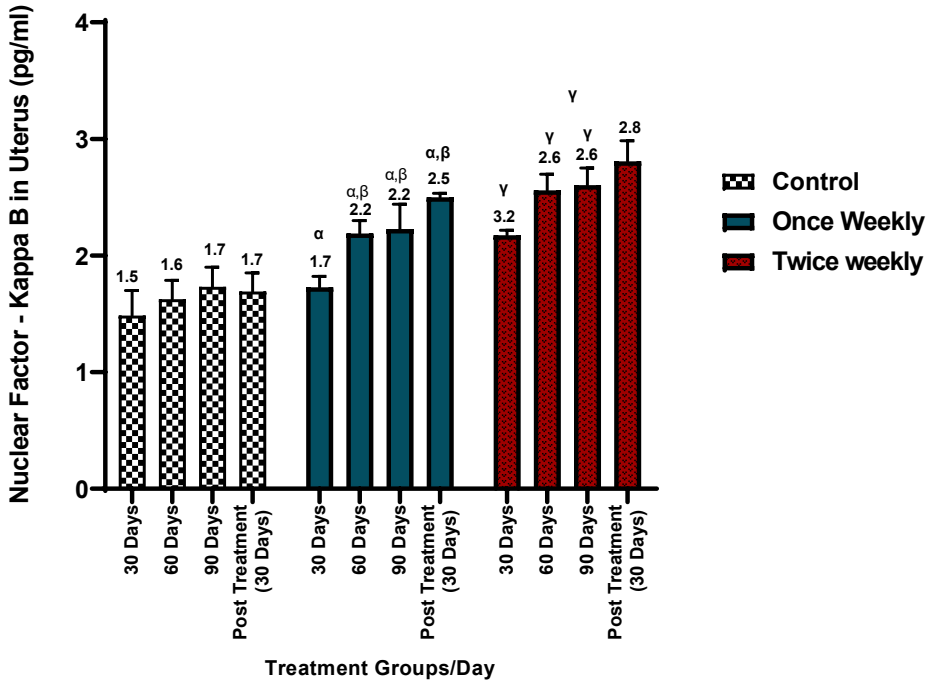

Figure 1a: Effect of LNG Intake on the Concentration of Nuclear Factor -Kappa B in Uterus of Wistar Rats

A significant increase that was dependent on the dose and period of administration was observed ($p < 0.05$). After the drug was stopped for 30 days (30 days recovery period), the concentration of NF-kB was not reversed in the uterus in both treated groups but further increased. Meanwhile in the ovary, the increase in NF-kB observed was reversed in both treated groups after drug withdrawal.

Effect of LNG Intake on the Concentration of Tumor Necrosis Factor-alpha (TNF-α) in the Reproductive Organs of Wistar Rats

The concentration of TNF-α determined in the uterus (Figure 2a) and ovary (Figure 2b) of the Wistar rats treated with LNG was compared with control. A significant increase that was dependent on the dose and period of administration was observed ($p < 0.05$). After the drug was withdrawn, the uterus experienced a remarkable decline in the level of TNF-α both in once weekly and twice weekly groups while the ovary, both treated groups maintained the same level of TNF-α as seen at 90 days.

Assessment of LNG Intake on Oxidative stress Biomarkers

Effects of Treatment on Catalase Activity in Reproductive Organs of Wistar Rats

The effect of LNG intake on reproductive organs (Figures 3a and 3b) that were harvested showed significant reductions occurred in both once weekly and twice weekly ($p < 0.05$). 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 ($p > 0.05$).

Effects of Treatment on the Concentration of Reduced Glutathione (GSH) in Reproductive Organs of Wistar Rats

Figures 4a and 4b illustrate respectfully the effects

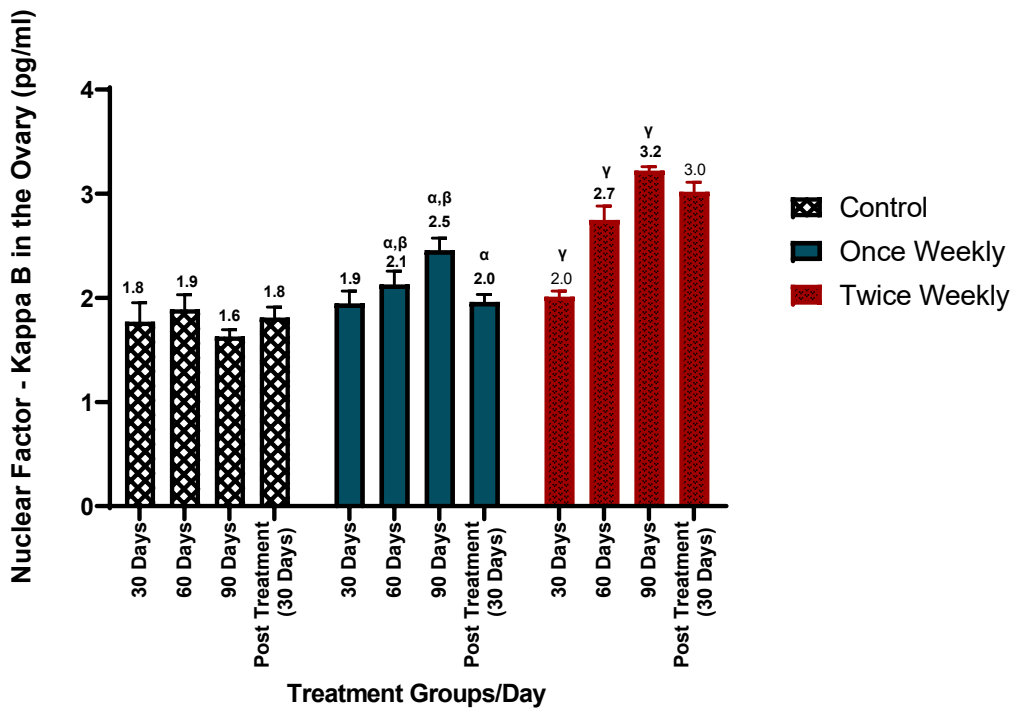

Figure 1b: Effect of LNG Intake on the Concentration of Nuclear Factor -Kappa B in Ovary of Wistar Rats

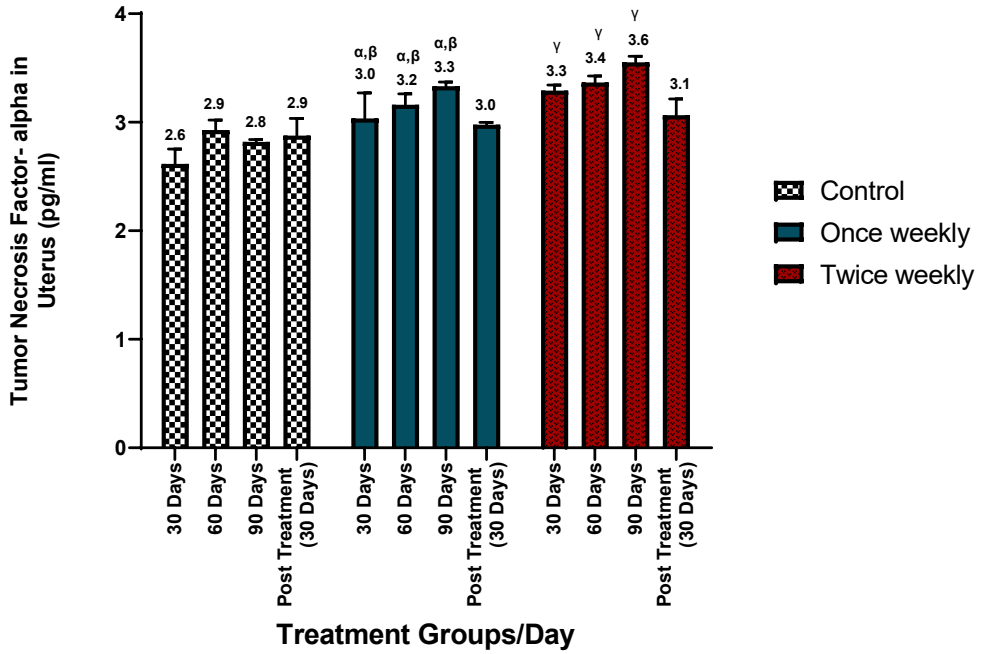

Figure 2a: Effect of LNG Intake on the Concentration of Tumor Necrosis Factor-alpha in Uterus

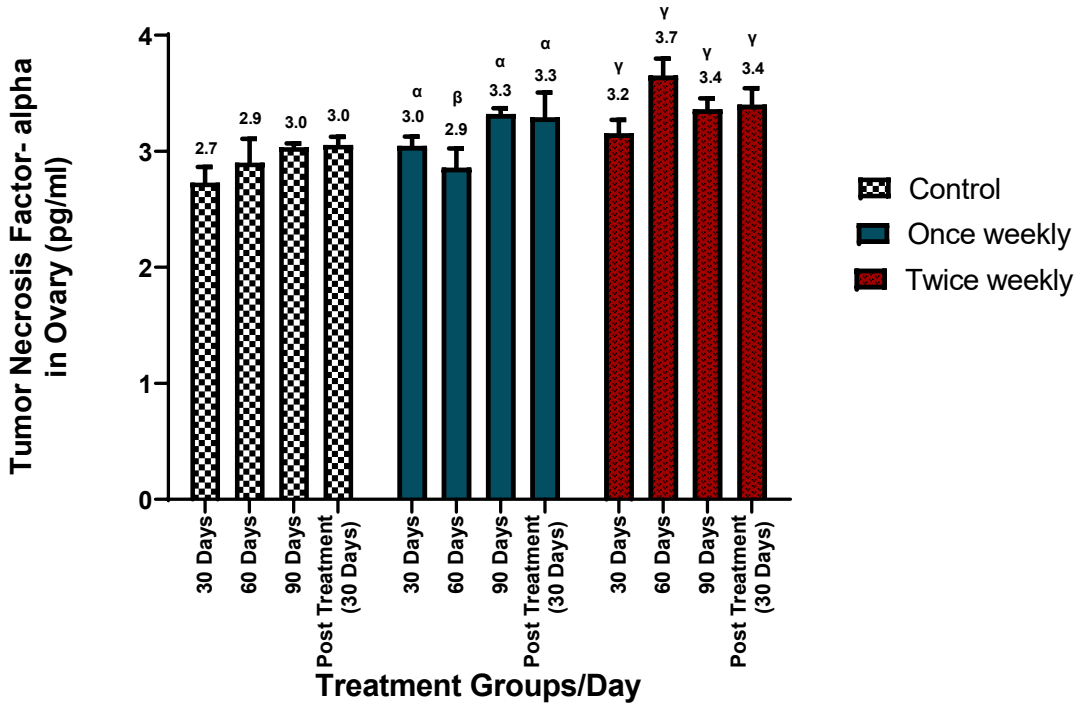

Figure 2b: Effect of LNG Intake on the Concentration of Tumor Necrosis Factor-alpha in the Ovary

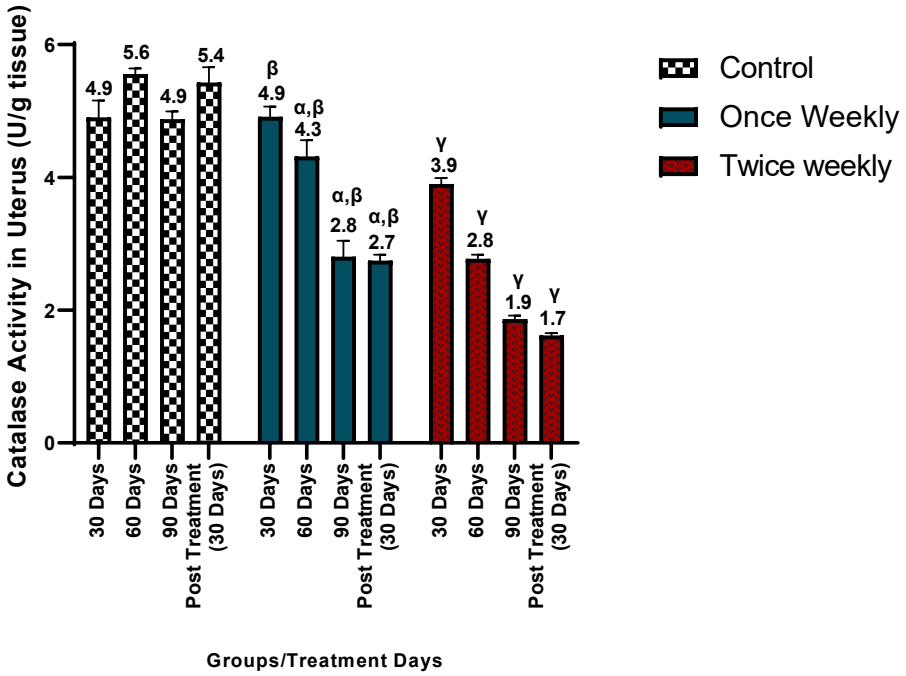

Figure 3a: Effect of LNG Intake on Catalase Activity in Uterus of Wistar Rats

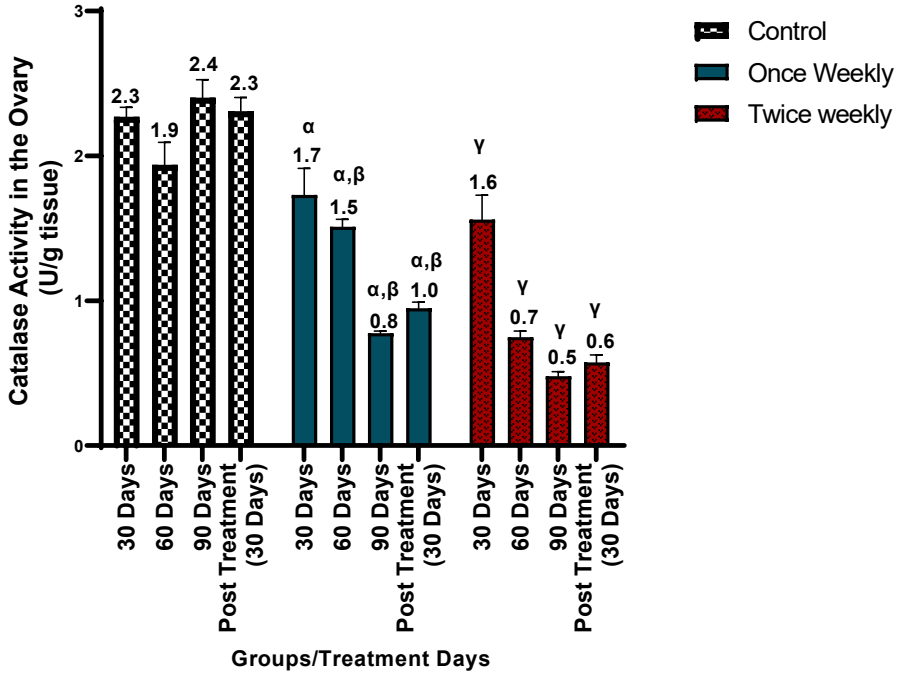

Figure 3b: Effect of LNG Intake on Catalase Activity in Ovary of Wistar Rats

of LNG intake on the level of Reduced Glutathione (GSH) in the uterus and ovary, 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 LNG 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 LNG 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 ($p < 0.05$).

Effects of LNG Intake on the Concentration of Glutathione Peroxidase (GPx) in Reproductive Organs of Wistar Rats

The effects of LNG intake on the level of Glutathione peroxidase (GPx) in harvested organs namely; uterus (Figure 5a) and ovary (Figure 5b), 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 LNG caused a significant decrease in GPx level in the uterus of once weekly and twice weekly ($p < 0.05$) in the first 30 days of treatment. The decrease continued in the second month ($p < 0.05$) and, third month ($p < 0.05$). Meanwhile, in ovary, intake of LNG did not cause any significant effect in the level of GPx in the treated

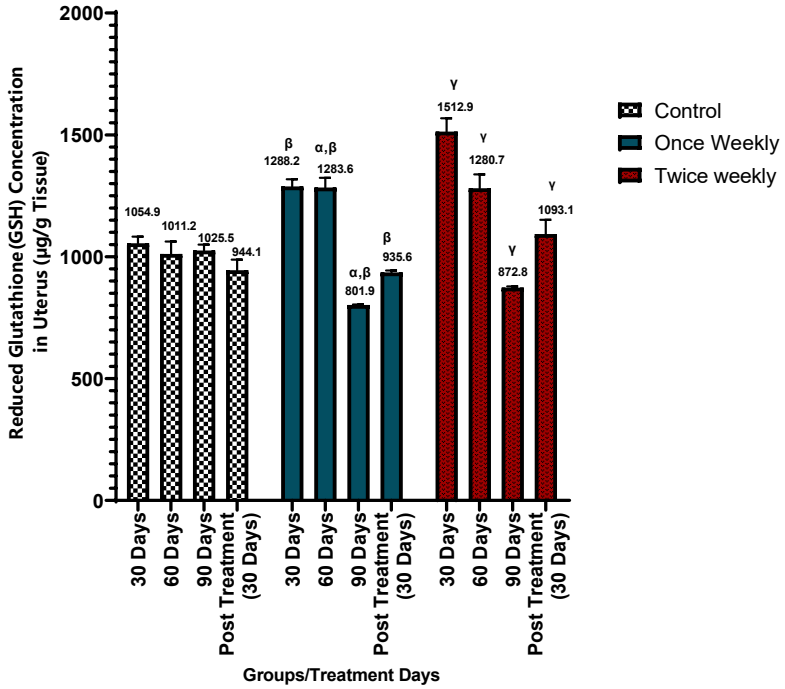

Figure 4a: Effect of LNG Intake on Reduced Glutathione (GSH) Concentration in Uterus of Wistar Rats

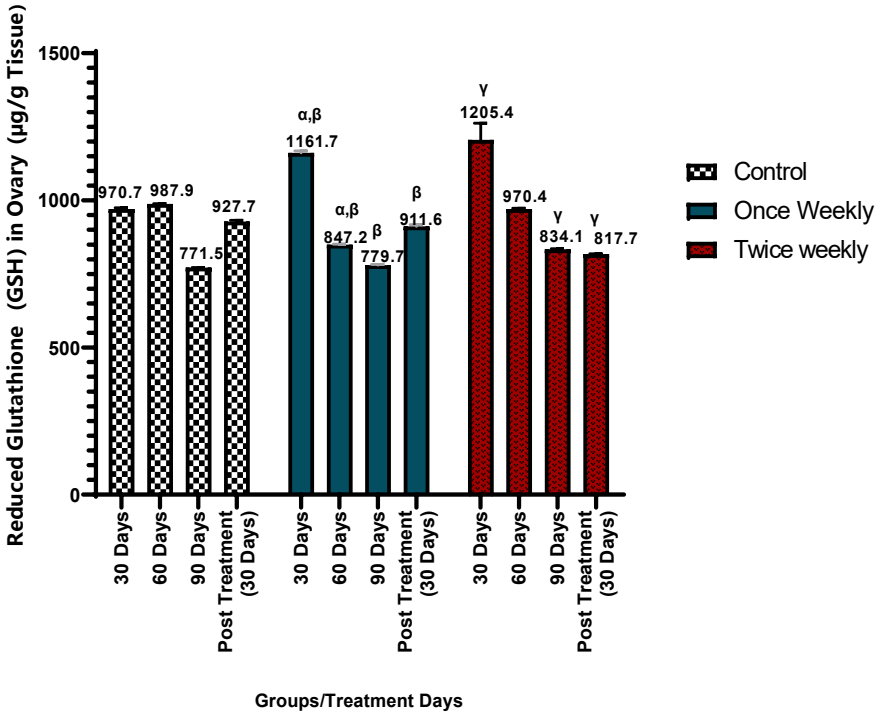

Figure 4b: Effect of LNG Intake on Reduced Glutathione (GSH) Concentration in the Ovary of Wistar Rats

groups in the first 30 days ($p > 0.05$). By 60 and 90 days, there was a reduction in concentration that was significant in both groups ($p < 0.05$).

Effects of LNG on the Concentration of Glutathione-S-Transferase in Reproductive Organs of Wistar Rats

The effect of LNG intake on the level of GST in the uterus and ovaries (Figures 6a and 6b) showed that at 30 days, 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, albeit, they were both significantly higher than control. At 90 days, both groups had decreased 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 GST in both treated groups which was still higher than in control ($p < 0.05$).

Effects of LNG on the Activity of Superoxide Dismutase (SOD) in Reproductive Organs of Wistar Rats

The effect of LNG intake on the activity of SOD in the uterus and ovary is shown in Figures 7a and 7b respectively. In both organs, there was a significant and progressive decrease in the activity of SOD with time and treatment. Similarly, decrease in SOD activity was also more pronounced in animals treated twice weekly when compared with once weekly ($p < 0.05$). When LNG was withdrawn (recovery period), slight improvement was observed in the uterus while the ovary experienced a further decrease in SOD activity

Effects of LNG Intake on the Level of Malondialdehyde (MDA) in Reproductive Organs of Wistar Rats

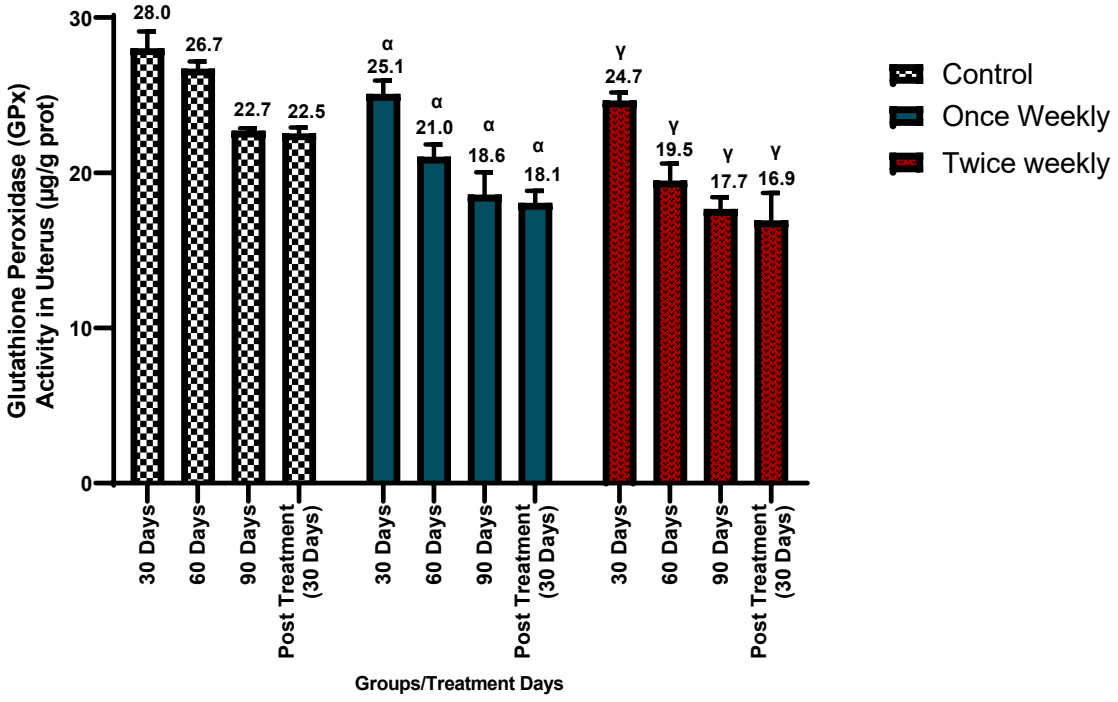

Figure 5a: Effect of LNG Intake on Glutathione Peroxidase (GPx) Activity in Uterus of Wistar Rats

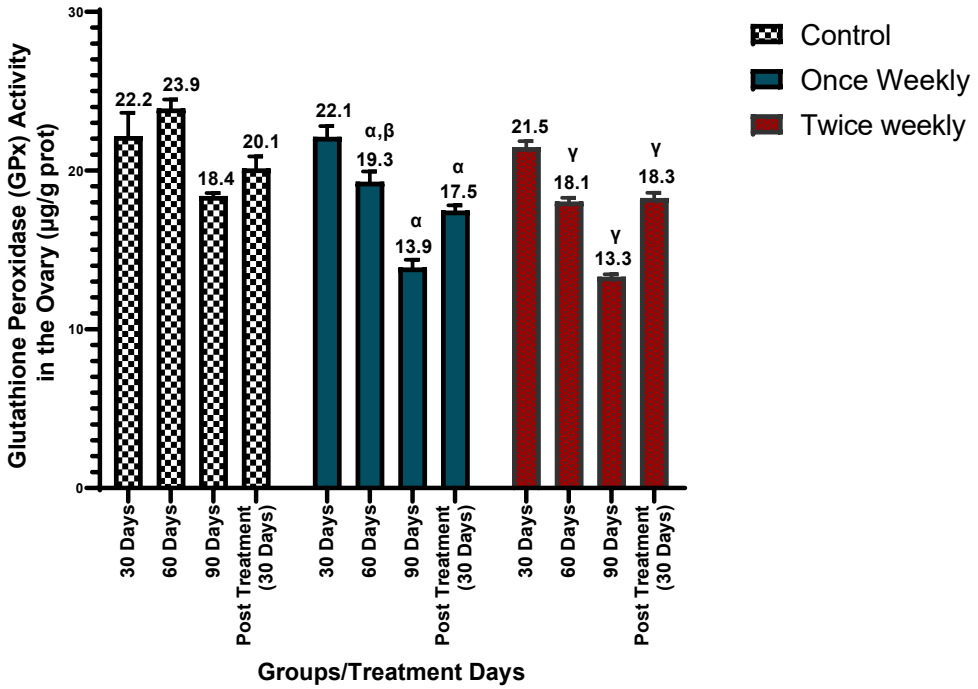

Figure 5b: Effect of LNG Intake on Glutathione Peroxidase (GPx) Activity in the Ovary of Wistar Rats

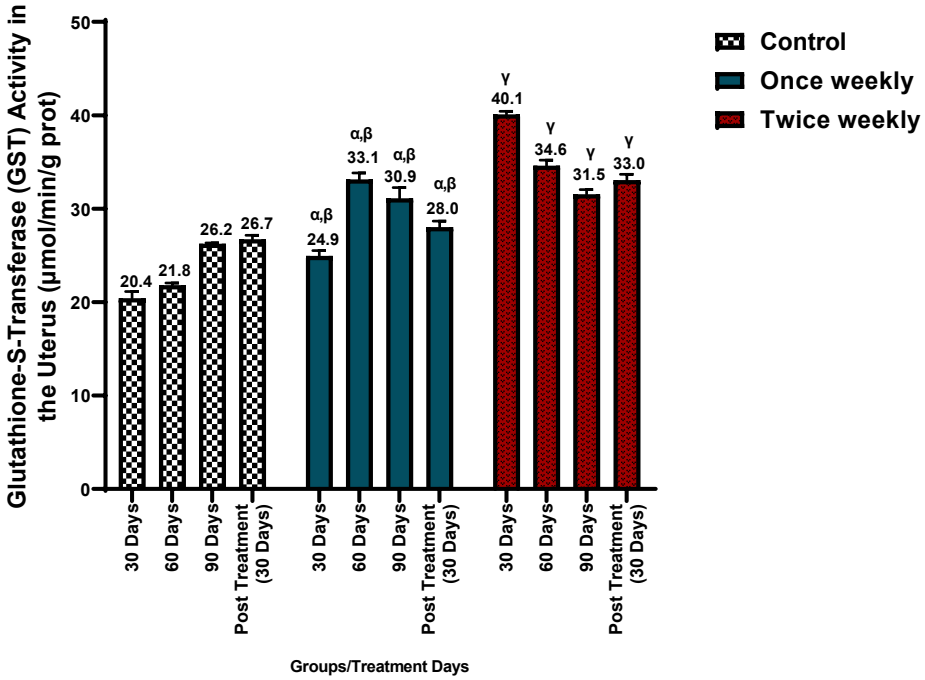

Figure 6a: Effect of LNG Intake on Glutathione-S-Transferase (GST) Activity in the Uterus of Wistar Rats

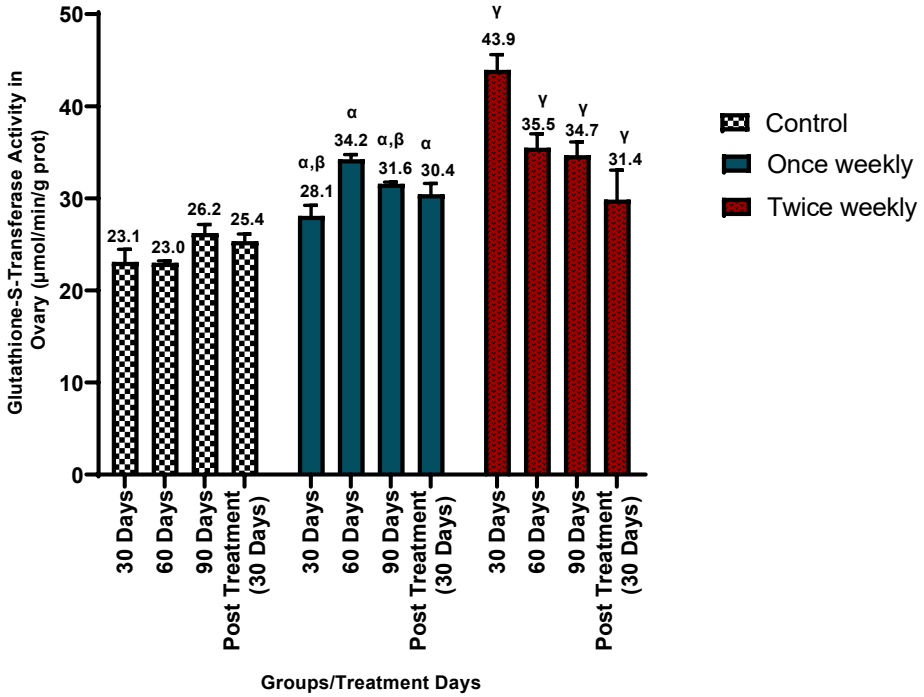

Figure 6b: Effect of LNG Intake on Glutathione-S-Transferase (GST) Activity in the Ovary of Wistar Rats

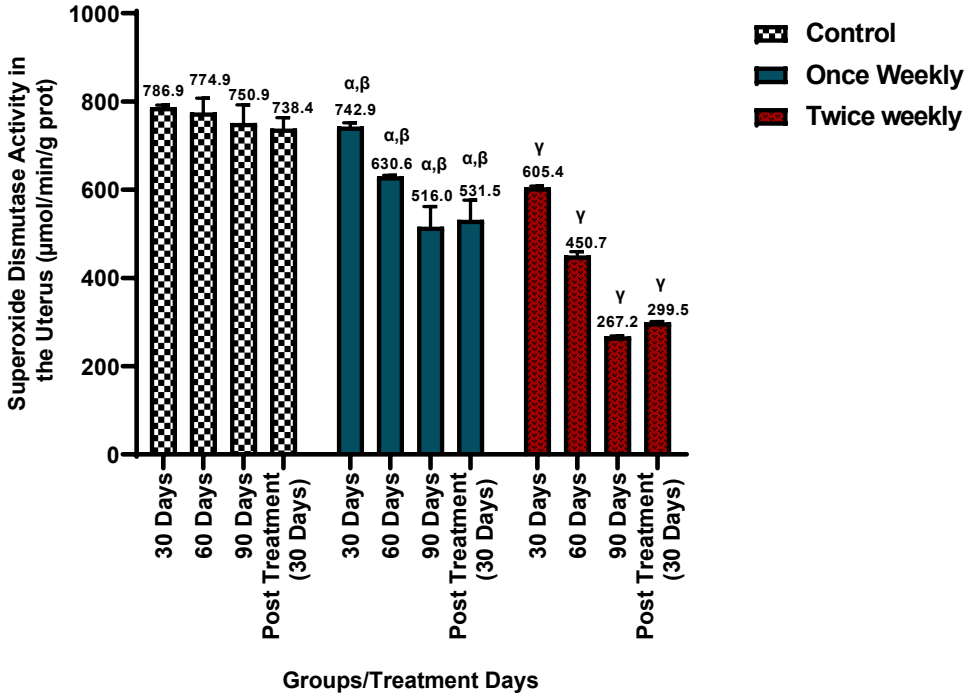

Figure 7a: Effect of LNG Intake on Superoxide Dismutase (SOD) Activity in Uterus of Wistar Rats

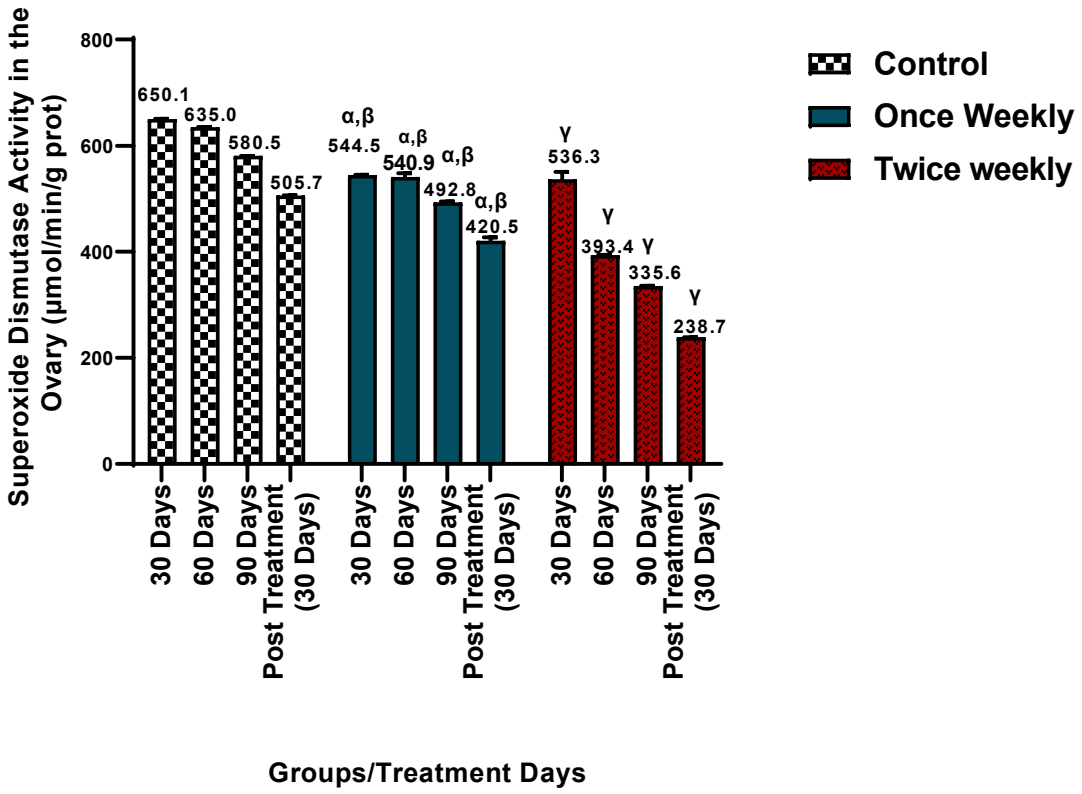

Figure 7b: Effect of LNG Intake on Superoxide Dismutase (SOD) Activity in Ovary of Wistar Rats

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 ($p < 0.05$). At 60 days, 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 LNG 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 ($p < 0.05$. The Recovery period brought about a reduction in MDA level especially in the uterus. The ovary only experienced recovery in the once weekly group while elevation in MDA concentration continued in twice weekly group.

Discussion

This study investigated the inflammatory and oxidative stress effects of prolonged intake of levonorgestrel (LNG) in female *Wistar* rats, with a focus on its impact on key proinflammatory cytokines, namely tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB) as well as oxidative stress biomarkers and enzymatic antioxidants in the uterus and ovaries. Results show a clear dose- and duration of intake-dependent proinflammatory and pro-oxidative response, which was only partially reversible after stopping LNG administration only in

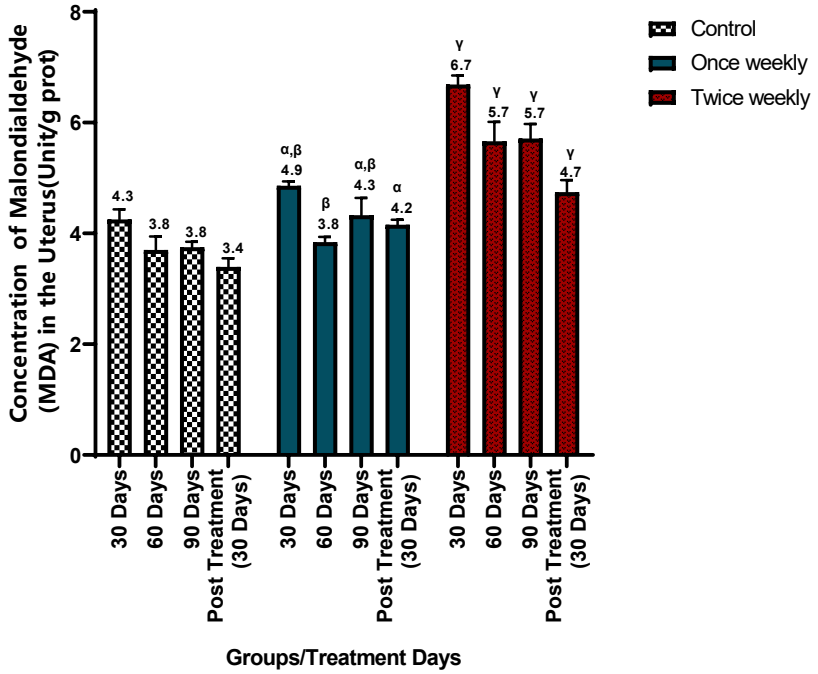

Figure 8a: Effect of LNG Intake on Malondialdehyde (MDA) Concentration in Uterus of Wistar Rats

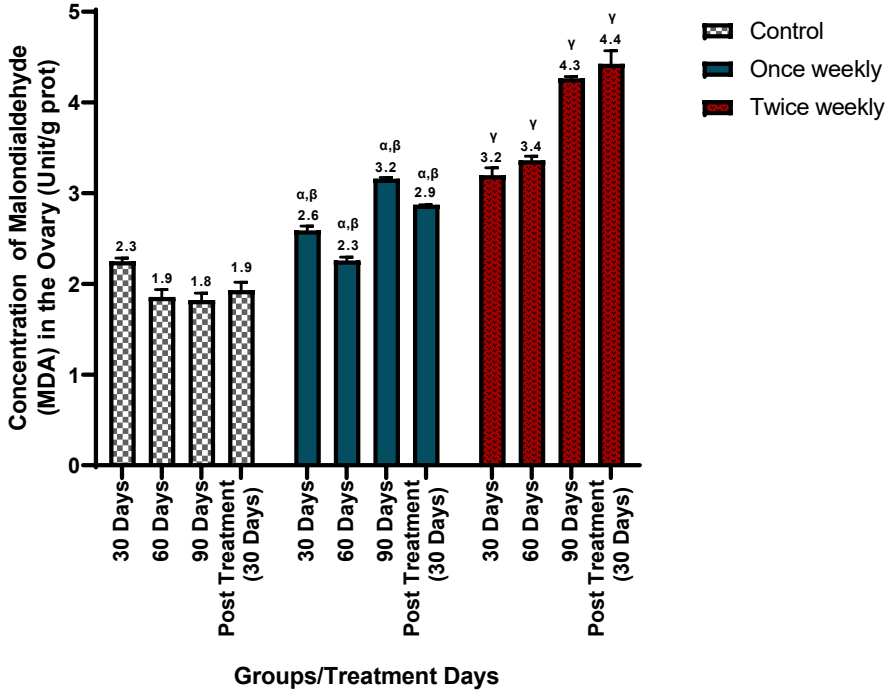

Figure 8b: Effect of LNG Intake on Malondialdehyde (MDA) Concentration in the Ovary of Wistar Rats

the once-weekly group.

The observed upregulation of TNF-α and NF-κB in the uterus and ovaries, especially in the twice-weekly LNG-administered group, indicates the activation of inflammatory pathways associated with prolonged hormonal stimulation. This is possible because LNG is a synthetic compound formulated to mimic the natural hormone progesterone, known as progestin (Mierzejewska *et al*., 2024). Its ability to bind to the receptors of both progesterone and androgen to cause a delay in the release of gonadotropin-releasing hormone from the hypothalamus inhibits the luteinizing hormone surge that occurs during the pre-ovulation stage. This is one among many ways of inhibiting normal body processes in an effort to prevent pregnancy, which could overwhelm the body's defence system, especially in the case of frequent use thereby contributing to the upregulation of these biomarkers.

TNF-α, produced predominantly by activated macrophages, plays a central role in initiating and sustaining inflammatory cascades in reproductive tissues. Elevated levels of this cytokine have been correlated with several reproductive disorders such as endometriosis, polycystic ovarian syndrome (PCOS), and immunological infertility (John-Olabode *et al*., 2025; Muisi *et al*., 2022). These could be contributed to or exacerbated by an individual's peculiarity and immune level. Although menstrual abnormalities, dysmenorrhea, amenorrhea, oligomenorrhea, headaches, and acne, are more frequently reported among users of LNG and these have been reported to greatly affect a woman's quality of life (Odongo *et al*., 2023). The persistent elevation of NF-κB observed even after the 30-day post-treatment period in the uterus further suggests potential for long-term inflammatory imprinting or impaired resolution mechanisms. NF-κB, being a transcription factor that regulates numerous genes related to immune and stress responses, may exacerbate tissue damage when hyperactivated, contributing to chronic reproductive dysfunction (Gao *et al.,* 2025).

These were further justified by the oxidative stress profile in both organs which revealed marked alterations in antioxidant enzyme activities and lipid peroxidation. This depletion of antioxidant defenses underscores the generation of reactive oxygen species (ROS) beyond physiological neutralizing capacity (Adeniji *et al*, 2022, Agarwal 2012). The concurrent increase in malondialdehyde (MDA), a reliable marker of lipid peroxidation, and glutathione-S-transferase (GST) activity further confirms the presence of oxidative stress-induced cellular injury in the reproductive tissues. Notably, the uterus appeared more susceptible than the ovary, which may reflect differences in tissue-specific metabolism, hormone receptor density, or inflammatory sensitivity.

Interestingly, while some degree of recovery was observed in TNF-α, GSH, and GPx levels, particularly in the once-weekly group, this improvement was either absent or minimal in the twice-weekly treatment group. This finding implies a threshold effect, where frequent exposure to LNG surpasses the organ's ability to recover fully, even after cessation of exposure. The persistence of SOD and MDA alterations post-treatment, especially in the uterus, suggests that oxidative damage may induce long-lasting or potentially irreversible cellular changes, such as mitochondrial dysfunction or membrane instability (Di-Callo and Sorrentino, 2024).

These results are consistent with prior evidence linking synthetic progestins to redox imbalance and reproductive toxicity. Studies have shown that chronic hormone administration can impair mitochondrial function, dysregulate apoptosis, and alter cytokine expression in estrogen- and progesterone-sensitive tissues (Shukla *et al*., 2017; Batres *et al*., 2018). Cauci *et al*., (2025) observed an increase oxidative stress and inflammation generated in response to intake of combined oral contraceptives. Also, Ibrahim *et al*., (2024) discovered that a higher concentrations of LNG disrupted developmental and reproductive processes as a result of increased oxidative stress response. A human study by Buyuk *et al.*, (2021) also observed increased oxidative stress among

women using LNG-containing intrauterine devices. Using *Dreissena polymorpha*, Contardo-Jara *et al*., (2011) observed enhanced oxidative processes as a result of second phase biotransformation of the drug. Our findings contribute further by delineating how different dosing frequencies of LNG impact the severity and reversibility of its effects.

From a translational perspective, the findings raise important concerns about the off-label or frequent use of emergency contraceptive pills among young women. While LNG is generally safe when used occasionally, repeated and unmonitored intake, as reflected in the twice-weekly group, may predispose individuals to chronic inflammation, oxidative damage, and reproductive dysfunction, potentially affecting fertility, endometrial health, and menstrual regulation. More importantly because despite advances in the diagnosis, prevention, and treatment of gynecological abnormalities, there are still increased mortality and morbidity in females as an outcome of high prevalence and incidence of cancers of the reproductive organs, unexplained infertility, uterine fibroids, endometriosis, and polycystic ovary syndrome (PCOS) (John-Olabode *et al.*, 2025). Inflammation and oxidative stress are two processes that significantly contribute to the onset of many diseases. Increased levels of these biomarkers are associated with increased risk and severity in these conditions. They have also been shown to play a role, potentially impacting their growth and progression.

Conclusion

Prolonged and frequent intake of levonorgestrel induces significant inflammatory and oxidative stress responses in the uterus and ovaries of female Wistar rats. These effects were more severe and less reversible with increased dosing frequency, underscoring the risks associated with the habitual use of emergency contraceptives. These findings advocate for increased public health education on the appropriate use of LNG, further research into its long-term reproductive safety, and potential mitigation strategies to safeguard female reproductive health

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