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Oxidative Stress and Hepatotoxic Effects of Levonorgestrel Treatment in Female Wistar Rats

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Abstract

Oral Levonorgestrel (LNG) is an emergency contraceptive pills, used in the prevention of unwanted pregnancy and it is effective when taken within first 72 hours of unprotected sexual intercourse. In order to evaluate its toxicological potential, female Wistar rats (140 ± 20 g; $n=15$) were exposed to LNG at varying doses (0.004 mg/kg b.w) once per week, (0.004 mg/kg b.w) or (0.008 mg/kg b.w) 3 times a week at 48 hours interval each for 6 weeks. Control rats ($n=5$) were exposed to 0.2 ml/kg b.w physiological saline three times per week at 48 hours intervals for the same period. Blood and tissue supernatants were collected for biochemical assays. The result showed significant ($p < 0.05$) increase in cardiac phospholipids concentration in a dose-dependent manner but at variance in the kidney. Meanwhile, MDA concentration was at variance with no significant different along with reduced GSH values. Serum ALT was significantly ($p < 0.05$) decreased with LNG 0.004 mg/kg/week when compared with control; however, ALT and AST activities were significantly ($p < 0.05$) amplified in LNG 0.004 mg/kg/3ce/week and LNG 0.008 mg/kg/3ce/week when compared with LNG 0.004 mg/kg/week. Serum total cholesterol significantly increased with LNG 0.004 mg/kg /week when compared with control while serum triglyceride significantly decreased with LNG 0.008 mg/kg/3ce/week when compared with LNG 0.004 mg/kg /week. These findings indicate that LNG evoked cellular injury without significant peroxidation. It exhibited some metabolic changes with adverse effect. Therefore, its availability should be restricted by prescription and administer as prescribed.

Keywords: Levonorgestrel, Emergency contraception, Oxidative stress, Hepatotoxicity, Lipid

Introduction

Levonorgestrel (LNG) is a synthetic progesterone analogue with extensive contraceptive and non-contraceptive properties. Oral preparation is specifically recommended for use as dedicated emergency contraceptives, in order to prevent unwanted pregnancy after unprotected sexual intercourse. It is said to be effective within the first 72 hours and safe in all situations. However, with violation of rules guiding its use, some metabolic dysregulations are anticipated with repeated use. The magnitude of injury mediated free radical lipid peroxidation can be measured by biological markers like malondialdehyde (MDA) [1,2]. Unsurprisingly, both lipid hydroperoxides and aldehydes are in biological system promoting oxidative stress through the increase of 2-thiobarbituric acid reactive substances (TBARS) that could be used to estimate MDA in tissues and fluids [3]. The objective of this study was to investigate the potential hepatotoxic and prooxidative/antioxidative effects of varying doses and repeated use of LNG in female Wistar rats.

Materials and Methods

Experimental Design

Twenty (20) female mature healthy Wistar rats (140 ± 20 g) were purchased from trusted animal vendor in Osogbo. The animals were kept under condition of uniform humidity and temperature on a 12 hour light-dark cycle. Animals were kept in dry aerated wooden cages and acclimatized for three (3) weeks in order to exclude pregnancy, adjust and adapt to new environment, physiologically and psychologically. The animal had unrestricted access to standard rat pellet diet and clean water *ad libitum*. As described by Bolu *et al.* (2009), the weight of the animals were determined on the first day of acclimatization and then weekly, throughout the period of study [4].

At the commencement of the experiment, the animals were randomly divided into 4 groups ($n=5$). Group A served as control group and received 0.2 ml/kg body weight normal saline three times per week at 48 hours intervals orally.

Group B was exposed to regular dose of LNG (0.004 mg/kg body weight) once a week orally via intragastric tube gavage and duration of drug administration was six (6) weeks.

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Group C was exposed to regular dose at increased frequency of LNG (0.004 mg/kg body weight, 3 times a week) at 48 hours interval orally via intragastric tube gavage and duration of drug administration was six (6) weeks. Group D was exposed to high dose and at increased frequency of LNG (0.008 mg/kg body weight 3 times a week) at 48 hours interval orally via intragastric tube gavage and duration of drug administration was six (6) weeks.

Collection of Blood Sample

At the end of the exposure, animals were fasted overnight and subjected to light ether anaesthesia. Blood was collected into plain bottles by cardiac puncture and then centrifuged at 4000 rpm for 10 minutes to obtain the serum and stores at -20°C until further analysis.

Preparation of Tissue Homogenate

Liver, kidney and heart were excised, rinsed with 1.15% KCl solution to remove haemoglobin. 1 g of each organ was homogenized in potassium phosphate buffer (0.1M, pH 7.4) and centrifuged at 5000 rpm for 10 min to obtain the homogenate. The homogenate was kept at -20°C until further analysis.

Biochemical Assays

Lipids were extracted from liver, kidney and heart as described by Folchet *al.* (1957) [5]. After washing with 0.05 M KCl solution, aliquots of the chloroform–methanol extract were then used for the determination of cholesterol, triglycerides and phospholipids concentrations. Concentrations of total cholesterol, triglycerides and phospholipids were determined with commercial kits (Spin React S.A., Santa Coloma, Sant Esteve De Bas, Spain). Lipid peroxidation was evaluated by measuring thiobarbituric reactive oxygen species (TBARS) in form of MDA using the method described by Vashney and Kale, 1990 [6]. The method described by Evelson *et al.*, 2001 was employed in evaluating the level of reduced glutathione in the serum and tissue homogenates [7]. Serum ALT and AST activities were assayed in the plasma using the Randox® reagent kits according to the method of Reitman and Frankel, 1957 [8].

Statistical Analyses

Statistical analyses were performed with One-way Analysis of Variance (ANOVA) using SPSS package program, version 20.0 (IBM Corporation, Armonk, NY, USA) software, followed by post-hoc Turkey multiple comparison test. Results were plotted using GraphPad Prism (GraphPad Prism Software version 5.01, San Diego, CA, USA). Data were presented as the Mean $\pm$ standard error of means (S.E.M). Statistical significance was considered at $p < 0.05$.

Results

Phospholipids concentration in Liver, Kidney and Heart

Hepatic, renal and cardiac phospholipids concentrations were depicted in figure 1. As illustrated, the LNG caused a significant ($p < 0.05$) induction of cardiac phospholipidosis when compared with the control. Meanwhile, in the liver, LNG treatment induced a non-significant ($p > 0.05$) increase in phospholipids concentration in a dose dependent manner. Furthermore, treatment of the animals with LNG 0.004 mg/kg/week and LNG 0.008 mg/kg/3ce/week led to a significant influence ($p < 0.05$) of renal phospholipids concentration. Contrarily, LNG 0.004 mg/kg/3ce/week decreased the renal phospholipid significantly when compared with the control.

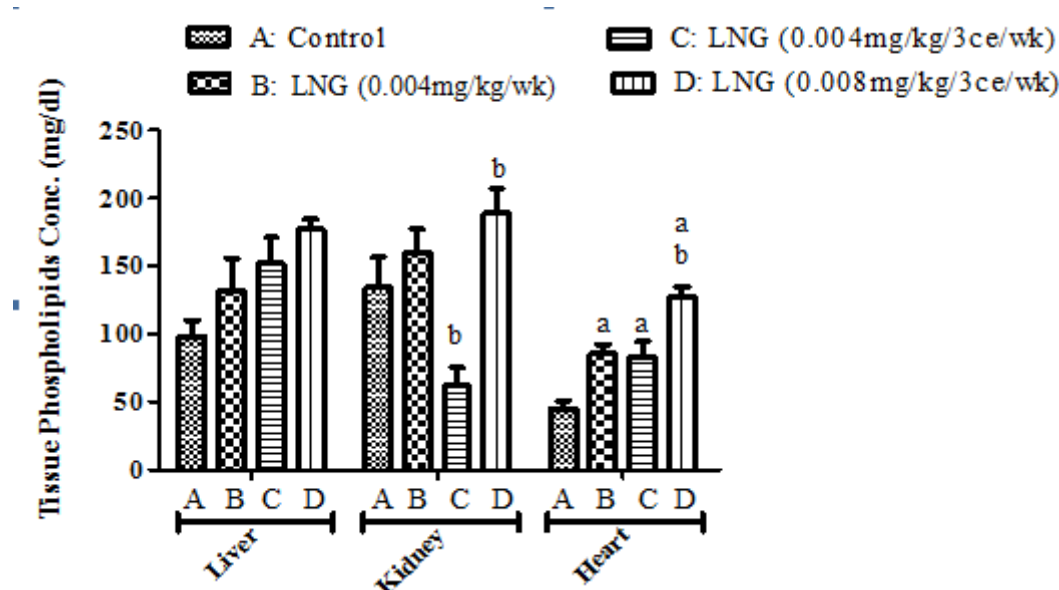


Figure 1. Effect of LNG on phospholipids concentrations in liver, kidney and heart in female Wistar rats. Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group; ^b Significant when compared with LNG 0.004 mg/kg/week at $p < 0.05$

Concentration of Malondialdehyde MDA in Liver, Kidney and Heart

As presented in figure 2, when compared with the control, LNG treatments in all the groups induce a non-significant ($p>0.05$) changes in the hepatic, renal and cardiac MDA level, which is the end product of lipid peroxidation.

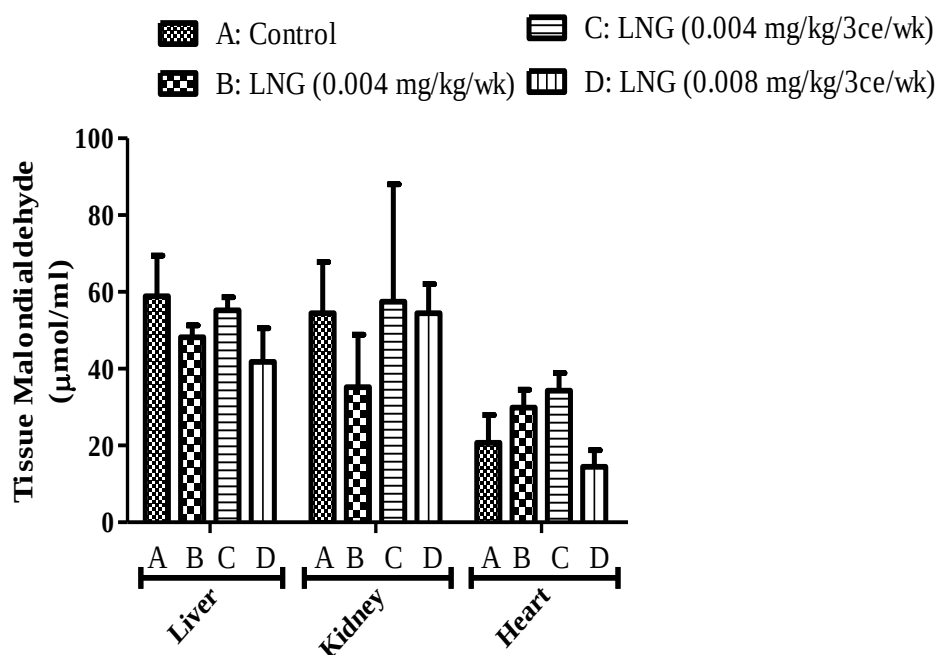


Figure 2. Effect of LNG on malondialdehyde concentrations in liver, kidney and heart.

Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group;

^b Significant when compared with LNG 0.004 mg/kg/week at $p<0.05$

Concentration of tissue Reduced Glutathione (GSH)

The mean hepatic, renal and cardiac GSH concentrations of the animals exposed to various doses of LNG is illustrated in figure 3. As presented in the figure, exposure to LNG 0.004 mg/kg/week induced a significant ($p<0.05$) reduction in the concentration of GSH in the liver. Contrarily, the treatment produced no significant ($p>0.05$) changes in the level of GSH in both the kidney and the heart.

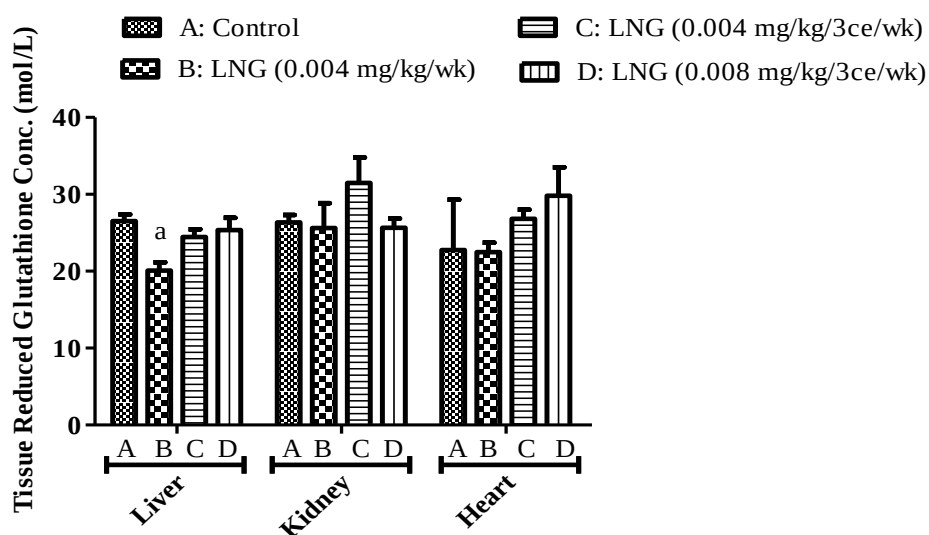


Figure 3. Effect of LNG on reduced glutathione in liver, kidney and heart in female Wistar rats. Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group; ^b

Significant when compared with LNG 0.004 mg/kg/week at $p<0.05$

Activities of Serum Alanine and Aspartate Aminotransferases

As depicted in figure 4, exposure of the animals to LNG 0.004 mg/kg/week significantly ($p < 0.05$) decreased the serum activity of ALT. However, when the frequency of exposure and dosage was increased to LNG 0.004 mg/kg/3ce/week and LNG 0.008 mg/kg/3ce/week respectively, there is a significant ($p < 0.05$) up-regulation of the activities of both transaminases in circulation.

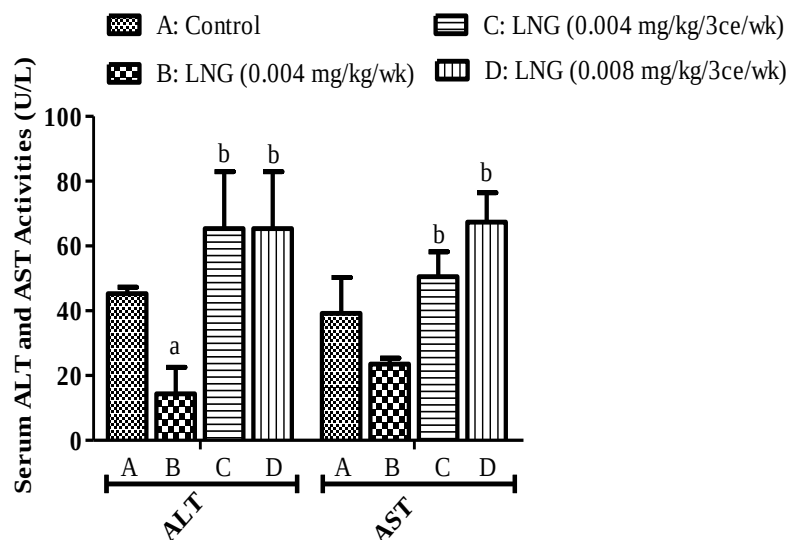


Figure 4. Effect of LNG on serum alanine and aspartate aminotransferases in female Wistar rats. Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group; ^b Significant when compared with LNG 0.004 mg/kg/week at $p < 0.05$

Change in serum Total cholesterol and Triglycerides concentrations

Treatment of the animals with various doses of LNG significantly ($p < 0.05$) induced hypercholesterolemia in the serum of the exposed animals when compared with the control (figure 5). On the other hand, when compared with the control animals, the only significant ($p < 0.05$) effect of LNG treatment on serum total triacylglycerol concentration was hypertriglyceridemia induced by exposure to LNG 0.008 mg/kg/3ce/week (figure 5).

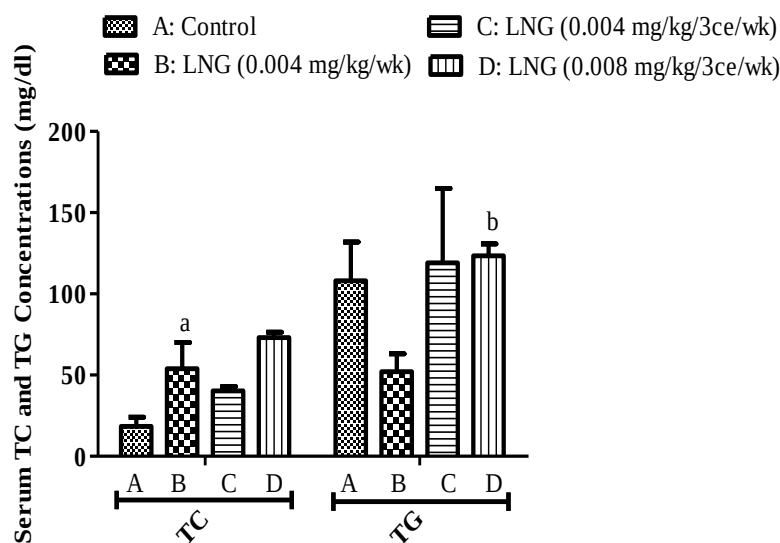


Figure 5. Effects of LNG on serum total cholesterol and triglycerides in female Wistar rats. Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group; ^b Significant when compared with LNG 0.004 mg/kg/week at $p < 0.05$

Change in serum Reduced glutathione concentration

As presented in figure (6), exposure to LNG 0.004 mg/kg/week caused a significant ($p < 0.05$) increase in the serum concentration of GSH. Contrarily, when the frequency of exposure and dosage was increased to LNG 0.004 mg/kg/3ce/week and 0.008 mg/kg/3ce/week respectively, the concentration of reduced glutathione in the serum was non significantly ($p < 0.05$) down – regulated.

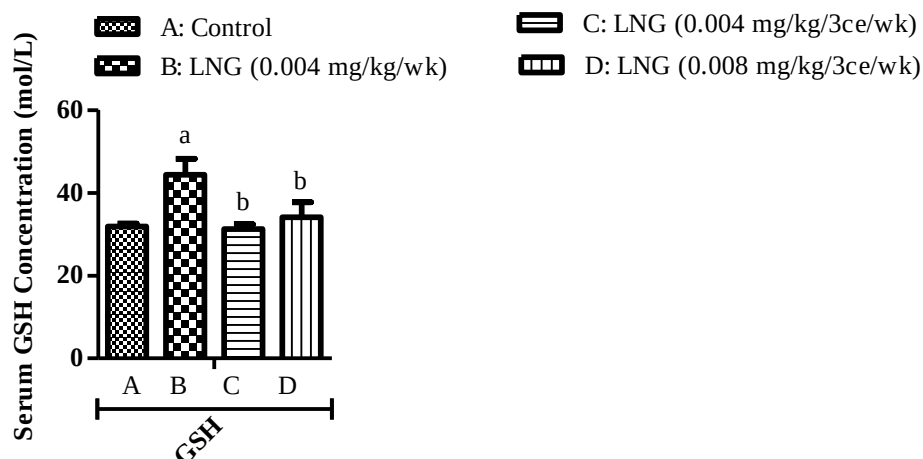


Figure 6. Effect of LNG on serum GSH concentration in female Wistar rats. Each bar represents Mean $\pm$ SEM of five animals in each group. ^a Significant when compared with control group; ^b Significant when compared with LNG 0.004 mg/kg/week at $p < 0.05$

Discussion

LNG modulates tissue's production of phospholipids

In this study, LNG caused mild decrease in tissue phospholipids concentrations (LNG 0.004 mg/kg/week and 0.004 mg/kg/3ce/week) respectively. However, increasing the dose and frequency of exposure tends to raise the liver and heart phospholipids concentration. The possible explanations for this observation are: at regular dose but increase frequency, LNG down - regulates the phospholipids synthesis and translocation from their site of production to target tissues [9, 10, 11]. Homeostatically, in the fed state, the followings processes are expectedly favoured:

1. Synthesis of storage fats (triacylglycerol) is favoured
2. Induction/repression of genes that synthesized transporter proteins that are responsible for transportation of phospholipids from site of synthesis to the plasma membrane and tissues
3. Decreased concentration and activities of translocases that are responsible for mobilization from circulation (extracellular space) to target cells and/or tissues
4. Altered degradation rate of phospholipids, mutation that can cause alteration in the orientation or conformational change in the distribution of phosphoglyceride molecules which may influence the asymmetric distribution of lipids in or within the cell
5. Exposure to physical or chemical insult with subsequent cellular adaptive response [12]

Injury to the cell plasma membrane may cause the breakdown and release of member of a membrane phospholipid called arachidonic acids whose presence is identifying as a marker of cellular damage. This marker is a free fatty acid (FFA) or polyunsaturated fatty acid (PUFA) [13].

Decrease tissue phospholipids may have implication on tissue oxidative damage because it may be consequent upon increase release of phospholipids due to increase tissue membrane damage [14]. The slight increase in tissue phospholipids as observed can therefore be linked to activation of endogenous antioxidant system.

However increased concentration of tissue phospholipids at double dose and increase frequency (LNG 0.008 mg/kg/3ce/week) may be ascribed to repressive effect of LNG at tissue level since it has been demonstrated by Lindenthal *et al.* (2015) that hormones (LNG synthetic analogue of progesterone) have high affinity for their receptors, very low concentrations are sufficient to produce responses in target tissues [15]. Conversely, this may be facilitated by the following events: increased concentration and activities of translocases that are responsible for mobilization from circulation (extracellular space) to target cells and/or tissues; altered degradation rate of phospholipids, mutation that can cause alteration in the orientation or conformational change in the distribution of phosphoglyceride molecules which may influence the asymmetric distribution of lipids in or within the cell prevent apoptosis [14,16].

LNG alters tissue's Pro-oxidant/Oxidant potential system

In this study, MDA concentration was at variance with no significant different along with reduced GSH values show that lipid peroxidation is enhanced and in contrast, the antioxidant system is weakened with LNG treatment. This observation was similar to finding obtained by Krauss and Burkman, 1992, which attributed this effect to enhanced hepatic synthesis and secretion of triglyceride rich particles, total cholesterol and modulation of LDL receptor activity [17].

LNG potentially induced hepatotoxicity

Our results suggest hepatotoxic potential of LNG in rats. Serum GSH an indicator of serum antioxidant potential was also elevated. However, regular dose and increase frequency of LNG (0.004 mg/kg/3ce/week), markedly increased serum hepatic function indices (ALT and AST) and serum GSH. At the highest dose and frequency (0.008 mg/kg/3ce/week), serum ALT and AST remain considerably elevated along with serum total cholesterol (TC). A rise in plasma ALT and AST is an indicator of damaged hepatic cell membrane specifically involving the cytoplasmic and /or mitochondria [18]. This finding revealed drug-induced hepatotoxic potential of LNG. Serum AST and ALT are important biochemical markers of hepatocyte membrane and biliary tract integrity, when there is insult to hepatocyte and biliary tract, the serum level of AST and ALT increases with increase release of these cytoplasmic and/or mitochondrial enzymes. These demonstrated the potential adverse effect of LNG on the liver function [19].

LNG potentially induced dyslipidemia

Similarly, LNG obviously tends to raise serum total cholesterol which reached a considerable value at the highest dose and frequency. This is a prelude and pointer to hypercholesterolemia and associated dyslipidemic condition like atherosclerosis. Since the observation in the present study reveals a marked difference at the highest dose and frequency (LNG 0.008 mg/kg/3ce/week), it can however be suggested that LNG may possess hypercholesterolemic properties and caution should be taken against its indiscriminate use by women of reproductive age who are fond of taken the medication outside recommended dose and frequency. Furthermore, the higher plasma cholesterol levels may lead to a greater increase in peroxide formation in low density lipoproteins (LDLs) [20]. Oxidized LDL may promote the development of plaque through a number of potentially atherogenic properties. It is a chemoattractant for leucocytes and stimulates cytokine production by macrophages. Oxidized LDL was also considered to play an important role in atherogenesis [21].

The serum triglyceride concentrations, without statistical significance, was also elevated in dose dependent manner among LNG administered groups compared to normal saline treated group. Triglyceride is known to be transported by VLDL to peripheral tissue during which LDL is produced.

LNG modulates serum free radical scavenger concentration

The serum antioxidant, GSH concentration which increased initially, reduced gradually to a level comparable to normal saline treated group. These shows that at the regular dose and frequency (LNG 0.004 mg/kg/week), the antioxidant defense system is very active and was able to protect the tissues against oxidative damage. However, the defense system got weakened with increase dose and frequency of LNG (LNG 0.004 mg/kg/3ce/week and 0.008 mg/kg/3ce/week) respectively. The reduced concentration of GSH was not significant thus the non-significant change in tissue lipid peroxidative damage as observed through the measurement of tissue MDA concentration. Oxidative stress is associated with the development of several disorders including cancer, gonadotoxicity, neurodegenerative and vascular diseases (22, 23, 24). Oxidative stress is defined as an imbalance between antioxidants and reactive oxygen species (ROS) in favour of the latter. ROS, which include free radicals, are continuously produced in the body and play an important physiological role at low concentrations [25].

Conclusion

Inferentially, LNG exhibited dyslipidemic effects by inducing elevated tissue and serum total cholesterol TC, decreased tissue but elevated serum triglycerides TG, and relative decreased tissue phospholipids, limit the generation of free radicals, thus enhanced immune defense system. The hepatotoxic potential was demonstrated by raised hepatic membrane enzymes due to elevated serum ALT and AST and/or mitochondria in the hepatocytes. Furthermore, these elevated parameters corroborate the cumulative potential of LNG on pro-oxidant/antioxidant system at revealed specific damage to cytoplasm and/ or mitochondria.

Recommendation: The oral levonorgestrel as emergency contraceptive pills exhibited some metabolic changes with adverse effect. Therefore, its availability should be restricted by prescription and administer once a week as prescribed.

Acknowledgements: I acknowledged my institution, University of Ilorin, Nigeria for the 'Staff Development Award' grant. The authors appreciate the mutual understanding of Adebowale Olabanji, Bridge Scientific Enterprises, Ilorin, Nigeria for his technical assistance during bench work and biochemical assays respectively.

Conflict of Interest: Authors have declared that no potential conflict of interest exist.

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