



RESEARCH ARTICLE

Sub-chronic Use of Levonorgestrel Alters Tissue Lipid Profile, Liver Function and Selected Tissue Cytoarchitecture in Female Wistar Rats

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ABSTRACT

Background and Objectives: Oral levonorgestrel is a post coitus contraceptive used to prevent unintended pregnancies. The uncontrolled availability of levonorgestrel over-the-counter for sexually active youth, as well as the increasing potential consequences of side effects with frequent use, necessitates research into the effects of repeated levonorgestrel use on selected tissues' cytoarchitectures and biomarkers in female Wistar rats.

Methods: Twenty rats (140 ± 20 g) were randomized into four groups: control received 0.2 mL/kg normal saline while others were exposed to *oral levonorgestrel* at 0.002 mg/kg, 0.004 mg/kg and 0.008 mg/kg, 3 times weekly at 48-hour interval for six weeks.

Results: Levonorgestrel increased hepatic total cholesterol, phospholipids and malondialdehyde; renal and cardiac total cholesterol were also found to be elevated in high dose (0.008 mg/kg) *levonorgestrel* treated rats. Regardless of administered dose, levonorgestrel has a varying effect on tissue triglycerides and GSH and increases serum AST and ALT. Furthermore, levonorgestrel significantly altered the histoarchitecture of selected tissues in a dose-dependent manner, indicating hepatic karyorrhexis, renal congestion, loss of cortical glomeruli in the kidney, cardiac hypertrophy, endometrial epithelial hypoplasia, disruption of glycoprotein zona pellucida, and uterine glandular atrophy.

Conclusion: These findings suggest that levonorgestrel is toxic at non-pharmacological doses by altering tissue lipid profiles and inducing cellular injuries through oxidative and non-oxidative mechanisms.

Keywords: Levonorgestrel; Lipid Profile; Liver Function; Cellular Injuries; Oral Contraceptives.

INTRODUCTION

Levonorgestrel (LNG) is a second generation synthetic female hormone, progesterone analogue, used for emergency contraception (EC) within 72 hours after an unprotected sexual intercourse to prevent an unwanted pregnancy (Trussell et al., 2014). Untimed and unplanned pregnancy poses a major public health challenge and social distress across the globe (Bastola, 2013). LNG is effective and safe in all situations, but because of the increasing

severity of side effects with frequent use, it has been recommended for use in humans four or fewer times per month (Curtis et al., 2016). Conventionally, it is prescribed to be taken in two divided-dose formulations 12 hours apart (two 0.75 mg pills) or as a single-dose formulation (1.5 mg pill) only after unprotected coitus and during an unsafe period (Gemzell-Danielsson and Chun-Xia, 2010).

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In clinical trials, a high dose of LNG as a progestin-only emergency contraceptive pill is justifiable for a single application with fewer side effects ranging from mild to tolerable, such as nausea and vomiting, abdominal pain, breast tenderness, headache, dizziness and fatigue (Harper et al., 2004). The side effects are commonly resolved after withdrawal but the same cannot be said for chronically administered LNG (Raymond et al., 2011). Since LNG is now available over-the-counter for both women and men of reproductive age or sexually active youth, improper use is expected (Gaffaney et al., 2016). This got the attention of biomedical researchers to the potentially toxic and harmful effects of this drug when used repeatedly (at sub or supra-therapeutic doses) over a long period of time.

LNG is metabolized in the liver, distributed to most organs of the body and eliminated by the kidney (Edelman et al., 2010). Earlier study revealed that organ failure may ensue with a prolonged repeated ingestion of high dose of LNG as emergency contraceptive in women of child-bearing age who are sexually active and subjected to unplanned unprotected sex exposure (Schmidt et al., 2020). In addition, others have also found that the ingestion of LNG causes dyslipidemia in women who used combined oral contraceptive pills (COCPs; ethinyl estradiol and norgesterol) for a long period of time (Sabatini et al., 2011; Al-Gazally et al., 2010). Most of these evaluations were conducted in the plasma. However, Leung et al. (2010) demonstrated that once orally administered, LNG is absorbed from gastrointestinal tract (GIT), it binds to sex hormone binding globulin (SHBG) and albumin, widely distributed initially to soft tissues such as liver, kidney, brain and spleen (Leung et al., 2010). Hence, we selected liver, heart, kidney, ovary and uterus (*endometrium*) to describe the lipid profile, liver function, cellular oxidative status, and histomorphological alteration in these tissues after administration of varying and repeated doses of oral LNG in female Wistar rats.

MATERIALS AND METHODS

Chemicals and Reagents

Levonorgestrel (LNG) (Shuangwei Pharmaceutical Company Limited, Ninghai, China) was freshly prepared in normal saline and administered orally at varying doses (0.002 mg/kg, 0.004 mg/kg, and 0.008 mg/kg) while all other chemicals and reagents used, except where otherwise stated, were of analytical grade and purchased from British Drug House (Poole, Dorset, UK). The doses of

LNG used in this study were chosen and modified based on previous research reported by Braden et al. (2017).

Animal

Nulliparous female Wistar rats (20) weighing 140.0±20g were purchased from Ladoke Akintola University of Technology, Ogbomoso Animal Holding Facility, kept under standard housing condition (12-hour light/dark cycle, 25±2°C and 65-75% relative humidity) in fenestrated white plastic cages, fed standard rat chow and tap water *ad libitum*. They were acclimatized for 7 days before the commencement of the study. Animals were well-cared for according to criteria outlined in the "care on use of animals in research and teaching" prepared by the U.S. National Institute of Health (NIH) and approved by the Ethical Research Committee of the University of Ilorin, Nigeria with approval number: UERC/ASN/2019/1806.

Experimental Design

The rats were randomly assigned into the following groups: control (normal saline: 0.2 mL/kg; p.o; n = 5), and LNG (n = 15). With the intention to ascertain the possible dose-dependent effect of LNG, the rats in LNG group were subdivided into three subgroups (n = 5 each/subgroup) of which some received 0.002 mg/kg, 0.004 mg/kg and 0.008 mg/kg body weight accordingly. In order to establish the toxic potential of this drug, treatments were given three times per week at 48 hours apart for six weeks orally via intra-gastric tube gavage.

Preparation of Tissue Homogenates

At the end of the experiment, animals were fasted overnight and subjected to light ether anaesthesia. Liver, kidney and heart were excised and rinsed with 1.15% KCl solution to remove haemoglobin. Tissue samples (1 g) from each organ were homogenized with potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 5000 rpm for 10 minutes to obtain the supernatants which were transferred into separate plain bottles and kept at -20°C prior to the biochemical analyses.

Biochemical Assays

An enzyme-based immunoassay (EIA) system was employed to measure the levels of total cholesterol, triglycerides, phospholipids, malondialdehyde and reduced glutathione in tissue samples. The EIA kits were procured from Fortress Diagnostics Limited (UK) and contained the respective EIA enzyme label, EIA substrate reagent along with EIA quality control sample. The

analyses were done with a spectrophotometer (Spectramax Plus; Molecular Devices, Sunnyvale, CA) according to the manufacturer's instruction. The serum alanine and aspartate aminotransferases (ALT and AST) were determined by a method previously described (Loomba et al., 2009).

Hematoxylin and Eosin (H and E) Staining

Excised tissue from liver, heart, kidney, ovary and uterus were fixed in Bouin's fluid for 24 hours before they were transferred into freshly prepared 10 % neutral buffered formalin for histological evaluation following the standard protocol. Light microscope (Nikon, Tokyo, Japan) with in-built camera attached to a monitor were used with the specimens sectioned at 4-5 μ m, mounted on clean slides (Superfrost Plus; Fisher Scientific), stained with H and E and photomicrograph of the slides were then taken.

Statistical Analysis

Statistical analysis and graphical representations were performed using Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corporation, Armonk, NY) and GraphPad Prism (GraphPad Prism Software version 5.01, San Diego, CA, USA) respectively. All data are expressed as Mean $\pm$ standard error of means (S.E.M). Comparisons among the groups were analyzed using One-way Analysis of Variance (ANOVA), followed by Tukey's *post hoc* multiple comparison tests. Values of $p < 0.05$ were considered as significant.

Results

Change in Tissue Levels of Total Cholesterol, Triglycerides, Phospholipids, Reduced Glutathione and Malondialdehyde in LNG Exposed Rats

The total cholesterol was significantly ($p < 0.05$) elevated in the selected tissues (liver, heart and kidney) in a dose-dependent manner when compared with control. The cardiac triglyceride significantly ($p < 0.05$) decreased when compared with control but the hepatic and renal triglyceride levels decreased insignificantly ($p > 0.05$) by all doses of LNG when compared to control (Figure 1A and B). LNG (0.008 mg/kg) treated rats exhibited significant ($p < 0.05$) increase in hepatic and cardiac phospholipids. MDA significantly increased in liver and decreased in heart after LNG (0.008 mg/kg) treatment when compared to control and LNG (0.002 mg/kg) respectively (Figure 1C and E). However, the tissue reduced glutathione was not altered significantly ($p > 0.05$) in LNG exposed rats when compared to control (Figure 1D).

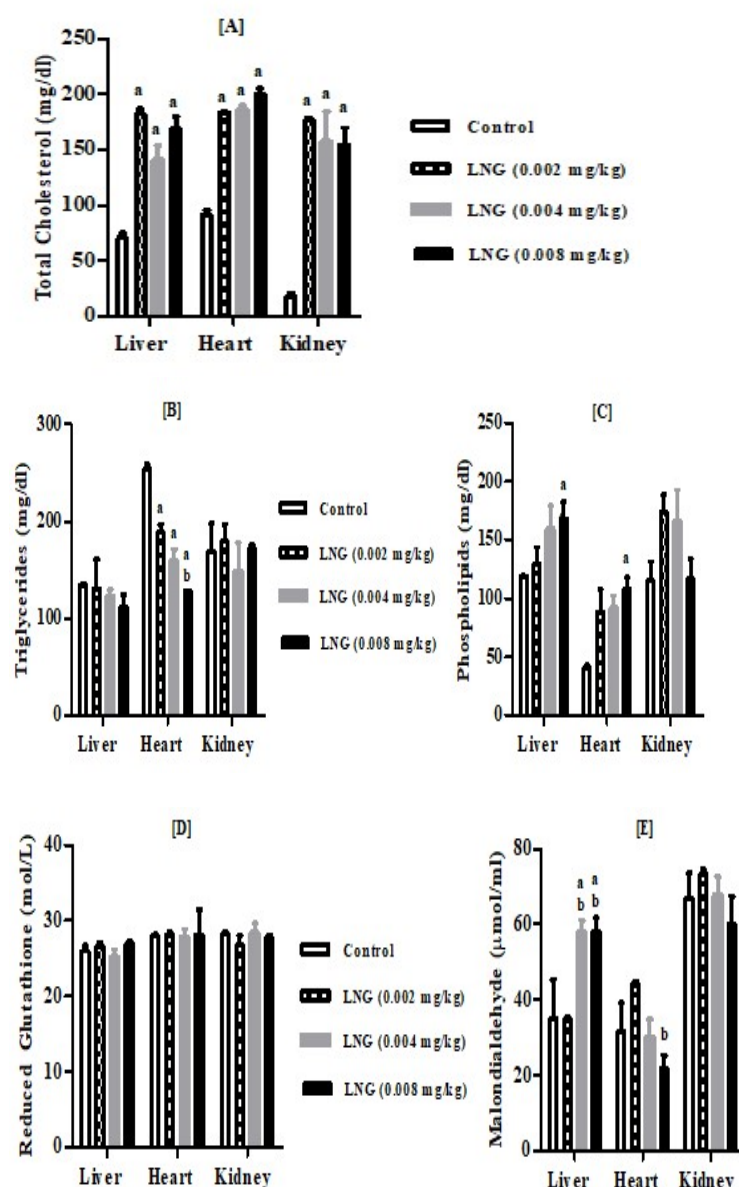


Figure 1. Effects of levonorgestrel on hepatic, cardiac, and renal total cholesterol, triglycerides, phospholipids, reduced glutathione and malondialdehyde in female Wistar rats. (A) Hepatic, cardiac and renal total cholesterol concentrations. LNG markedly raised tissue cholesterol. (B) Hepatic, cardiac and renal triglycerides concentrations. LNG produced no marked change in tissue triglycerides except decrease in the heart. (C) Hepatic cardiac, and renal phospholipids concentrations. LNG markedly increased hepatic, cardiac and renal phospholipids. (D) Hepatic, cardiac and renal malondialdehyde concentrations. LNG increased hepatic triglycerides. (E) Hepatic, cardiac and renal reduced glutathione concentrations. LNG did not affect tissue reduced glutathione. The results are expressed as the mean $\pm$ S.E.M. ($n=5$), ^a $p < 0.05$ vs the

control group, ^b $p < 0.05$ vs the LNG (0.002 mg/kg) treated rats.

Change in Serum Alanine and Aspartate Aminotransferases Activities in LNG Exposed Rats

Liver function test showed significant increase in the serum levels of liver enzymes' activities. Irrespective of doses of levonorgestrel administered, ALT and AST significantly ($p < 0.05$) increased when compared to control group (Table 1).

Table 1: Effect of Levonorgestrel on Serum ALT and AST in Female Wistar Rats

	Control	LNG 0.002mg/k g	LNG 0.004mg/k g	LNG 0.008mg/k g
AS T	29.75±8.3 0	67.03±6.16 a	56.47±6.91 a	76.42±1.52 a
ALT	45.28±2.9 0	73.88±5.63 a	71.93±5.08 a	73.33±4.18 a

The results were expressed as the mean $\pm$ S.E.M. ($n=5$), ^a $p < 0.05$ vs the control group. LNG, Levonorgestrel; AST(U/L), Aspartate aminotransferase; ALT(U/L), Alanine aminotransferase

Histological Assessment of the Liver, Heart, Kidney, Ovary and Uterus in LNG Exposed Rats

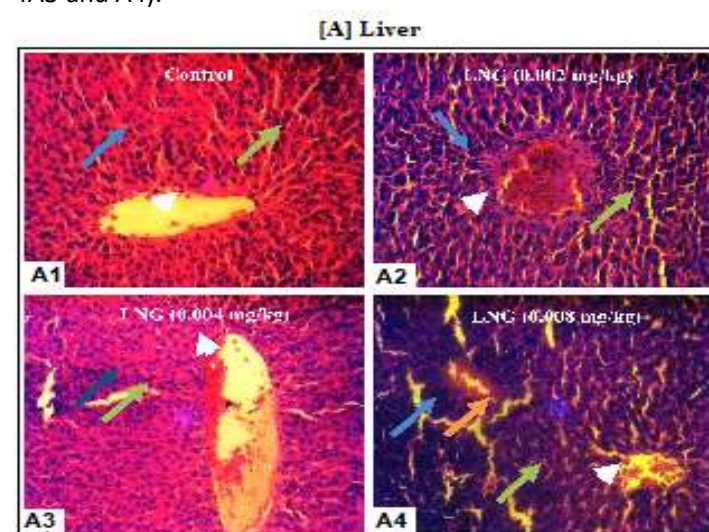
The effects of LNG on specific organs studied were observed to be toxic in a dose-dependent manner as observed through histomorphological assessment. The photomicrographs are shown in Figures 2, 3 and 4. Normal histoarchitecture was observed in control and LNG (0.002 mg/kg) treated rats but altered in LNG (0.004 mg/kg and 0.008 mg/kg) treated rats.

In the liver, the photomicrograph of control and LNG (0.002 mg/kg) treated rats showed normal histoarchitecture of central vein, sinusoid and hepatocytes (Figures 2A1 and A2). Noticeably, there is abnormal histoarchitecture with normal central vein, vacuolation of hepatocytes and engulfment of dying/dead cells by phagocytosis in LNG (0.004 mg/kg) exposed rats. Furthermore, the photomicrograph of LNG (0.008 mg/kg) treated rats revealed distortion of hepatocytes, basophilic nuclei and central vein collapse (degeneration of hepatocytes) (Figure 2A3 and A4). The cardiac histomorphology showed no histoarchitectural (myocytes, fibre and interstitial space) abnormalities in the control and LNG (0.002 mg/kg) treated rats (Figures 2B1 and B2). However, there was induction of ischemic necrosis with

inflammatory cells infiltrates in LNG (0.004 mg/kg) exposed rats, in addition to the expansion of interstitial space and hypertrophy of viable fibres (increased size of individual viable cells) which were observed in LNG - 0.008 mg/kg treated rats (Figure 2B3 and B4).

Kidney histology for the control and LNG (0.002 mg/kg) treated rats showed normal renal corpuscles, tubular cells and *interstitium* (Figure 3A1 and A2). LNG (0.004 mg/kg) treated rats exhibit loss of tubules with eosinophilia, a characteristic feature of partial tubular necrosis. In addition, renal corpuscular distortion or degeneration and congestion of renal tubule and *interstitium* were observed in LNG (0.004 mg/kg) treated rats (Figure 3A3 and A4). Ovarian histomorphology revealed normal histoarchitecture with intact zona pellucida (a thick homogeneous layer of glycoprotein and acid proteoglycan) and stroma cells in the control and LNG (0.002 mg/kg) treated rats (Figure 3B1 and B2). However, there were distortions of these structures in the ovary of LNG (0.004 mg/kg and 0.008 mg/kg) treated rats as ischemic necrosis with global follicular degeneration and distortions of zona pellucida (Figure 3B3 and B4).

The endometrial lining of uterine sections of control and LNG (0.002 mg/kg) treated rats revealed normal histoarchitecture (Figure 4A1 and A2). A repressed cellular differentiation and secretory glandular epithelium were observed in LNG (0.004 mg/kg) treated rats, while distortion of stroma, degeneration of basement membrane and loss of epithelium (glandular atrophy) were observed in LNG (0.008 mg/kg) exposed rats (Figure 4A3 and A4).



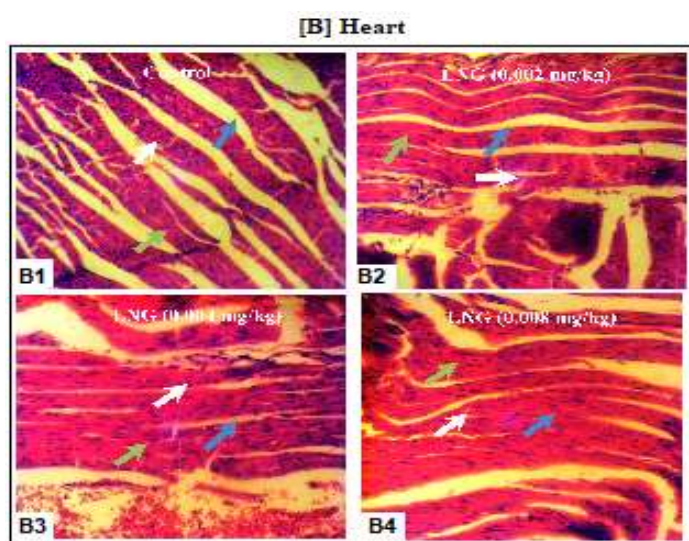


Figure 2. Representative photomicrograph of liver (A) and heart (B) tissues stained with hematoxylin and eosin ($\times 200$). Control and LNG (0.002 mg/kg) showed normal histoarchitecture of liver (A1 and A2). LNG (0.004 mg/kg) induced cellular hyperplasia, vascular congestion and vacuolation in the liver (A3), while LNG (0.008 mg/kg) caused distortion of hepatocytes, basophilic nuclei (orange arrow) and central vein collapse in the liver (white arrow) (A4). White arrow head; central vein, blue stealth arrow; hepatocytes, green straight arrow; sinusoid (A1-A4).

In the heart tissue, both Control and LNG (0.002 mg/kg) exhibited normal histoarchitecture of myocytes, fibre and interstitial space (B1 and B2). LNG (0.004 mg/kg) induced ischemic necrosis with inflammatory cells infiltrate (B3), while LNG (0.008 mg/kg) caused ischemic necrosis with inflammatory cells infiltrate; expansion of interstitial space and hypertrophy of viable fibres (increased size of individual viable cells) (B4). White arrow; myocytes, green stealth arrow; muscle fibre, blue straight arrow; interstitium (B1-B4).

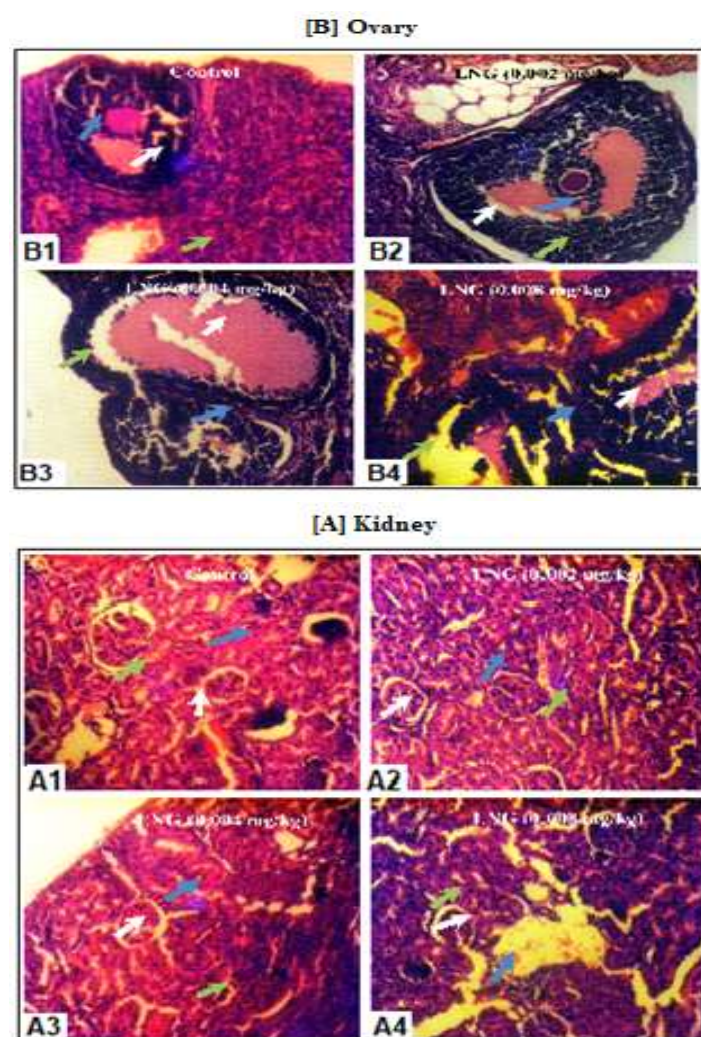


Figure 3. Representative photomicrograph of kidney (A) and ovary (B) tissues stained with hematoxylin and eosin ($\times 200$). Both Control and LNG (0.002 mg/kg) showed normal histoarchitecture of renal corpuscles, tubular cells and interstitium (A1 and A2). LNG (0.004 mg/kg) induced partial tubular necrosis characterized by loss of tubules with eosinophilia (A3), while it (0.008 mg/kg) caused renal corpuscular distortion (degenerating renal tubule and congestion of interstitium) (A4). White stealth arrow; renal corpuscles, blue stealth arrow; renal tubules, green stealth arrow; interstitium (A1-A4). In the ovary tissue, normal histoarchitecture was observed in both Control and LNG (0.002 mg/kg) (B1 and B2). LNG (0.004 mg/kg) administration induced follicular degeneration by loss of zona pellucida and distortion of granulosa (B3), while LNG (0.008 mg/kg) induced follicular degeneration by distortion and degeneration of stroma (B4). White stealth arrow; follicular antrum, blue stealth arrow; zona pellucida

are surrounded by granulosa, green stealth arrow; stroma (B1-B4).

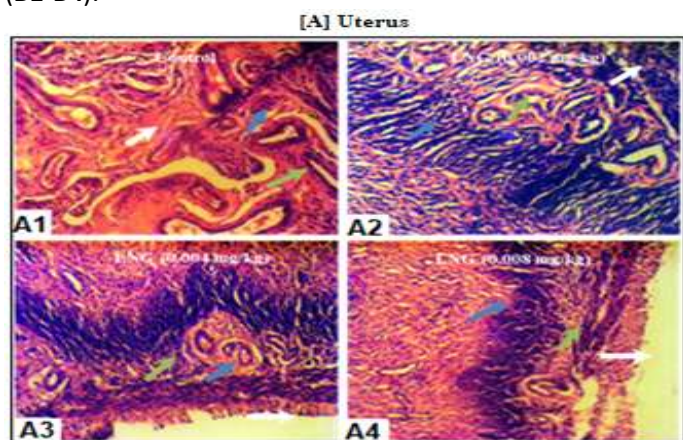


Figure 4. Representative photomicrograph of endometrial lining of the uterus stained with hematoxylin and eosin ($\times 200$). Normal histoarchitecture was observed in both Control and LNG (0.002 mg/kg) (A1 and A2). LNG (0.004 mg/kg) repressed cellular differentiation and secretory glandular epithelium while LNG (0.008 mg/kg) induced distortion of stroma, degeneration of basement membrane and loss of epithelium (glandular atrophy) (A3 and A4). White stealth arrow; luminal epithelium, green stealth arrow - interstitium, blue stealth arrow; glandular epithelium.

DISCUSSION

Previous studies show that the toxicity of synthetic steroids can cause injury or damage to the plasma membrane (a lipid-rich barrier), resulting in lipid peroxidation and free radical generation, and thus induced oxidative stress, if the antioxidant system fails to mop up the free radicals. (Pomara et al., 2015; Dornelles et al., 2017; Mokra et al., 2019). Therefore, we investigated the toxic effects of LNG on selected tissue biochemical markers and histomorphological changes in female Wistar rats. The current findings showed that the administration of LNG caused elevation of hepatic, cardiac and renal cholesterol levels with concomitant dose-dependent decrease in cardiac triglycerides concentration. Impliedly, the long term use of synthetic steroid (LNG) could potentially enhance tissue hypercholesterolemic condition, whereby excess cholesterol may be deposited in the tissues (e.g blood vessel) leading to plaques formation, narrowing of the arterial lumen, restriction of blood flow to the heart, inducing hypoxic-ischemic damage and stimulating inflammatory responses to cause cardiovascular diseases such as atherosclerosis, myocardial infarction and coronary artery disease

(D'Souza et al., 2009). Meanwhile, the hypotriglycerides observed in the heart may be due to increased mobilization of stored triglycerides (lipolysis) and utilization to provide metabolic fuel in order to meet increased calorie demand. In conformity with our findings, reports have shown that LNG raises plasma total cholesterol (Al-Gazally et al., 2010), reduces or fails to alter tryglyceride concentration. Furthermore, Tkocz et al. (1988) demonstrated that LNG inhibits the synthesis of tryglyceride in isolated rat hepatocytes.

LNG (0.008mg/kg) predominantly elevated hepatic and cardiac phospholipids and lipid peroxidation marker (MDA) without change in reduced glutathione. These results are in agreement with some recent studies in which tissue damage was said to be mediated by the reactive oxygen species which act as two-edged swords, waging war against cell membrane through the peroxidation of unsaturated fatty acids. Increased expression of phospholipids and production of MDA are associated with hepatic tissue dysfunction. Liver, as a key metabolic machinery or organ, is proved to be most susceptible and sensitive when exposed to chronic use of drugs or toxic substances (Gómez-Lechón et al., 2016). The current study showed that there was a slight but insignificant increase in renal phospholipids after LNG (0.008mg/kg) treatment.

Additionally, the results also revealed drug-induced hepatotoxic potential of LNG, as it elevated serum ALT and AST. These are important biochemical markers of hepatocyte membrane and biliary tract integrity, when there is an 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 side effect of LNG on the liver function (Al-Aubaidy et al., 2013).

Microscopically, the selected tissues further confirm the potential toxic effect of LNG at the cellular level with frequent and repeated use in a dose-related response. LNG causes hepatotoxicity ranging from induced cellular hyperplasia, vascular congestion, and vacuolation at 0.004 mg/kg to hepatocyte degeneration (basophilic nuclei, central vein collapse) at 0.008 mg/kg. These trigger cellular and vascular responses leading to parenchymal cell injury and induced chronic inflammatory processes (Iredale, 2007; Forbes and Rosenthal, 2014; Adler et al., 2020).

It is worthy of note that elevation of cardiac total cholesterol, as discovered in this study, seems to exceed the physiological and metabolic needs as revealed by the marked histomorphological distortion evidenced by cardiac hyperplasia and hypertrophy when LNG at 0.004mg/kg and 0.008mg/kg was administered respectively. This suggests the occurrence of hypoxic-ischemic injury, with inflammatory response that leads to necrosis and death of some muscle fibres. The remaining viable muscle fibres tend to enlarge and increase their work rate (hypertrophy) to compensate *via* cellular adaptation (Villapol et al., 2014; McArdle et al., 2019). This explains our findings that LNG induced hyperlipidemic condition with coagulation necrosis showed a strongly eosinophilic anucleated myocardial fibre, inflammatory cell infiltrates as an evidence of cellular response and adaptation to injury. Meanwhile, the morphological changes observed in this study may be reversed when the treatment is withdrawn or discontinued. It has been reported that organ failure may ensue with prolonged repeated administration of oral LNG as an emergency contraceptive in women of child-bearing age who are sexually active and subjected to unplanned unprotected sex exposure (Schmidt et al., 2020).

There was no marked alteration of biochemical parameters assessed in the kidney except the total cholesterol that was found to be elevated with LNG (0.008mg/kg) treatment; others were not in parallel to the microscopic appearance of renal tissue. However, the observation in the rats administered LNG (0.004mg/kg and 0.008mg/kg) revealed abnormal histoarchitecture of kidney. LNG induced tubular necrosis and congestion of interstitium which can be linked with the elevated tissue total cholesterol. Perhaps through mineralocorticoid receptor, LNG acts directly on the renal tubules to increase sodium and water retention. This increases extracellular fluid volume and therefore increases arterial blood pressure (Ma et al., 2007; Sheldon et al., 2015). Thus, this may explain the distension of interstitium observed at LNG (0.008 mg/kg) dose and the elevated tissue total cholesterol being steroid also enhances water retention.

The present study also considered the effect of LNG on the microstructure of primary target organs: ovary and endometrium of the uterus. A sustained and repeated administration of LNG induced follicular degeneration with loss of zona pellucida (noncellular glycoprotein envelope). The dysregulation of zona pellucida glycoprotein receptors

(ZP3) interrupts acrosomal reaction of the sperm leading to loss of transmembrane signaling transduction that is necessary for fusion between the released mature ovum and the spermatozoa. This observation is corroborated by a report that LNG induced a dose-dependent effect on zona-binding capacity and sperm velocity in an *in-vitro* study (Yeung *et al.*, 2002). From this study, it may be hypothesized that LNG prevents pregnancy by dissolving zona pellucida in a dose-dependent manner.

Changes in the endometrial epithelium is evidenced by the appearance of cellular degeneration and necrosis of the glands and lining epithelium and loss of endometrial brush border of the uterus as the LNG dosage increases suggestion of atrophy. This is an adaptive response in which size and function of cells decreases. This finding correlates with the native structural metamorphosis of the endometrium that defines the start of estrus in rats. Estrus phase of estrous cycle is characterized by loss of mitotic activity and leucocyte infiltration of the endometrium. Similarly, just like natural progesterone, this effect may be an adaptive response. Further studies should focus on molecular mechanisms of levonorgestrel on lipid metabolism and its regulation in experimental animals.

CONCLUSION

Sustained exposure to LNG caused dyslipidaemia and induced tissue damage in different organs of the body *via* oxidative and non-oxidative mechanisms.

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CONFLICT OF INTEREST:

Authors have declared that no competing interests exist.

FINANCIAL SUPPORT INFORMATION: NIL

AUTHORS' CONTRIBUTIONS

ASA, EBO and HAA conceived the study. HAA and JOF designed the study. HAA, NOA and JOF carried out the study. ASA, EBO and JOF supervised the study. ABN and NOA analyzed and interpreted the data. HAA and NOA drafted the manuscript. ABN and NOA revised the

manuscript. All authors read and approved the final manuscript to be published.

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