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Postinor®-2 and Norinyl®-1/35 Impairs Hepatic Functions and Morphology in Rat Models: Evidences of Oxidative Stress as a Mechanism Pathway

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Abstract

Oral contraceptives such as Postinor®-2 and Norinyl®-1/35 are easily accessible contraceptive pills by women of reproductive age (15 – 49 years). The hepatic effects of these drugs were evaluated in rats.

A total of 25 adult female Wistar rats purchased from the animal house, College of Health Sciences, Benue State University, Makurdi were randomly divided into five groups (1 – 5) of five rats each, and acclimatized for 14 days. After which the groups were treated as follows: group 1 (100mg/kg body weight of normal saline), group 2 (0.10mg/kg body weight of Postinor®-2), group 3 (0.20mg/kg body weight of Postinor®-2), group 4 (0.10mg/kg body weight of Norinyl®-1/35) and group 5 (0.20 mg/kg body weight of Norinyl®-1/35) for 14 days, and then the rats were fasted overnight and sacrificed. Blood samples and organs (liver) were collected for biochemical and histological analysis respectively.

Postinor®-2 and Norinyl®-1/35 had no significant effect on the body weight and liver weight of rats across groups. For liver function enzymes; groups 2, 3, 4 and 5 all showed a statistically significant increase in mean compared to the control group for ALT. For ALP, only group 4 (86.69±2.54) showed a statistically significant increase in mean in comparison to group 1, while for AST, groups 2 and 4 showed a significant decrease in mean compared to group 1. For oxidative stress markers MDA and SOD, groups 2 and 3 showed a statistically significant increase and decrease respectively, when compared to the control group. Group 1 showed normal liver histology, while those in groups 2 and 3 showed a markedly distorted liver histology, with widely dilated sinusoids, degenerated endothelial cells with neutrophilic and lymphocytic infiltrates in portal spaces.

the results obtained in this study pointed clearly that of the two drugs tested, Postinor®-2 had more deleterious effects than Norinyl®-1/35 on the liver of the adult female Wistar rats. It may therefore be inferred from the result of this study that Postinor®-2 should be taken according to prescription or should be avoided, because its frequent consumption and abuse can result in tissue damage in human organs.

Keywords: liver, Postinor®-2, Norinyl®-1/35, Levonorgestrel, Ethinyl estradiol, Norethindrone, comparison, Necrosis, Vacuolation, Cytoplasm, cell

1. Introduction

The liver, the biggest organ in the body, is essential for metabolism and plays a role in detoxification, glycogen storage, red blood cell breakdown, plasma protein synthesis, and other metabolic processes (Opoku et al., 2007). Due to its strategic positioning and function in the body, it is continually and variably exposed to xenobiotics, environmental contaminants, and chemotherapeutic drugs. Being exposed to both the parent medication, which is transported from the gastrointestinal tract via the portal vein, and any metabolites created, which subsequently

reach the systemic circulation via the hepatic vein, renders the liver a susceptible organ. Hepatic damage will result if the liver's natural defenses are overwhelmed by all of these exposures (Ibrahim et al., 2008).

Levonorgestrel, a contraceptive pill made by a Hungarian company, is sold under the trade name Postinor®-2 (Adelaide, 2007). The post-coital medicine Postinor is advertised in Nigeria as preventing conception. It has not been demonstrated to be secure or successful in preventing conception. It has been suggested that Postinor®-2 be marketed as an emergency contraceptive, the only application for which has been demonstrated to be successful. However, until the causes of women's usage are addressed, this use may not alter. Each pill of Postinor® contains 0.75 mg of the progesterone levonorgestrel. Currently, a packet of 10 Postinor®-2 pills may be purchased in Nigeria (Bottomly, 1992).

It seems to be offered by bigger pharmacies in Nigerian cities. Inactive substances include potato starch, maize, silica colloidal anhydrous magnesium stearate, talc, and lactoc. One blister sheet containing one levonorgestrel is also included (Jolin & Rapkin, 2002). When other methods of birth control have failed or after unprotected sexual activity, it is used as an emergency contraceptive to avoid undesired pregnancy. In addition to controlling menstrual irregularities, Postinor®-2 is also utilized to treat endometriosis (Palter & Olive, 2002).

The progesterone in Levonorgestrel Postinor-2 (1.5 mg) is the same progesterone used in common birth control tablets, but in a stronger concentration (Bottomly, 1992). If one has unprotected sex in the first two weeks of the month before ovulation, when the probability of fertilization is the highest, it is believed to operate mostly by preventing ovulation and fertilization. By inhibiting the uterine lining's endometrium from growing and becoming thicker, which is necessary for the implantation of the fertilized egg, it also lessens the likelihood that the fertilized egg will implant and develop.

Postinor-2 is no longer effective if fertilization and implantation have already occurred (Kalantaridou et al., 2006). If given within an acceptable amount of time, Postinor-2 is designed to work similarly to the feminine hormone progesterone, preventing conception. Postinor®-2 should typically be taken no later than three (3) days after engaging in unprotected intercourse. It has, however, shown to be more successful in situations when therapy was initiated sooner after the sexual experience (Bottomly, 1992).

Norinyl®-1/35 is a combination birth control pill that blocks ovulation (the release of an egg from an ovary) by combining two female hormones, ethinyl estradiol and norethindrone. The cervical mucus and uterine lining are also affected by this medicine, thickening the mucus and making it more difficult for sperm to enter the uterus and for a fertilized egg to adhere to the uterus (Mevdev, 2004). The contraceptive Norinyl®-1/35 is used to prevent pregnancy. Women who are at least 15 years old, have begun menstruating, and want to use birth control pills can also use norinyl-1/35 to treat mild acne (Okunde, 2008).

2. Materials and Methods

2.1 Experimental Animals

A total of twenty-five (25) adult female Wistar rats weighing average of 100grams used for this study were obtained from the animal house, College of Health Sciences, Benue State University, Makurdi. The rats were divided randomly into five groups of five rats each and kept in spacious, well-ventilated plastic cages and allowed to acclimatize for fourteen (14) days in the animal house, while being fed with standard supreme feed and tap water *ad libitum.*

At the end of the fourteen days acclimatization period, they were treated with normal saline and the test drugs for the next 14 days (two weeks).

2.2 Experimental Drugs: Postinor®-2 and Norinyl®-1/35

The drugs Postinor®-2 (20 tablets of 0.75mg levonorgestrel each) and Norinyl®-1/35 (100 tablets of 0.15mg ethinyl estradiol/norethindrone each) were obtained from Mernax Pharmacy and Veterinary Drugs Store, shop four, Vaipama plaza, opposite College of Health Sciences, Gboko Road, Makurdi, Benue State and stored at room temperature.

2.3 Preparation of Drug Solution (Dilution)

20 tablets of Postinor®-2 (0.75mg each) making 15mg of Levonorgestrel was dissolved in 500mls of distilled water to obtain a concentration of 0.03mg/ml (Adigun *et al.*, 2016). 100 tablets of Norinyl®-1/35 (0.15mg each) making 15mg of ethinyl estradiol was also dissolved in 500mls of distilled water, making a concentration of 0.03mg/ml (Adelaide, 2007). The two drug solutions were stored in the refrigerator for optimum temperature regulation at 0^0C and ready for use.

2.4 Ethical Clearance

All experimental procedure was duly followed in agreement with the guidelines on animal experiment as prescribed by the Ethics committee of College of Health Sciences, Benue State University, Makurdi, Nigeria. A

copy of the proposal was submitted to the ethical committee to be examined for approval.

2.5 Experimental Design

The total of 25 adult female Wistar rats were weighed and randomly divided into five groups of five rats in each group labeled 1 – 5, and then acclimatized for fourteen (14) days, being fed once daily with standard supreme feed and tap water.

After the period of acclimatization, the rats were treated for two weeks (fourteen days) with doses of Postinor®-2 and Norinyl®-1/35 as follows:

Group 1 (Control Group): 100mg/kg body weight of Normal Saline once daily for 14 days

Group 2: 0.10mg/kg body weight of Postinor®-2 once daily for 14 days

Group 3: 0.20mg/kg body weight of Postinor®-2 once daily for 14 days

Group 4: 0.10mg/kg body weight of Norinyl®-1/35 once daily for 14 days

Group 5: 0.20mg/kg body weight of Norinyl®-1/35 once daily for 14 days

2.6 Animal Sacrifice

At the end of the experiment (day 28), all the animals were sacrificed by chloroform inhalation. Blood was collected through the left ventricle of the heart of the animals in heparinized centrifuge tube under a deep anaesthesia with chloroform. The blood collected was centrifuged using centrifuge machine at 10,000 rmp for five minutes and the serum collected was subjected to liver function test (AST, ALP and ALT) and estimation of oxidative stress enzymes (SOD and MDA). The liver tissue was harvested for histological examination.

2.7 Histological Tissue Processing

The fixed specimens were processed overnight for dehydration, clearing, and impregnation using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan) and serial sections of 5um thickness were cut using a microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). We used dan autostainer (Model 5020, Leica Biosystems, Wetzlar, Germany) for Hematoxylin & Eosin staining of the sections. The mounted specimens were observed and were scored under light microscopy at x40.

2.8 Statistical Analysis

Statistical data were analyzed using the statistical software, Statistical Package for the Social Sciences (IBM SPSS version 23.0) and results were expressed as Mean±SEM (Standard Error in Mean). Group means were compared using one-way ANOVA, and mean differences among groups were determined using LSD Post-Hoc test. Mean differences across groups were considered statistically significant at $P<0.05$.

3. Results

3.1 Physical Observations: Body Weight and Liver Weight

An increase in body weight was observed across groups, with group 1 which received 100mg/kg body weight of normal saline showing the highest increase in body weight (21.20±5.19), followed by group 2 (20.74±8.41) which received 0.10mg/kg body weight of Postinor®-2. No group showed a statistically significant difference in mean when compared to the control group (group 1) on one-way ANOVA.

The Liver weight of rats in this research remained fairly constant across groups, with no group showing a statistically significant difference in mean when compared against the control group (group 1) on one-way ANOVA.

It is therefore inferred that both Postinor®-2 and Norinyl®-1/35 had no significant effect on the body weight and liver weight of the experimental animals across groups in this research.

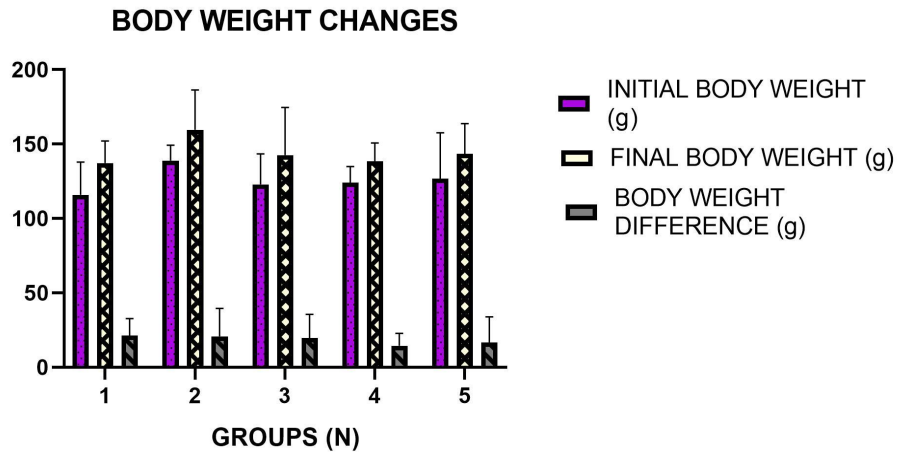

Figure 1. Simple Bar Chart Showing the Mean Body Weight Changes across Groups Compared on One-Way ANOVA

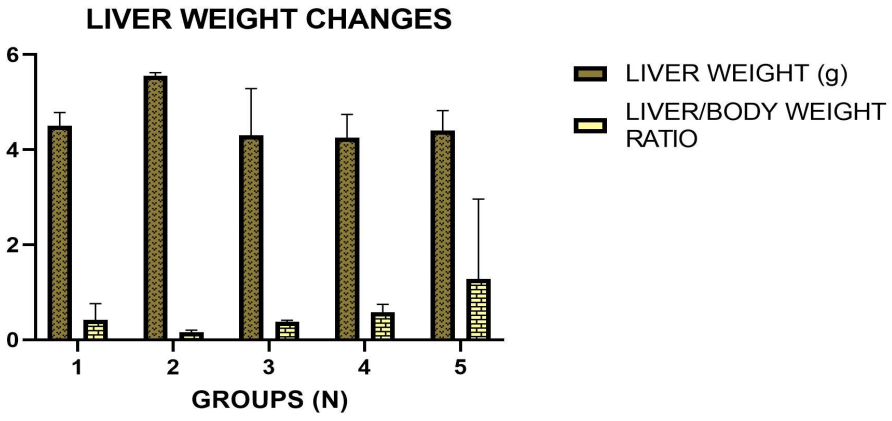

Figure 2. Simple Bar Chart Showing the Mean Liver Weight and Liver/Body Weight Ratio across Groups Compared on One-Way ANOVA

3.2 Biochemical Analysis

3.2.1 Liver Function Enzymes: Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP) & Aspartate aminotransferase (AST)

The mean liver function parameters of rats across groups were compared on one-way ANOVA. For ALT; groups 2-5 all showed a statistically significant difference (increase) in mean to the control group (group 1), for ALP; only group 4 showed a statistically significant difference (increase) in mean to the control group while for AST; groups 2 and 4 showed a statistically significant difference (decrease) in mean to the control group (group 1) when compared on one-way ANOVA (* = significant at p-value<0.05 when compared to group 1).

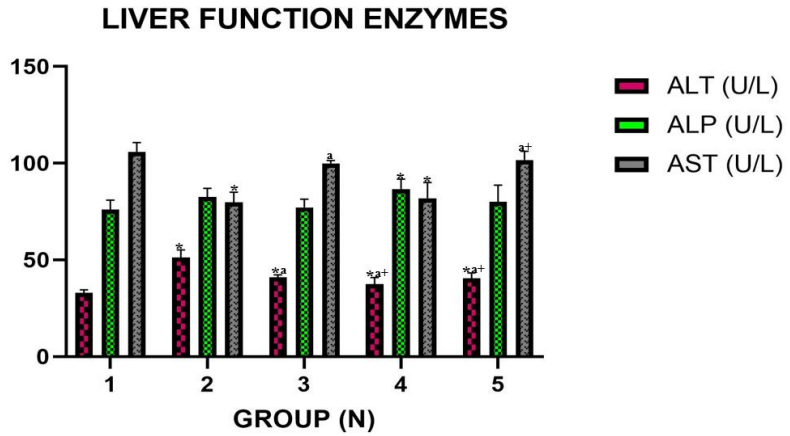

Figure 3. Simple Bar Chart Showing the Mean Liver Function Enzymes across Groups Compared on One-Way ANOVA

Note: $*^{a+}$ = statistically Significant at $p<0.05$ when compared to group 1, group 2 and the Postinor®-2 groups (groups 2 and 3) respectively

3.2.2 Oxidative Stress Markers: Superoxide Dismutase (SOD) & Malondialdehyde (MDA)

The mean oxidative stress and anti-oxidant markers across groups were compared by one-way ANOVA. For MDA; groups 2-3 showed a statistically significant difference (increase) in mean compared to the control group while for SOD; groups 2-4 showed a statistically significant difference (decrease) in mean compared to the control group (group 1) on one-way ANOVA (* = significant at p-value<0.05).

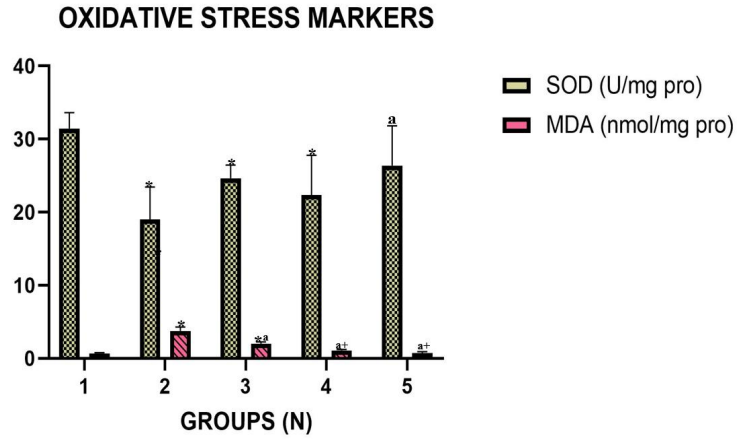

Figure 4. Bar Chart Showing the Mean Oxidative Stress Markers across Groups Compared on One-Way ANOVA

Note: $*^{a+}$ = statistically significant at $p<0.05$ when compared to group 1, group 2 and the Postinor®-2 groups (groups 2 and 3) respectively

3.3 Histological Observations

Histological examination in the liver of control rats showed normal architecture.

The group 2 cross-sections showed very lightly dilated sinusoids with lymphocytic infiltrates in the portal spaces of the liver, while lightly to moderately dilated sinusoids with mixed lymphocytic and neutrophilic infiltrate in the portal spaces were observed in the group 3. Lightly dilated sinusoids with mixed lymphocytic and neutrophilic infiltrate in the portal spaces, around biliary ducts, were observed in the group 4. Additionally, scattered mononuclear cells in sinusoids were present in zone 1 of the acinus. Clear hepatocyte cytoplasm with condensed chromatin in nuclei was observed in the group 5, while venous thrombus was present in only one of

the examined tissues. Pattern of changes observed in group 3 to 5 were less prominent compared to group 2.

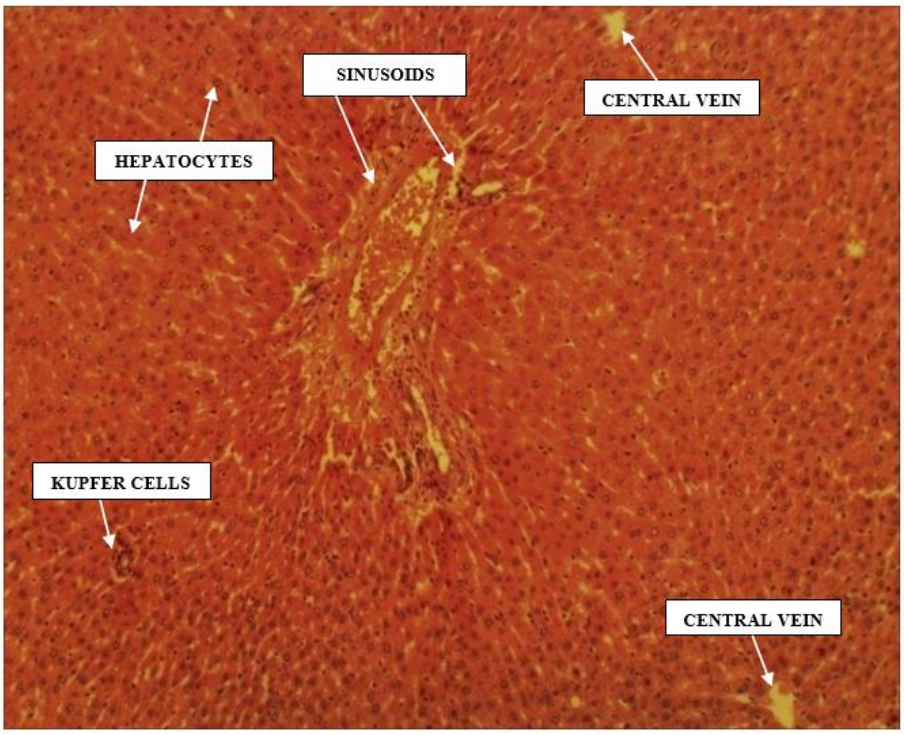

Figure 5. Liver section of group 1 showing: normal liver histoarchitecture; Central vein I; Hepatocyte (H); Sinusoids (S), polyhedral shaped cells and slightly vacuolated cytoplasm. (H & E, x 400)

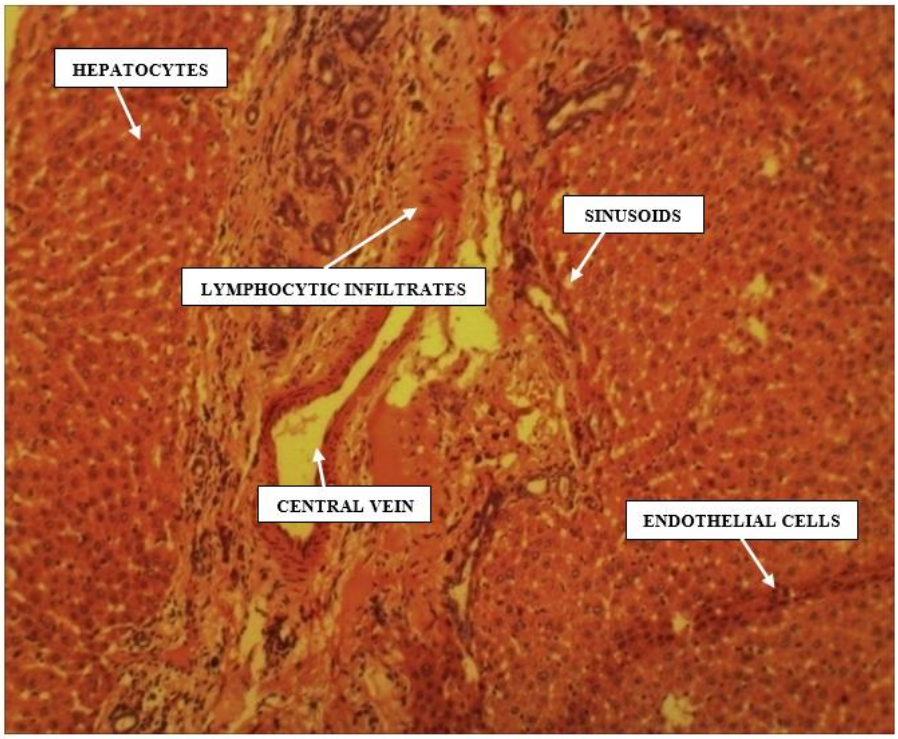

Figure 6. Liver section of group 2 showing: very lightly dilated sinusoids with lymphocytic infiltrates in the portal spaces of the liver (H & E x400)

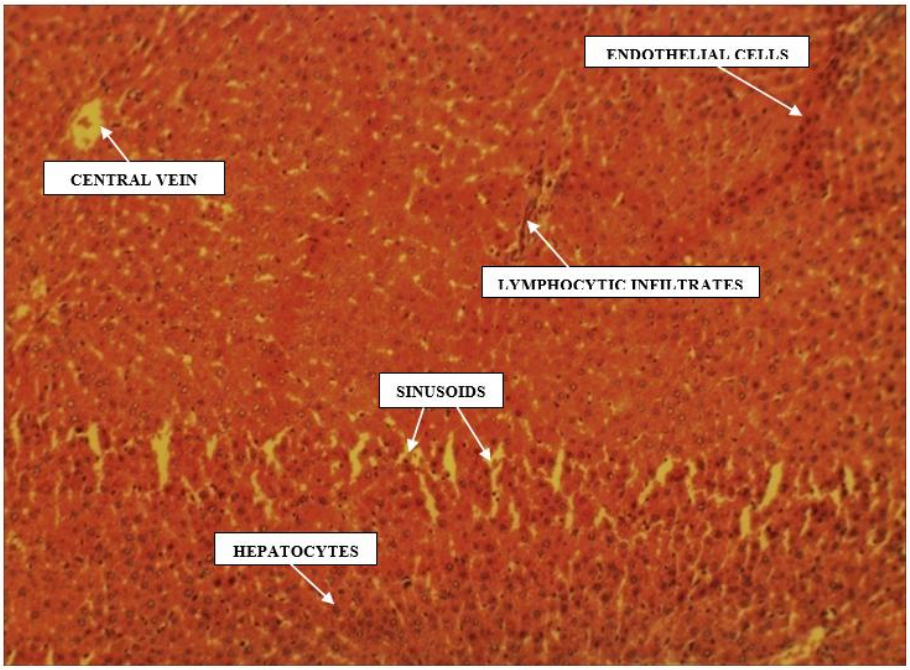

Figure 7. Liver section of group 3, showing: lightly to moderately dilated sinusoids with mixed lymphocytic and neutrophilic infiltrate in the portal spaces (H & E x400)

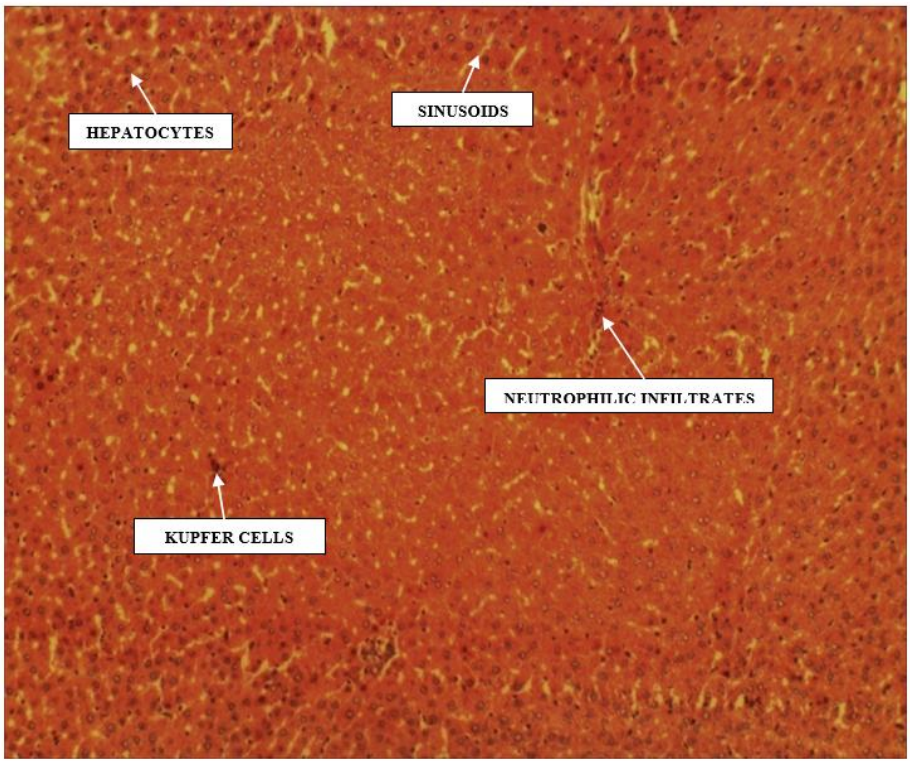

Figure 8. Liver Section of group 4 showing: lightly dilated sinusoids with mixed lymphocytic and neutrophilic infiltrate in the portal spaces around biliary ducts and scattered mononuclear cells in sinusoids of zone 1 of the acinus (H & E x400)

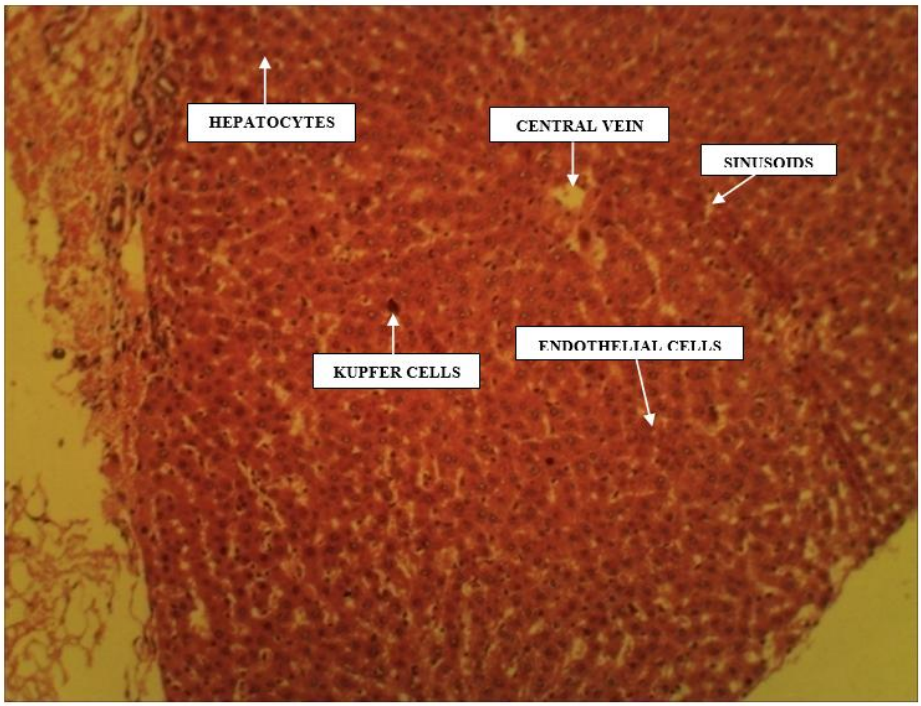

Figure 9. Liver section of group 5, showing: clear hepatocyte cytoplasm with condensed chromatin in nuclei (H & E x400)

4. Discussion

The liver, the biggest organ in the body, plays a crucial role in metabolism, including the storage of glycogen, breakdown of red blood cells, production of plasma proteins, and detoxification. Due to its strategic positioning and function in the body, it is continually and variably exposed to xenobiotics, environmental contaminants, and chemotherapeutic drugs. Women of reproductive age (15–49) have easy access to oral contraceptives like Postinor®-2 and Norinyl®-1/35.

Rats' body weight increased in all groups, but since group 1 (the control group), which received just normal saline, saw the greatest gain, it is illogical to link this to the test medications (Postinor®-2 and Norinyl®-1/35). As seen in figure 1, using one-way ANOVA, there was no statistically significant difference in the mean weight for any of the groups when compared to the control group (group one).

It goes on to say that the administration of Postinor®-2 and Norinyl®-1/35 dosages in this study had no appreciable impact on the rats' body weights since the rise in weight might have been caused by a variety of other variables, including dietary intake, growth, physical activity, ambient circumstances, etc. The body weight and liver weight of the experimental animals in this study were not significantly affected by the administration of these widely available oral contraceptives. As shown in figure 3, there was an increase in the levels of the liver function enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP), and asparatate aminotransferase (AST) in the serum. This increase was more evident in the groups receiving Postinor®-2.

The liver contains the enzyme alanine aminotransferase (ALT), which aids the liver's hepatocytes in converting protein into energy (Burt et al., 2012). ALT and AST are the two main cytosolic transaminases found in the liver, with ALT being the most liver-specific of the two (Ross & Pawlina, 2011). According to Abdel et al. (2010), when there is liver injury (hepatotoxicity), the liver releases ALT into the circulation, which raises the bloodstream ALT level. According to Burt et al. (2012), an increased ALT level is a sign of primary liver disease, viral hepatitis, alcoholic liver disease, biliary obstruction, or pancreatitis.

Since all pharmaceuticals are processed in the liver, the rise in ALT level seen in this study may be connected to this fact. High dosages of drugs taken over an extended period of time are thus expected to cause some degree of liver damage, which in turn raises the plasma ALT level in Wistar rats. An enzyme that breaks down proteins called alkaline phosphatase (ALP), which is abundant in the liver and bones, is crucial (Omini, 2005). It belongs to a group of zinc metalloenzymes that are localized at high levels in the microvilli of the bile canaliculus as well as in a number of other tissues, including the placenta, gut, and bones (Viney, 2017).

According to Burt et al. (2012), an elevated ALP level is a sign of pathologies including biliary obstruction,

primary liver disease, interstitial hepatic disease, bone disorders, hyperparathyroidism, and hyperthyroidism. One of the liver's transaminases that metabolizes proteins is aspartate aminotransferase (AST); the other is alanine aminotransferase (ALT) (Burt et al., 2012). Although it is not only exclusive to the liver like ALT, it aids in protein metabolism (Gray et al., 2000). By accelerating the transfer of amino groups from aspartic acid to oxaloacetic acid, AST takes part in gluconeogenesis (Hamilton, 2014).

It may be found in the liver, cardiac muscle, skeletal muscle, kidneys, brain, pancreas, lungs, leucocytes, and red blood cells as a cytosolic and mitochondrial isoenzyme (Maton et al., 1993). The AST level in the blood increases as a result of liver diseases, although less so than the ALT level (Abdel et al., 2010). A significant decrease in mean SOD level was seen across groups, and groups 2-4 showed a statistically significant difference (decrease) in mean SOD level compared to the control group (group 1), which showed a mean SOD level of 31.45 U/mg pro. The mean oxidative stress marker levels of rats across groups were compared in figure 4 using one-way ANOVA.

The mean MDA level increased significantly across all groups, and it increased much more significantly in groups 2 and 3 compared to group 1, the control group, which had a mean MDA level of 0.68 nmol/mg pro. Group 4 (22.36U/mg pro), which received 0.1mg/kg body weight of Norinyl®-1/35, trailed group 2 (18.97U/mg pro), which received 0.10mg/kg body weight of Postinor®-2, while group 3 (0.2mg/kg body weight) showed the least decrease in mean SOD level.

The mean MDA level in group 2, which received 0.10mg/kg body weight of Postinor®-2, increased the most (3.74nmol/mg pro), followed by group 3, which received 0.20mg/kg body weight of Postinor®-2 (2.00nmol/mg pro), and group 5, which received 0.20mg/kg body weight of Norinyl®-1/35, showed the least elevation (0.70nmol/mg pro). These findings suggest that the drugs (Postinor®-2 and Norinyl®-1/35) significantly decreased the SOD levels of the rats in each group, with Postinor®-2 having the highest decreasing effect as the lowest SOD level (18.97U/mg pro) was found in group 2 that received Postinor®-2 treatment.

Superoxide dismutases (SODs) are oxygen-dependent universal enzymes or organisms (Jolin & Rapkin, 2002). According to Elias et al. (1952), they catalyze the transformation of superoxide anions (O2-) into oxygen (O2) and hydrogen peroxide (H2O2). Superoxide anions are the result of numerous metabolic activities, including mitochondrial respiration, as well as the intended product of certain signaling enzymes. The amount of various reactive oxygen species (ROSs) and reactive nitrogen species (RNSs) is regulated by SOD activity, hence reducing the potential toxicity of these molecules (Viney, 2017). A decrease in SOD level exposes the cell to a wide range of effects of ROSs and RNSs, which is indicative of destruction to the cell or cellular pathology (Glay, 2001).

The observed decrease in SOD level of rats across groups and significantly in groups 2 and 3 may be as a result of cellular toxicity triggered by the administered doses of Postinor®-2 and Norinyl®-1/35 respectively.

One of the byproducts of polyunsaturated fatty acid peroxidation in cells is malondialdehyde (MDA). MDA is overproduced as a result of an increase in free radicals (Fortherby, 1996). Malondialdehyde levels are frequently used as indicators of the antioxidant state and oxidative stress (Zakim et al., 2002). All aerobic organisms produce and destroy reactive oxygen species (ROS), either to physiological concentrations necessary for proper cell activity or in excessive amounts, which is the condition known as oxidative stress (Friedman, 2015). ROS are potentially harmful, mutagenic, or carcinogenic due to their high reactivity. Free radicals have recently been demonstrated to perform a number of essential physiological roles in biological systems and are implicated in numerous pathological situations and at subtoxic doses (Maton et al., 1993).

Key players in the immune responses that are antiparasitic, antimicrobial, and tumoricidal include macrophages, neutrophils, and other phagocytic cells. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide radicals (O2-), hydrogen peroxide (H2O2), and hydroxyl radicals (OH-), can be produced in enormous quantities by these cells. The pathogenesis of infectious pathogens may include ROS and RNS. Indicators of oxidative stress in cells and tissues include lipid peroxidation, a well-established cause of cellular harm (Long, 2015).

An increase in MDA levels indicates disease or toxicity since it indicates a high rate of lipid peroxidation at the cellular level (Maton et al., 1993). The doses of Postinor®-2 that were administered may have caused some level of toxicity to the rats' immune cells, which would have increased the level of lipid peroxidation, a sign of high oxidative stress, and led to the significant increase in MDA levels in groups 2 and 3.

Control rat livers shown by histopathological analysis had normal architecture. Very minor sinusoidal dilation and lymphocytic infiltrates were seen in the liver portal spaces of group 2 cross-sections, whereas group 3 cross-sections revealed light to moderate sinusoidal dilation and mixed lymphocytic and neutrophilic infiltrates in the liver portal spaces. Group 4 showed mildly dilated sinusoids with mixed lymphocytic and neutrophilic infiltration in the portal regions, close to the biliary channels. Additionally, zone 1 of the acinus had sporadic

mononuclear cells in sinusoids. In group 5, clear hepatocyte cytoplasm was seen along with condensed chromatin in the nuclei, but venous thrombus was only seen in one of the tissues that were investigated. Pattern of changes observed in group 3 to 5 were less prominent compared to group 2.

The groups treated with Postinor®-2 (groups 2 and 3) showed more severe changes in the liver histological architecture as seen here.

It is clear from the foregoing that Postinor®-2 has a more widespread effect on the visceral organ, as seen in the liver of female Wistar rats, which is consistent with research done by Newman et al. (2004) who reported that Postinor®-2, when administered in high doses, can have harmful effects on the liver, including inflammatory disorders and sarcoidosis. The findings of this study on the liver histology are consistent with those of Trussell (1998), Adelaide (2007), and Adigun et al. (2016), who all revealed distortion in the normal liver histoarchitecture of their experimental animals.

5. Conclusion

The results obtained in this study shows clearly that; of the two drugs tested, Postinor®-2 has more deleterious effects than Norinyl®-1/35 on the liver of the adult female Wistar rats. One may infer from the result of this study that Postinor®-2 should be taken according to prescription or should be avoided, because its frequent consumption can result in tissue damage in human organs.

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