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Research Article

Reproductive: Hepatic, and Renal Toxicity Induced by Low Doses of N-Hexane in Male Wistar Rats after Sub-Chronic Oral Exposure

Imane Bouakkaz[1*], Kamel Khelili[1], Tarek Rebai[2]

[1]Animal Ecophysiology Laboratory, Badji Mokhtar University, Annaba, Algeria; [2]Histology-Embryology Laboratory, Sfax Medicine College, Sfax, Tunisia

ABSTRACT

The research done on toxicity caused by oral exposure to n-hexane is rare to find, as opposed to those that study exposure by inhalation. The oral toxic effects caused in regarding the solvent n-hexane on the male hormonal, reproductive system, hepatic, and renal functions after sub-chronic exposure were conducted on male Wistar rats at low doses of 300, 600, and 1200 mg/kg respectively. This study was conducted each day for eight weeks and compared the control group and the positive control group. The toxicity observed was characterized by significant increases in the relative weight of the liver and kidneys. Moreover, the evaluation of the effects of n-hexane on the biochemical parameters was marked by an increase in serum levels of transaminases hepatic, renal functions, serum creatinine, uric acid, and urea levels, as well as an increase in tissue levels of testicular, hepatic, and renal GSH. While the level of glucose, cholesterol, and triglycerides decreased significantly, we also noted that n-hexane caused a significant decrease in sperm concentration and mobility; an insignificant decrease in testosterone with an insignificant increase in the Luteinizing Hormone (LH). On the histological and histochemical profile, tissue damage in testicles, liver, and kidneys was observed in rats treated with n-hexane. When compared to the control group and to the positive control group, this confirmed the biochemical observations. Our results prove that oral exposure to even just low doses of n-hexane will cause physiological damage in the body.

Keywords: Solvents; N-hexane; Toxicity; Reproduction; Physiology; Oral administration

INTRODUCTION

Human beings are exposed daily, directly or indirectly, to a wide range of chemicals within the contaminated environment [1], consumed products (food, cosmetics, pharmaceuticals), and work out of industries. Nowadays, exposure to these chemicals has become inevitable, they pose a threat to the ecosystem, human health, and well-being [2]. Organic solvents are among the chemicals that cause a risk to human health [3]. One such toxic solvent is n-hexane.

N-hexane is an oil-soluble aliphatic hydrocarbon. It is considered the best solvent used for the extraction of natural lipophilic elements. This can range through aromas, carotenoids, and vegetable oils [4,5].

The toxicity of n-hexane is related to its lipophilic nature and its high volatility. The lipophilic products diffuse and easily spread in biological membranes, including common routes of entry (lung, intestine, skin) [6]. N-hexane is recognized by its peripheral neurotoxicity [7], chronic exposure causes peripheral polyneuropathy [8], which is the result of peripheral nerve damage [9], the latter of which being the most sensitive effect caused by n-hexane. The data on hepatotoxicity and nephrotoxicity caused by n-hexane are limited and very old. In 1992, the experimental pathology laboratory revealed that exposure of rats to n-hexane at doses of 125 and 500 ppm caused an increase in the absolute and relative weights of the liver and kidneys in rats exposed to the high dose with the presence of tissue damage.

Concerning reprotoxicity, effects on fertility are illustrated by a reduction in testicular weight and atrophy of the seminiferous tubes after chronic exposure to high doses of n-hexane, whether by inhalation or through oral admission [10].

Most of the previous research and results have been obtained after exposure to n-hexane by inhalation. Oral exposure to n-hexane is possible in the extracted oils. Residues of n-hexane have been found in vegetable oils. N-hexane attention in five brands of extra-virgin olive oil ranged from 19.1 ng/mL to 95.3 ng/mL; for peanut

Correspondence to: Imane Bouakkaz, Animal Ecophysiology Laboratory, Badji Mokhtar University, Annaba, Algeria, E-mail: imanebouakkaz23@gmail.com

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and sunflower oil the mean concentrations of n-hexane were <0.9 mg/kg and <1.5 mg/kg respectively [11].

It became essential to take into consideration the harmful effects of pollutants that surround us, to increase life expectancy and protect our health [12]. The objective of this study is to evaluate the sub-chronic toxicity of n-hexane at low doses orally in each of the male reproductive system, the hepatic system, and the renal system.

MATERIALS AND METHODS

The toxic solvent studied

The solvent used is commercial n-hexane with a purity of 95% (Sigma-Aldrich, St. Louis, MO, USA) [13].

Laboratory animals used in research

The experiment was carried out on thirty-five (35) adult Wistar male rats from Pasteur Institute in Algeria, with an average body weight of 300 ± 18 g. The rats were subjected to a one-month adaptation period, making sure to be kept in natural living conditions. They were fed with water ad libitum and food in the form of 20 g croquettes for each rat made of wheat, corn, and barley; all of this was purchased from the agro-food complex (El Kseur, Bejaia).

Experimental protocol

To study the toxic effect of n-hexane on the physiology and reproduction of a laboratory animal model (Wistar rat), the rats were divided into five groups, seven rats per each (n=7), two control groups (a control group, and a positive control group), and three treated groups that received n-hexane dissolved in 0.5 mL of cottonseed oil, at doses of 300, 600, and 1200 mg/kg by gavage daily for eight (8) weeks. After the experimentation period, blood was collected, and the organs (liver, kidney, testicles) of each animal were removed, weighed, and immediately stored in formalin (C=10%).

- **Control:** Exposure to 1 mL of water.
- **Positive control:** Exposure to 0.5 mL of cottonseed oil.
- **Group 1:** Exposure to 300 mg/kg n-hexane dissolved in 0.5 mL of cottonseed oil.
- **Group 2:** Exposure to 600 mg/kg n-hexane dissolved in 0.5 mL of cottonseed oil.
- **Group 3:** Exposure to 1200 mg/kg n-hexane dissolved in 0.5 mL of cottonseed oil.

Studied parameters

Dry tubes: The blood collected underwent a centrifugation process at 5000 rpm for 15 minutes, and the resulting serum was stored at -20 °C to measure:

- **Transaminases and alkaline phosphatase:** They were assayed by the kinetic method, using a Spinreact reagent kit.
- **Glucose:** It was measured by a glucometer using reagent strips.
- **Creatinine:** It was assayed by the enzymatic colorimetric method, using a Spinreact reagent kit.
- **Urea, uric acid, cholesterol, and triglycerides:** They were assayed by the kinetic method, using a Spinreact reagent kit.

Heparin tubes

The collected blood was centrifuged at 5000 rpm for 15 minutes; the plasma obtained was used to determine:

- **Gonadotropin (LH):** It was assayed by the enzyme immunoassay method, using a Diametra ELISA reagent Kit.
- **Steroid hormone (testosterone):** It was assayed by the enzyme immunoassay colorimetric method, using a Diametra ELISA reagent Kit.

Tissue glutathione

Glutathione (GSH) determination was performed in the liver, kidney, and testis according to the method of Weckbecker and Cory [14], and expressed as µM/mg protein. 200 mg of each tissue was homogenized in 8 ml of 0.02 M Ethylenediaminetetraacetic acid (EDTA) solution and then mixed with 0.25% Sulfosalicylic Acid (SSA). After centrifugation of 1000 rpm for 5 minutes with the addition of 25 µl 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1 ml Tris-EDTA, the absorbance was measured at 412 nm.

Spermogram

The spermogram is the analysis that assesses the quality of sperm based on the characteristics of spermatozoa: Concentration, mobility, speed, and viability. The sperm was taken from an opening made at the level of the tail of the epididymis and was diluted in a physiological saline solution (NaCl 0.9%).

Sperm count

The sperm count was measured using a Malassez counting chamber [15]. Once the semen sample was diluted, we introduced a drop into the Malassez slide and covered it with a coverslip. This study is based on counting sperm in 5 squares at (40X) magnification under a microscope. The number of spermatozoa was calculated using the following equation:

Concentration (106/ml)=(D.V.n)/N

Where, D: Dilution factor (50), V: Volume of the Malassez counting chamber (hemocytometer), n: The number of spermatozoa counted in 05 fields, N: The number of small squares of the slide.

Sperm mobility

Mobility was determined by the number of motile spermatozoa in three fields using the light microscope after placing a drop of semen between a slide and a coverslip. From those results, the average percentile of motile sperm is calculated [15].

Histological study

Simple histological sections with Hematoxylin and Eosin (H&E) staining were carried out at Ibn Roched Hospital and El-Bouni University Hospital of Annaba, Department of Pathology, following the technique described by [16]. The technique involves the steps explained in the appendix.

Hematoxylin and eosin staining technique works to recognize different types of tissues and their morphological changes. Hematoxylin is dark blue-purple in color and stains nucleic acids. Eosin is pink and stains proteins in a non-specific way. The nuclei are stained in blue in a typical tissue, while the cytoplasm and extracellular matrix have varying degrees of pink staining.

Histochemical study

The specific histochemical sections with Masson`s trichrome staining and Periodic Acid Schiff (PAS) were carried out in Tunisia at the Faculty of Medicine of Sfax, at the histology-embryology laboratory. The techniques involve the same steps as a simple histological study. Despite this, the histochemical study is characterized by specific staining and with a reading using a Leica light microscope, which made it possible to identify different structures and abnormalities present in the liver, kidney, and testis.

- **Masson`s trichrome staining:** Masson's trichrome stains the nucleus, cytoplasm, and collagen [17].
- **PAS staining:** PAS staining is used for the demonstration of glycogen (oxidation followed by staining with Schiff's reagent) [18].

Statistical study

The results obtained are expressed as the average plus or minus the standard deviation (average ± SD) and were subjected to a one-way Analysis of Variance (ANOVA), followed by a Tukey's test for multiple comparisons. GraphPad Prism software (Version 7) was used. Significant differences were found at *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001. Note: Significant when (*P ≤ 0.05).

- Highly significant compared to control (**P ≤ 0.01)
- Very highly significant compared to control (***P ≤ 0.001)
- P: Significance threshold

RESULTS

Relative weight of organs

Evaluation of the relative liver weights revealed a significant increase (*P ≤ 0.05, and **P ≤ 0.01) respectively, in rats treated with n-hexane at 600 mg/kg and 1200 mg/kg, and a significant increase (*P ≤ 0.05) in relative kidneys weight, only in rats exposed to n-hexane at 1200 mg/kg, compared to the control group and positive control group. The relative weights of the testicles and epididymis, however, did not register any significant change in any of the treated groups (Table 1).

Hematological parameters

The evaluation of the effect of n-hexane on the levels of Red Blood Cells (RBC), Hemoglobin (HB), Hematocrit (HCT), and the Mean Corpuscular Volume (MCV) did not show any notable change compared to the control group and the positive control group.

Regarding the Platelet Count (PLT) in rats exposed to n-hexane at 600 mg/kg and 1200 mg/kg doses, there was a significant decrease (**P ≤ 0.01 and ***P ≤ 0.001) respectively, compared to the control group and the positive control group (Table 2).

Liver function

Aminotransferases: Rats treated with n-hexane at doses of 600 mg/kg and 1200 mg/kg showed a statistically significant increase (*P ≤ 0.05 and **P ≤ 0.01) respectively, in the enzymatic activity of Aspartate Aminotransferase (AST), while the 300 mg/kg dose group showed no significant change when compared to both the control group and to the positive control group. For Alanine Aminotransferase (ALT), our results recorded a significant increase (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001) respectively. This was in the three treated groups to n-hexane, as compared to the control group and the positive control group (Figures 1 and 2).

Alkaline phosphatase: The results of the enzymatic activity of the Alkaline Phosphatase (ALP) revealed a significant increase (*P ≤ 0.05 and ***P ≤ 0.001) respectively, in all groups treated compared to the control group and the positive control group (Figure 3).

Glucose: A significantly decreased level of glucose (*P ≤ 0.05 and **P ≤ 0.01) was demonstrated in this study in groups treated with n-hexane at the doses of 600 mg/kg and 1200 mg/kg. On the other hand, the 300 mg/kg dose showed no significant change compared to the control group and the positive control group (Figure 4).

Glutathione: The tissue level of GSH in the liver was found to be significantly reduced (*P ≤ 0.05 and **P ≤ 0.01) in the 600 mg/kg and 1200 mg/kg groups respectively, whereas the rats treated at the dose of 300 mg/kg showed no significant differences, compared to the control group and the positive control group (Figure 5).

Hematoxylin and Eosin Staining (H&ES): The damage recorded after single staining (H&E staining) in the liver in rats exposed to n-hexane at the three (03) doses studied 300 mg/kg group (B), 600 mg/kg group (C), and 1200 mg/kg group (D) was represented by the existence of foci of hepatocellular necrosis, congestion, and polynuclear inflammation, compared to the control group and the positive control group (Figure 6).

Masson's trichrome histochemical staining: Histological staining for sections of the liver with Masson's trichrome showed the presence of several necrotic-inflammatory foci of hepatocytes in rats exposed to the dose of 300 mg/kg group (B). The alterations observed in rats exposed to the two highest doses 600 mg/kg group (C1, C2) and 1200 mg/kg group (D) were more severe than the dose 300 mg/kg group (B). They are characterized by necrotic-inflammatory foci of hepatocytes and dilation of the centrilobular vein, compared to the control group and the positive control group. There were no observable effects on collagen fibers in the liver of rats treated with n-hexane at doses of 300 mg/kg and 600 mg/kg. The histological representation of these two groups presented identical characteristics in collagen, while the liver of rats exposed to n-hexane at a dose of 1200 mg/kg showed the appearance of collagen fibers around the centrilobular vein (green color) (Figure 7).

Periodic acid Schiff histochemical staining: The histological sections stained by the Periodic Acid Schiff (PAS) allowed us to study the presence of glycogen in the cytoplasm of hepatocytes. Microscopic observations in rats exposed to n-hexane at the three treated doses 300 mg/kg group (B), 600 mg/kg group (C), and 1200 mg/kg group (D) showed a negative appearance of PAS staining in perivenous and periportal hepatocytes, determined by a cellular degranulation and the disappearance of glycogen pink pigments. Furthermore, histological examination showed necrotic inflammation of hepatocytes with cellular degeneration in the three groups of rats exposed to n-hexane at doses 300 mg/kg group (B), 600 mg/kg group (C) but more extensive at the dose 1200 mg/kg group (D), compared to the control and the positive control groups (Figure 8).

Table 1: Relative organ weight after exposure to n-hexane compared to control groups.

Organs (g/100 g)	Control	Positive control	Group 1 (300 mg/kg)	Group 2 (600 mg/kg)	Group 3 (1200 mg/kg)
Liver	2.78 ± 0.07	2.66 ± 0.05	2.84 ± 0.10	3.30 ± 0.14	3.44 ± 0.08
Kidney	0.281 ± 0.02	0.282 ± 0.01	0.284 ± 0.02	0.285 ± 0.009	0.327 ± 0.01
Testicle	0.515 ± 0.05	0.498 ± 0.03	0.483 ± 0.02	0.482 ± 0.02	0.482 ± 0.03
Epididymis	0.182 ± 0.02	0.184 ± 0.01	0.181 ± 0.01	0.174 ± 0.01	0.171 ± 0.02

Table 2: Change in haematological parameters (RBC, HB, HCT, MCV, PLT) after exposure to n-hexane compared to the control groups.

Settings	Control	Positive control	Group 1 (300 mg/kg)	Group 2 (600 mg/kg)	Group 3 (1200 mg/kg)
RBC (1012/L)	9.126 ± 0.02	9.134 ± 0.02	9.128 ± 0.008	9.138 ± 0.01	9.936 ± 0.01
HB (g/L)	148.8 ± 4.65	148.2 ± 5.31	149.2 ± 5.76	151.8 ± 11.62	154.2 ± 8.25
HCT (%)	40.344 ± 2.44	40.018 ± 1.84	41.032 ± 1.36	42.192 ± 1.478	45.758 ± 2.84
MCV (μm^3)	45.4 ± 1.02	44 ± 2	44.8 ± 0.74	44.2 ± 0.74	45.2 ± 1.47
PLT (109/L)	420.8 ± 20.53	411.2 ± 32.90	404.46 ± 22.34	230 ± 22.22	193 ± 24.92

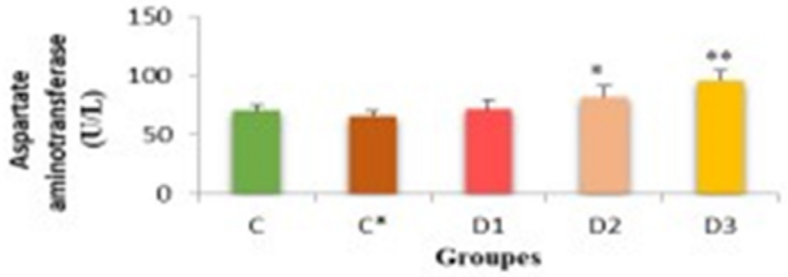

Figure 1: Change in the activity of aspartate aminotransferase in rates exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

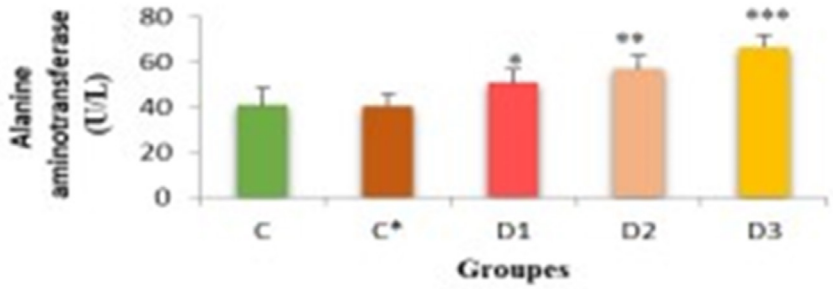

Figure 2: Change in alanine aminotransferase activity in rates exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

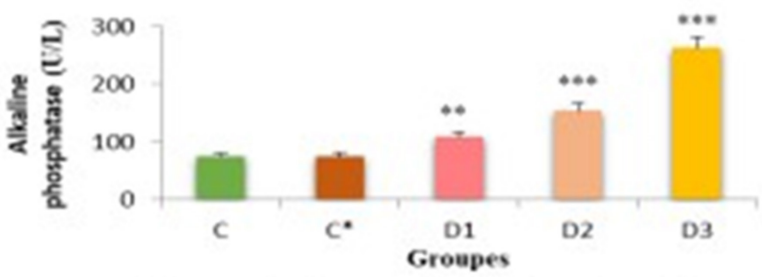

Figure 3: Change in alanine phosphatase activity in rats exposed to n-hexane for eight weeks (08), compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

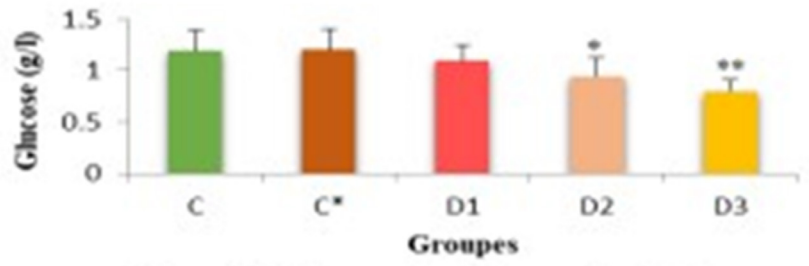

Figure 4: Change in the glucose levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

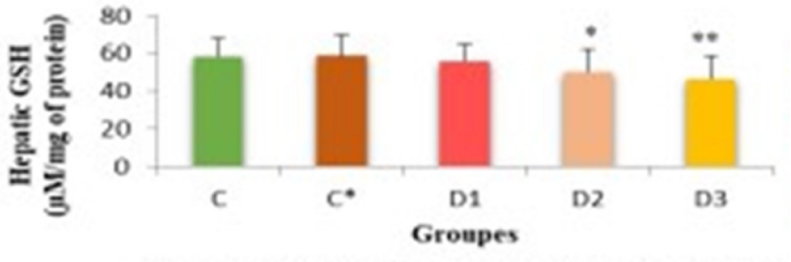

Figure 5: Change in alanine phosphatase activity in rats exposed to n-hexane for eight weeks (08), compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

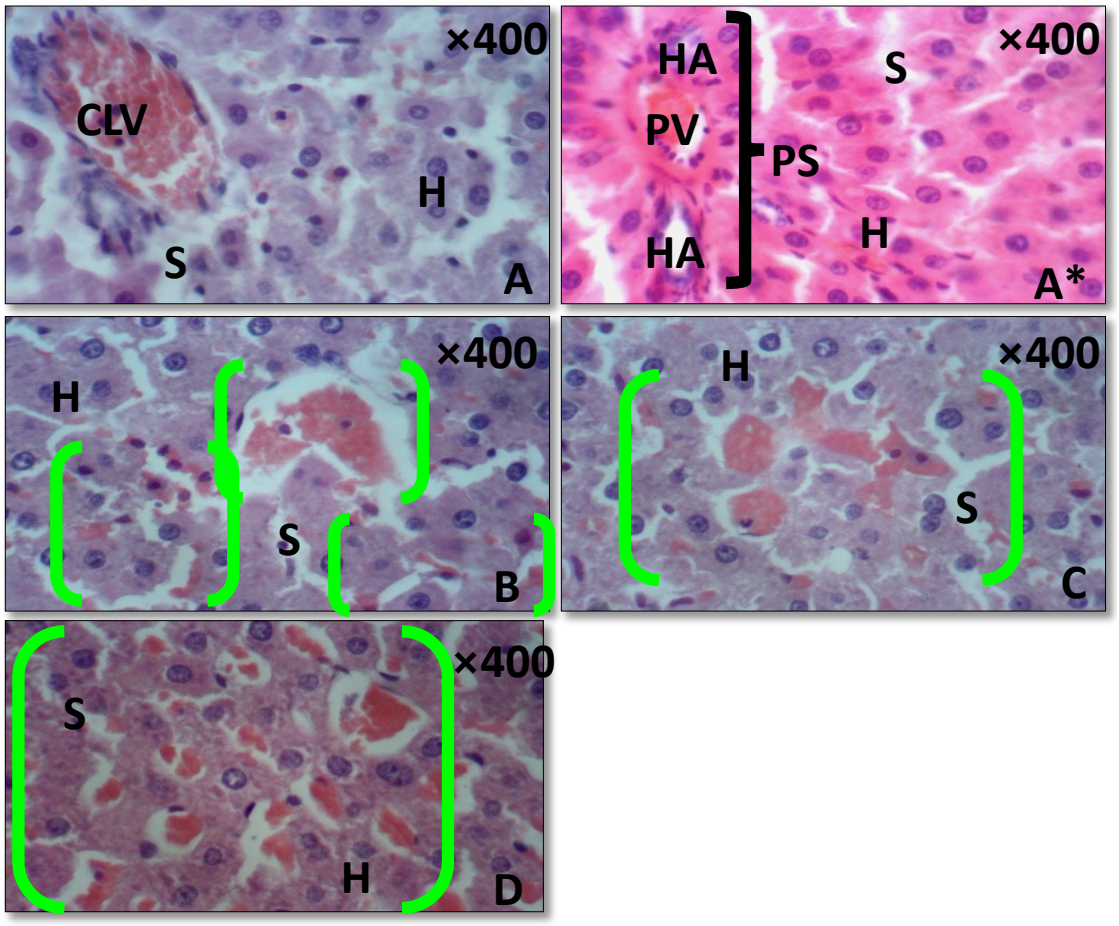

Figure 6: Photograph of histological sections of the H&E-stained liver of male Wistar rats of the three (03) groups treated with n-hexane compared to the control group and the positive control group. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the liver; (B): Group treated with n-hexane at a dose of 300 mg/kg; (C): Group treated with n-hexane at a dose of 600 mg/kg; (D): Group treated with n-hexane at a dose of 1200 mg/kg. **Note:** ( ): Foci of cellular necrosis with polynuclear inflammation; H&E staining, magnification 400X; CLV: Centrilobular Vein; H: Hepatocyte; S: Sinusoid; PV: Portal Vein; HA: Hepatic Artery; PS: Porte Space.

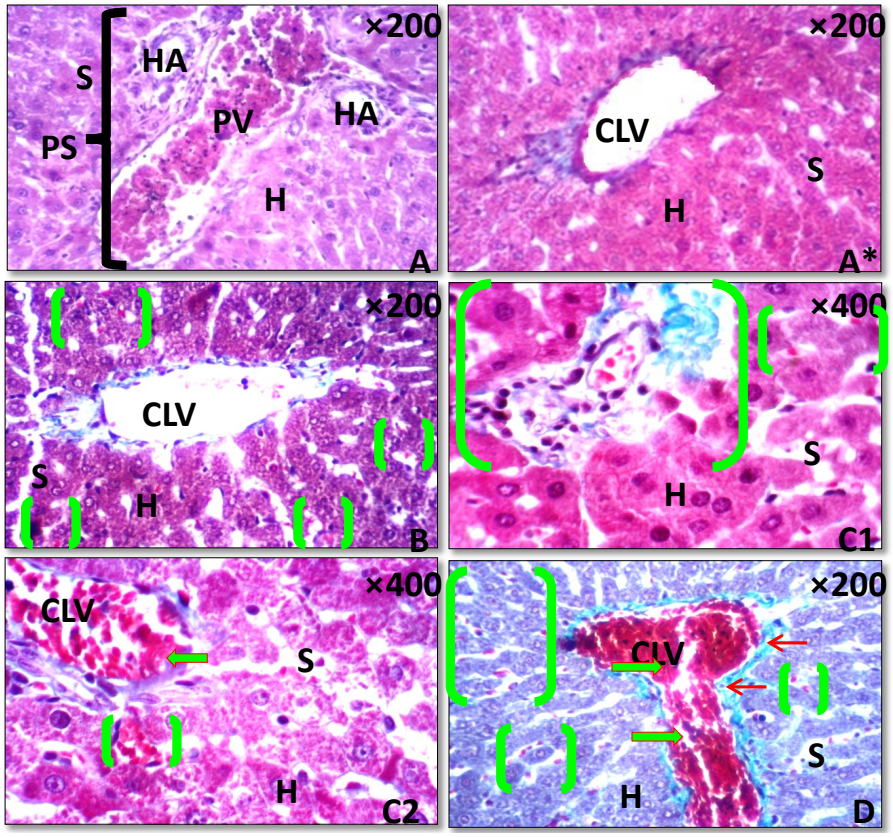

Figure 7: Photograph of histological sections of Masson's trichrome-stained liver of male Wistar rats from the three groups treated with n-hexane compared to the control group and the positive control group. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the liver; (B): Group treated with n-hexane at a dose of 300 mg/kg; (C1 and C2): Group treated with n-hexane at a dose of 600 mg/kg; (D): Group treated with n-hexane at a dose of 1200 mg/kg. **Note:** ( ): Several necrotic-inflammatory foci; (➡): Dilation of the centrilobular vein; (←) Collagen fibers. Masson's trichrome-stained, Magnification 200X and 400X. CLV: Centrilobular Vein; H: Hepatocyte; S: Sinusoid; PV: Portal Vein; HA: Hepatic Artery; PS: Porte Space.

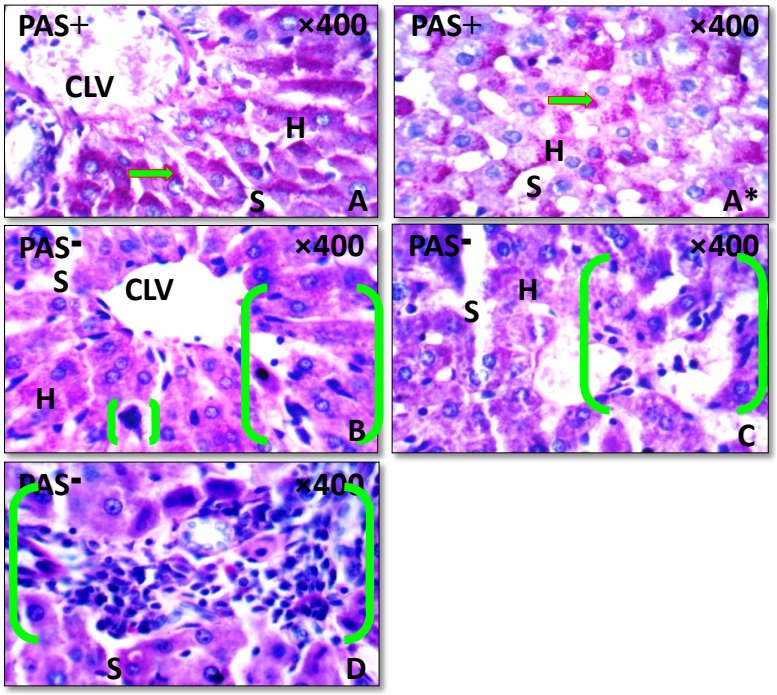

Figure 8: Photograph of histological sections of the liver with Periodic acid Schiff staining (PAS) of male Wistar rats of the three groups treated with n-hexane. This is compared to the control group and the positive control group. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the liver; (B): Group treated with n-hexane at a dose of 300 mg/kg; (C): Group treated with n-hexane at a dose of 600 mg/kg; (D): Group treated with n-hexane at a dose of 1200 mg/kg. **Note:** (➡): PAS-positive staining; ( ): PAS-negative staining and necrotic inflammation of hepatocytes with cell degeneration. Periodic acid Schiff staining, Magnification 400X. CLV: Centro-Lobular Vein; H: Hepatocyte; S: Sinusoid.

Triglyceride and cholesterol

Treatment of rats with n-hexane at doses of 600 mg/kg and 1200 mg/kg induced a significant decrease in triglyceride and cholesterol levels (*P ≤ 0.05 and **P ≤ 0.01) when compared to the control group and the positive control group (Figures 9 and 10).

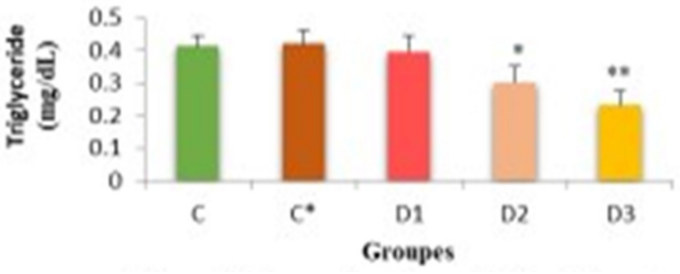

Figure 9: Change in the triglyceride levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

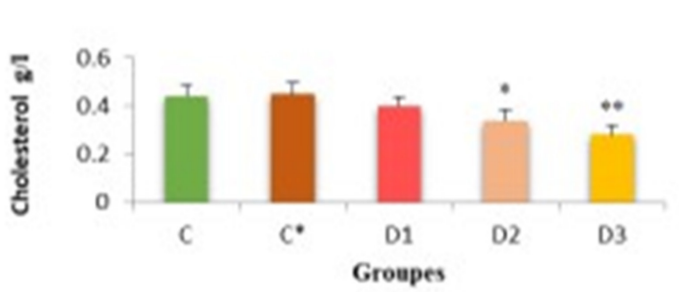

Figure 10: Change in the cholesterol levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

Kidney function

Urea: From Figure 11, we found that n-hexane caused a significant increase in urea levels (*P ≤ 0.05 and **P ≤ 0.01) respectively, in rats treated with the doses 600 mg/kg and 1200 mg/kg, while the rats treated at the dose 300 mg/kg did not register any significant difference, compared to the control group and the positive control group.

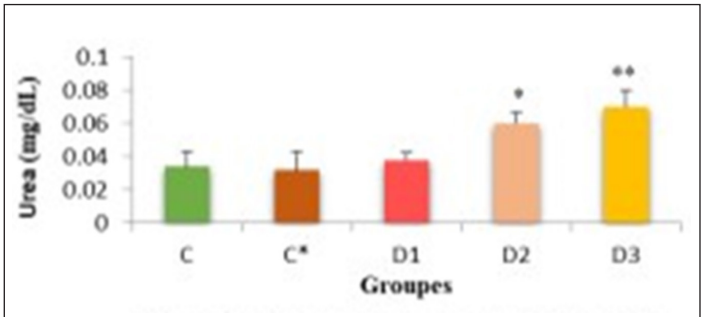

Figure 11: Change in the urea levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

Uric acid: Treatment of rats with n-hexane caused a significant increase in uric acid levels in all treated groups (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001) respectively, compared to the control group and the positive control group (Figure 12).

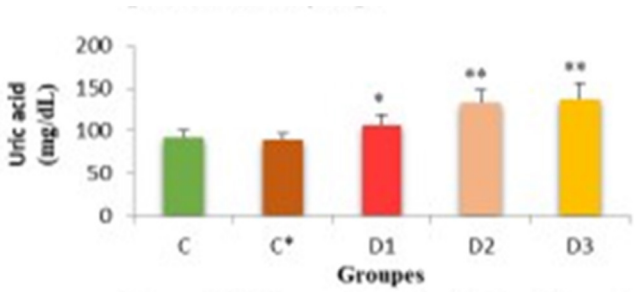

Figure 12: Change in the uric acid levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

Creatinine: Our results showed a significant increase in creatinine levels in all groups treated with n-hexane (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001) respectively, compared to the control group and the positive control group (Figure 13).

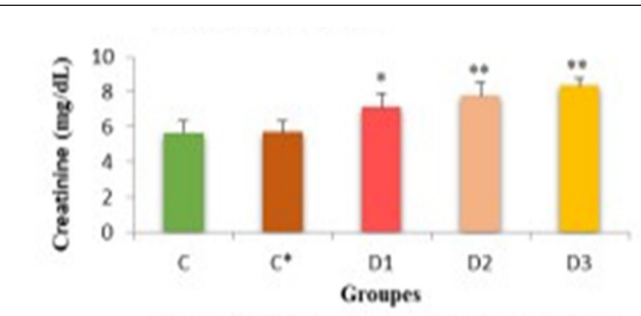

Figure 13: Change in the creatinine levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

Glutathione: The n-hexane caused a significant decrease in the levels of GSH in all treated groups (*P ≤ 0.05 and **P ≤ 0.01). This is compared to the control group and the positive control group (Figure 14).

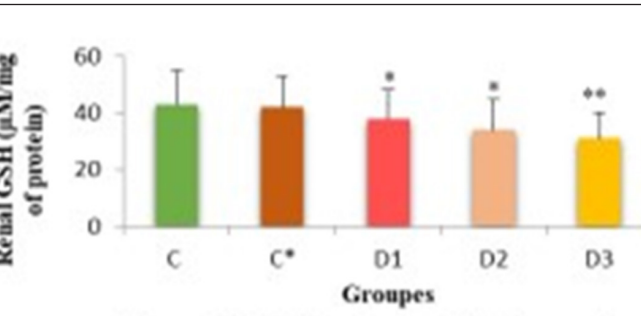

Figure 14: GSH levels in kidneys of rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (■): C; (■): C*; (■): D1 ; (■): D2; (■): D3, *: Positive control group.

Hematoxylin and eosin staining: Observations of histological sections of H&E-stained kidneys showed the presence of lesions defined by exfoliation and degeneration of proximal and distal renal tubules and a decrease in brush border space in rats exposed to n-hexane at the doses 300 mg/kg and 600 mg/kg. The alterations observed in rats treated with the highest dose of n-hexane 1200 mg/kg were like those observed in the groups exposed to the two lower doses 300 mg/kg and 600 mg/kg in the presence of other damage, defined by a series of tubular lesions and retraction of

the glomerular flocculus. No inflammatory response was observed after exposure to n-hexane, compared to the control group and the positive control group (Figure 15).

Masson's trichrome histochemical staining: Regarding the microscopic examination of histological sections with Masson's trichrome staining, the observations showed results that were like those observed in histological sections with H&E staining. We recorded desquamation and degeneration of proximal and distal renal tubules, lesions of the brush border illustrated and represented by the reduction of this border with the absence of the accumulation of collagen fibers in all groups that had exposure to n-hexane at the three studied doses: 300 mg/kg, 600 mg/kg and 1200 mg/kg. No inflammatory response was observed after exposure to n-hexane, compared to the control group and the positive control group (Figure 16).

Male fertility

Testosterone and luteinizing hormone: No significant change was detected in the levels of testosterone and Luteinizing Hormone (LH) in any group exposed to n-hexane, compared to the control group and the positive control group (Figures 17 and 18).

Glutathione: The testicular GSH levels recorded a significant decrease (*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001) in the three (03) groups treated with n-hexane at 300 mg/kg, 600 mg/kg, and 1200 mg/kg respectively, as compared to the control group and the positive control group (Figures 19 and 20).

Spermogram: The rats exposed to n-hexane demonstrated a significant decrease (**P ≤ 0.01 and ***P ≤ 0.001) in sperm count in all treated groups compared to the control group and the positive control group (Figure 20). Concerning sperm mobility our study revealed a statistically significant decrease (**P ≤ 0.01 and ***P ≤ 0.001) in the three groups of rats treated with n-hexane, even compared to the control group and the positive control group.

Hematoxylin and eosin staining: Observations of histological sections of H&E-stained testicles showed that n-hexane has a deleterious effect on the testicles. The results observed in rats exposed to n-hexane at the three (03) treated doses (300 mg/kg, 600 mg/kg, and 1200 mg/kg) revealed an empty appearance of the lumens of the seminiferous tubes, a decrease in spermatogonia, in the sertoli cells and maturity of germ cells with a decrease in the space of the testicles epithelium, compared to the control group and the positive control group.

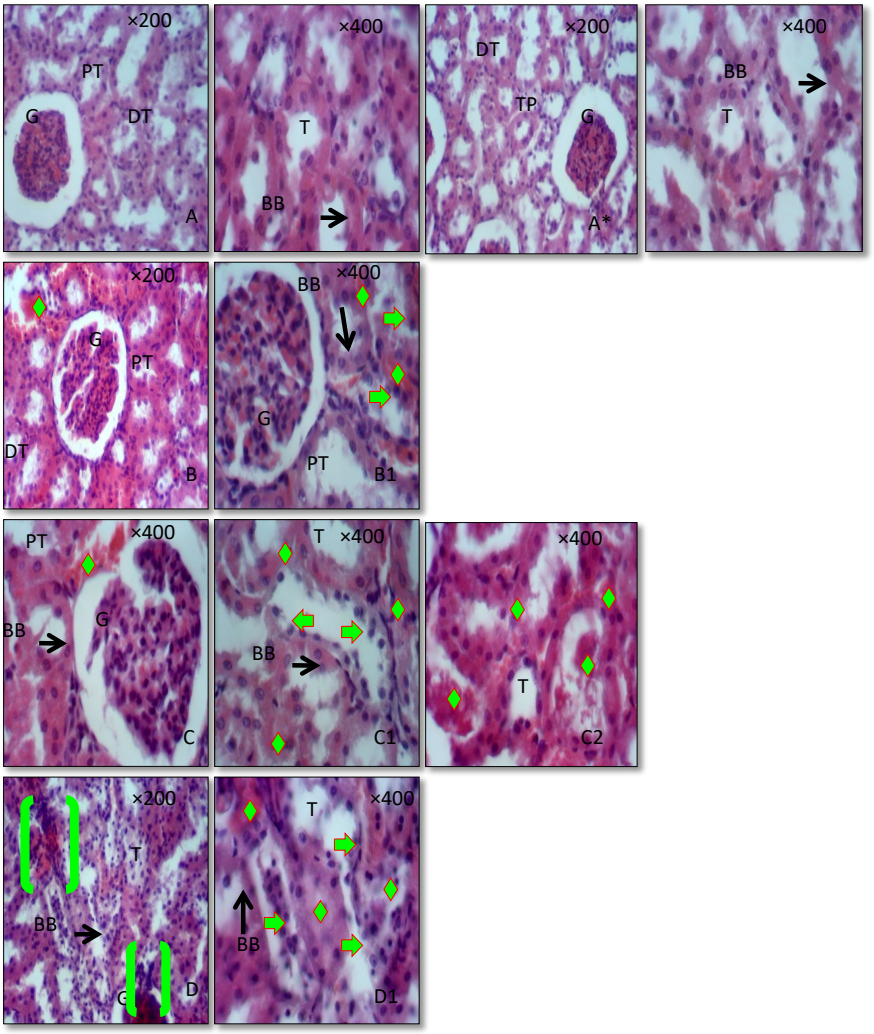

Figure 15: Photograph of histological sections of H&E-stained kidneys of male Wistar rats of the three (03) groups treated with n-hexane compared to the control group and the positive control group. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the glomerulus, proximal and distal tubules; (B and B1): Group treated with n-hexane at a dose of 300 mg/kg; (C, C1 and C2): Group treated with n-hexane at a dose of 600 mg/kg; (D and D1): Group treated with n-hexane at a dose of 1200 mg/kg; (♦): Exfoliation and degeneration of proximal and distal renal tubules. **Note:** (➡): Reduction in the space of the brush borders; (( )): Retraction of glomerular floccules; H-E staining, magnification 200X and 400X; G: Glomerulus; PT: Proximal Tubule; DT: Distal Tubule; RT: Renal Tubule; BB: Brush Border.

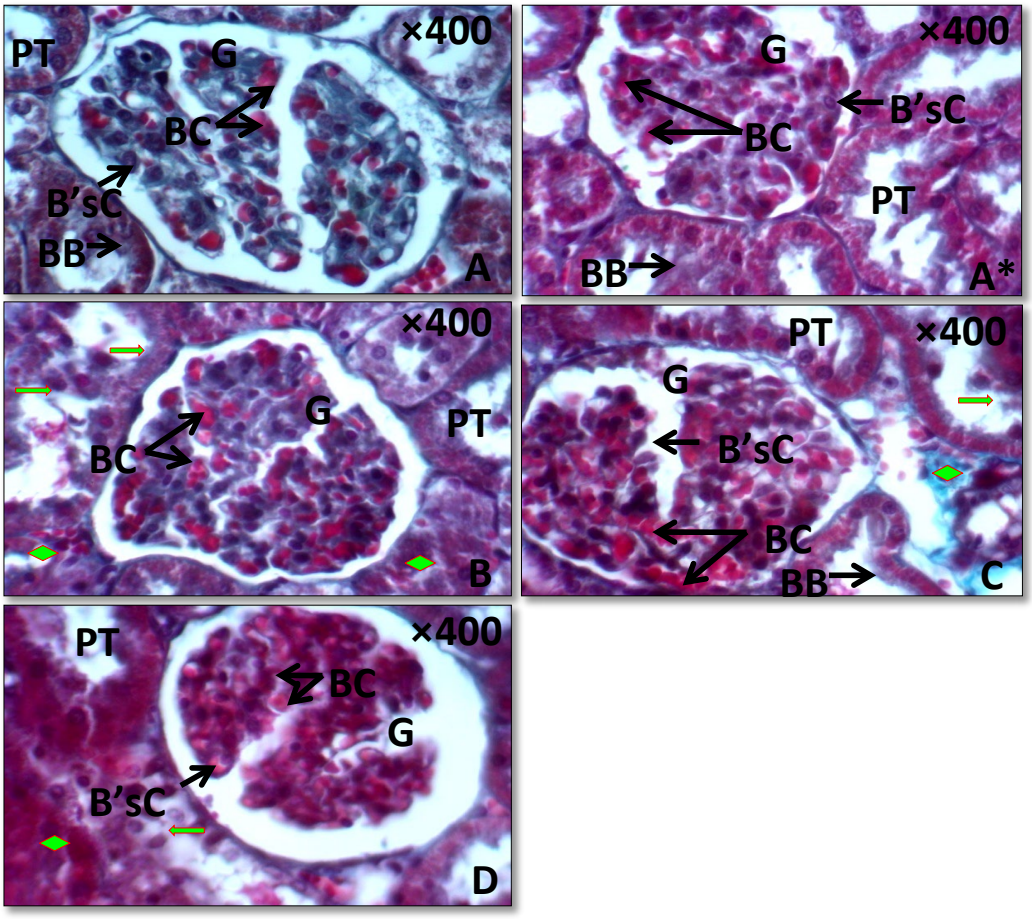

Figure 16: Photographs of histological sections of Masson's trichrome stained kidneys of male Wistar rats from the three groups treated with n-hexane compared to the control group and the positive control group. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the glomerulus, Bowman's capsules, and proximal and distal tubules; (B): Group treated with n-hexane at a dose of 300 mg/kg; (C): Group treated with n-hexane at a dose of 600 mg/kg; (D) Group treated with n-hexane at a dose of 1200 mg/kg. **Note:** (♦): Exfoliation and degeneration of proximal and distal renal tubules; (➡): Reduction in the space of the brush borders. Masson's trichrome stain, magnification 400X. G: Glomerulus; PT: Proximal Tubule; DT: Distal Tubule; BsC: Bowman's Capsule; BC: Blood capillaries; BB: Brush Border.

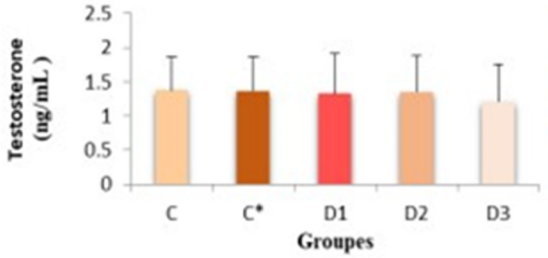

Figure 17: Change in the testosteron levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (): C; (): C*; (): D1 ; (): D2; (): D3, *: Positive control group.

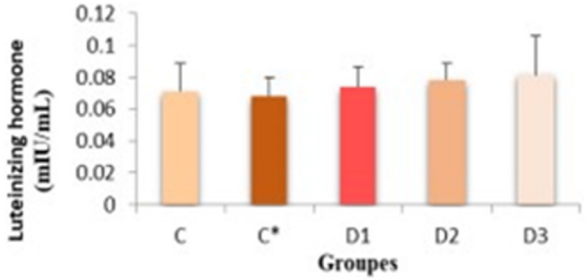

Figure 18: Change in the luteinizing Hormone (LH) levels in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (): C; (): C*; (): D1 ; (): D2; (): D3, *: Positive control group.

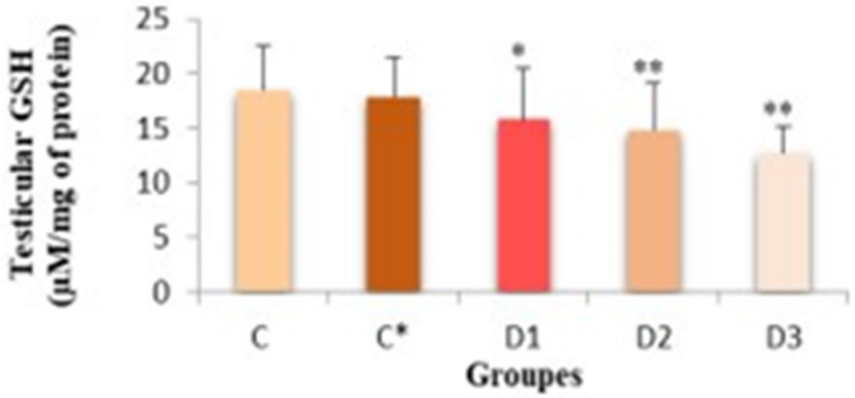

Figure 19: GSH levels in the testes in rats exposed to n-hexane for eight (08) weeks, compared to the control group and the positive control group. **Note :** (): C; (): C*; (): D1 ; (): D2; (): D3, *: Positive control group.

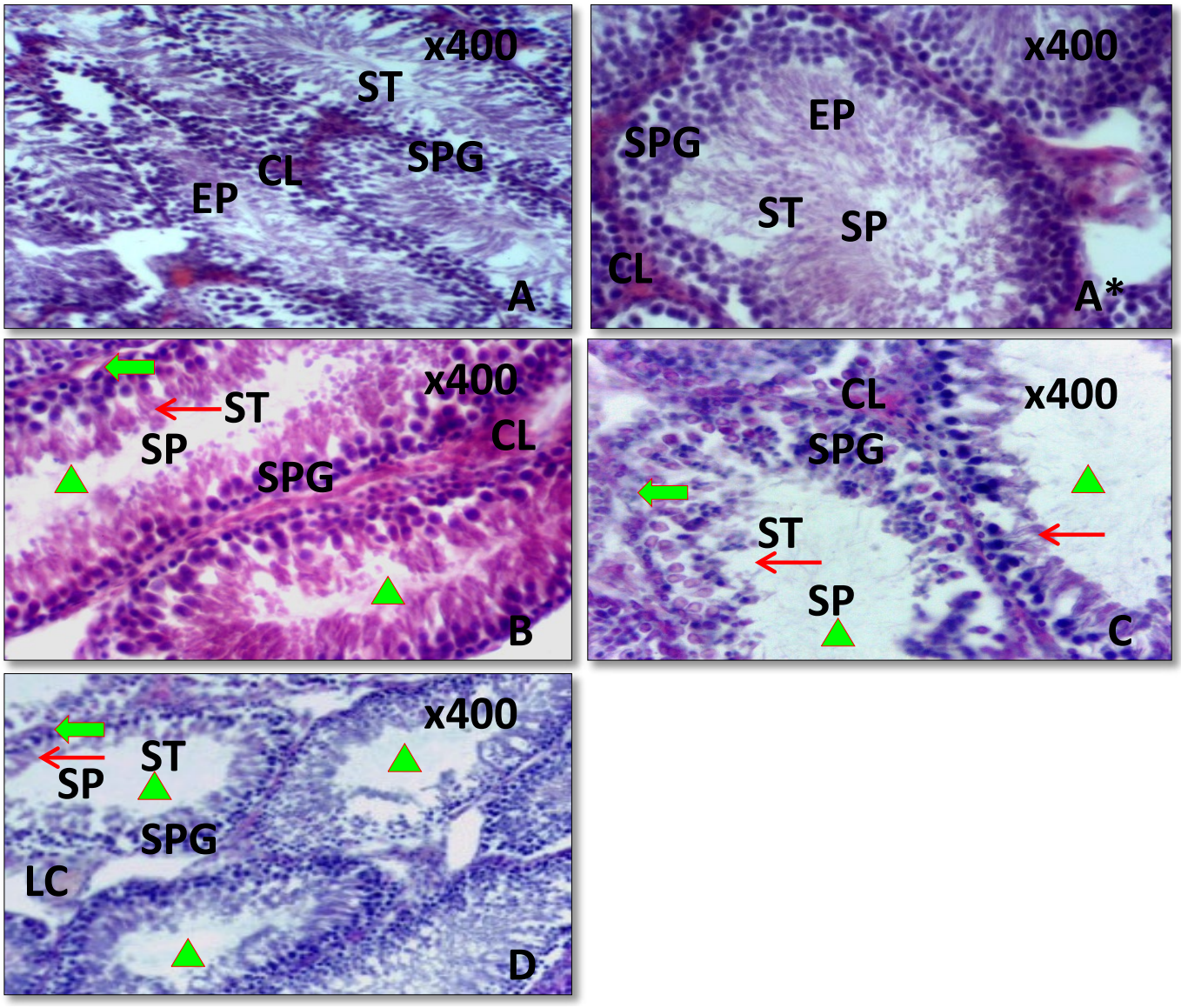

Figure 20: Photograph of histological sections of H&E-stained testicles of male Wistar rats of the three groups treated with n-hexane compared to the control and the positive control groups. (A): Control group; (A*): Positive control group-architecture and normal histological appearance of the testes; (B): Group treated with n-hexane at a dose of 300 mg/kg; (C): The group was treated with n-hexane at a dose of 600 mg/kg; (D): The group was treated with n-hexane at a dose of 1200 mg/kg. **Note:** (▲): Lights of empty seminiferous tubules; (➡): Decreased spermatogonia and germ cell maturity; (➡): Decrease in the space of the testicular epithelium. H-E-staining, magnification 400X. ST: Seminiferous Tubule; LC: Leydig Cell; EP: Testicular Epithelium; SPG: Spermatogonia; SP: Spermatozoa.

DISCUSSION

Toxic products can reach humans in several unique ways. Profoundly, the body tries to defend itself against these dangerous substances in any instance of ingestion or encounter with these products, making sure to decrease their harmful impacts on the target organs. Although these organs are likely to resist toxins, their functions can be attacked by these toxins which result in cellular damage.

Analysis of the group exposed to two high doses of n-hexane showed a significant decrease in body weight with an increase in the relative weight of the liver, and kidneys, and no significant change was presented in the relative weight of the testicles and epididymis. Adenuga et al. [19], and Dunnick [20], revealed that exposure to hydrocarbon solvents and n-hexane induced an increase in the relative weight of the liver and kidneys. The increase in relative weight represents inflammation and organ swelling [21,22], indicating general and organic toxicity. The increase in organ weight may have been caused due to the accumulation of n-hexane in the organs. The relative weight of the testes and epididymis is an important sign of reprotoxicity [23].

In our work, we did not observe a significant change in the relative weight of male organs. This could be related to the dose-response effect, the period (season), and the duration (time) of the treatment. Male Sprague-Dawley rats were exposed to 2,5-hexanedione at doses of 250, 500, and 600 mg/kg/day for four, five, and seven weeks respectively. The results obtained showed that the n-hexane metabolite caused a decrease in the relative weight of the testicles at the highest dose of 2,5-hexanedione [24].

As for the blood profile, Goel et al. [25], reported a non-significant increase in red blood cells and hemoglobin. According to these researchers, n-hexane does not induce a toxic effect on the hematopoietic process. Evaluation of the hematotoxic effect of benzene in Bulgarian petrochemical workers showed no change in the levels of red blood cells, hemoglobin, and hematocrit, compared to unexposed workers [26]. Moreover, there was no significant change in the levels of red blood cells, hemoglobin, and hematocrit that was observed in the study by Kim et al. [27]. This study exposed male and female Sprague-Dawley rats to n-heptane by inhalation for thirteen weeks.

Uzma et al. [28], assessed the exposure of gas station workers to organic solvents. The results obtained revealed a significant increase in the levels of red blood cells and hemoglobin. Similarly, Upreti and Shanker [29], observed an increase in the levels of red blood cells and hemoglobin following the exposure of mice to the n-hexane metabolite, 2,5-hexanedione. Similar observations have been reported after exposing thirty (30) albino mice to injected toluene at doses of 1700 and 1000 mg/kg for twenty-five (25) days [30].

The effect of n-hexane on the hematopoietic system may be indirect. We believe that the increase in the levels of red blood cells and hemoglobin is the result of the toxic effect of n-hexane on the lungs. In our study, shortness of breath was noted in rats treated with n-hexane. The attempt to increase the respiratory rate in these treated rats proves that there is a lack of circulating oxygen in the blood and therefore cellular hypoxia occurs. According to this hypothesis, anoxia stimulates the production of red blood cells and hemoglobin to capture more oxygen and distribute it to the tissues. Red blood cells can detect cellular oxygen deficiencies by vasodilator mediators that regulate blood flow during hypoxia [31].

There are other earlier studies, however, that reported anemia in species that were exposed to n-hexane and gasoline. Egeli et al. [32], observed a decrease in the levels of red blood cells, hemoglobin, hematocrit, and mean corpuscular volume in Swiss albino rats that were exposed to n-hexane *via* intraperitoneal injection at doses of 0.125 ml/kg and 0.250 ml/kg for twenty-four weeks. Likewise, a decrease in the levels of red blood cells and hemoglobin was observed in female rats exposed to gasoline vapor at a dose of 17.8 ppm and orally at a dose of 2 mg/kg and 4 mg/kg [33]. Amarnath et al. [34]; and Valentine et al. [35], found that the 2,5-hexanedione, a metabolite of n-hexane, alters the axonal membrane proteins of red blood cells.

As for platelets, workers' exposure to benzene, toluene, xylene, ethylbenzene, and n-hexane in petrochemical plants led to a decrease in platelet counts [36]. Another study carried out on gas station workers revealed a decrease in the number of platelets [28].

Hooshmand et al. [37], showed that the exposure of male Wistar rats to paint vapor and smell caused a decrease in platelet count. According to Zhang et al. [38], the decrease in the number of platelets may be due to the damage solvents caused to the myeloid progenitor cells. In contrast, the platelet count was significantly increased in male *Oryctolagus cuniculus* rabbits following oral administration of ethanol at concentrations of 20%, 25%, and 30%, each day for six weeks [39].

The analysis of hepatic biochemical parameters showed significant changes in the rats exposed to n-hexane during eight weeks of the experimental period. Adedara et al. [40], revealed a significant increase in the level of hepatic biomarkers (AST, ALT, ALP) after exposure for twenty-one days to the n-hexane metabolite, 2,5-hexanedione, at concentrations of 0.025%, 0.5% and 1% in drinking water. In a study that aimed to determine the volume of gasoline that may cause toxicity, male rats were given 0.5 ml/kg of gasoline. The results recorded a significant elevation of ALT, AST, and ALP [41,42].

The increase in the level of these enzymes reveals tissue and cell damage, more particularly the increase in the ALT enzyme indicates liver cell necrosis, the altered hepatic cells release these enzymes as a signal of liver dysfunction [43, 44].

As for glucose, the administration of n-hexane to rats induced a reduction in the glucose level. The work carried out by Khedun [45], revealed a decrease in glucose in rats exposed to n-hexane. Adenuga et al. [19], showed that the exposure of Sprague- Dawley rats (male and female) to aliphatic hydrocarbon solvents orally at 0, 500, 2500, and 5000 mg kg/day for 90 days caused a decrease in the rate of glucose produced. Reduced glucose levels may be due to a dysfunction of the intestinal nutrient absorption processes and food consumption [46], or the use of glucose under stressful conditions [47]. Furthermore, one of the toxicological characteristics of aliphatic hydrocarbon solvents is the inhibition of enzymes involved in the phenomenon of gluconeogenesis [48]. This contrasted with the findings that no significant change in glucose levels between the control group of people and shoe manufacturers, who were exposed to several organic solvents (benzene, toluene, xylene, and n-hexane) [49].

A decrease in cholesterol was reported in our work in rats exposed to the two highest doses of n-hexane. Total cholesterol is mainly synthesized in the liver and intestines [50]. It is a constituent of

mammalian cell membranes and is an essential constituent in the manufacturing of steroid hormones, bile acids, and several fat-soluble vitamins [51].

Our results agree with the observations obtained by Goel et al. [52], who mentioned a decrease in serum cholesterol levels in rats after exposure to n-hexane and n-heptane intraperitoneally for forty-five (45) days at a dose of 1 ml/kg. These researchers explained this decrease in cholesterol by the presence of an abnormality in the functioning of specific enzymatic activities, caused by n-hexane and n-heptane. Another study done on *Oryctolagus cuniculus* rabbits registered a decrease in cholesterol under the effect of the solvent, Ethylene Glycol Monomethyl Ether (EGME), orally administered five days a week for four weeks [53]. It has been presumed that the reduction in serum cholesterol level can be explained by a dysfunction in the metabolism and production of the lipase enzyme [54].

The decrease in cholesterol level according to Peretti [55], is due to intestinal malabsorption of lipids, illustrated by a lowering in the absorption surface, by an alteration of the intestinal epithelium in the case of intestinal inflammations. He explained that the malabsorption of cholesterol can be a result of a genetic origin. The intestinal absorption is under the control of the Chylomicrons whose synthesis and functioning demand the presence of the apolipoprotein B-48 which is an integral part of the structure of chylomicrons. They both have a role in the absorption and transport of cholesterol. The mutation of the *APOB* gene causes cholesterol malabsorption.

In a health magazine, doctor Amselem et al. [56], published an explanatory article on the causes leading to a decrease in the level of cholesterol, and hypocholesterolemia. According to this doctor, the drop in serum cholesterol may be due to intestinal disorders and liver failure.

However, our results do not agree with the observations revealed in the study by Adedara et al. [40]. This study noted an increase in cholesterol levels following the exposure of male rats to e n-hexane metabolite, 2,5-hexanedione. Further work was done to assess some biochemical and hematological parameters after exposing workers to organic solvents such as benzene, toluene, xylene, and n-hexane during shoe manufacturing, compared to the control group. An increase in blood cholesterol levels was reported [49].

In addition, a significant reduction in serum triglyceride levels was noted in the two groups of rats exposed to the two (02) highest doses of n-hexane. Rare studies have been done on the assessment of triglyceride levels and none of them recorded a decrease in triglyceride levels after exposure to n-hexane [57]. Although glucose is the primary source of energy, triglycerides are comparably an energy store. They come from food and the liver [58]. The decrease in triglyceride levels may be due to their use as an energy source [59]. In contrast, an increase in serum triglycerides was observed in Wistar rats that were orally exposed to n-hexane at doses of 0.2 to 5 g/kg for thirteen (13) weeks [60].

Histological analysis of the liver proved the liver damage induced by n-hexane. The observed tissue alterations confirm the biochemical observations indicated in our study. Liver damage may be due to the accumulation of n-hexane or its metabolite, 2,5-hexanedione in the liver. Our results are in accordance with the research done by Ashmawy et al. [61], who indicated that n-hexane induced liver damage that is marked by focal hepatocellular necrosis.

Oral exposure of male Sprague-Dawley rats to n-hexane at a dose of 15 mmol/kg and a dose of 10 ml/kg of 2,5-hexanedione caused central-lobular injury, cell swelling, the appearance of pyknotic nuclei and hepatocyte degeneration [62]. Adedara et al. [40], reported that the 2,5-hexanedione, a metabolite of n-hexane, caused inflammation and liver congestion. The damage observed in the liver of treated rats suggests that n-hexane affects the structure and function of the liver.

Reported liver damage may be due to oxidative stress induced by n-hexane. According to Ratziu et al. [63], and Marra et al. [64], oxidative stress alters the liver tissue. n-hexane intoxication could interfere with liver proteins and enzymes by triggering an antioxidant defense system that leads to an inflammatory response [65]. Other results reported on the effect of gasoline [41], and on the effect of DBP solvent [54], showed the presence of hepatic damage, respectively illustrated by cellular inflammation and hepatocellular necrosis. The formation of collagen fibers observed on histochemical sections of rats exposed to n-hexane at the high dose (1200 mg/kg) was potentially due to the inflammation and lesions induced by n-hexane. These reactions cause an increased blood supply to the affected part, which enhances the beneficial effects of the immune system cells. Collagen fibers are formed during tissue damage where they intervene to regenerate damaged tissue [66]. These fibers play an essential role in the healing of injured tissues, subsequently working to strengthen them [67].

To highlight hepatic glycogen storage, PAS staining was performed in our study. Histochemical observation showed a decrease in glycogen content accompanied by necrotic inflammation and cell degeneration in rats exposed to n-hexane at the three (03) doses treated.

According to Cullen, et al. [68], the lack of glycogen suggests anorexia. The lowering of glycogen could be due to the malabsorption of glucose in the intestines or to the alteration of enzymes involved in the process of glycogenesis/glycolysis. The negative staining of periportal hepatocytes could indicate that glycogenesis was affected [69-71]. No studies have been described to evaluate the histochemical effects of n-hexane in organs.

The toxic effect of n-hexane on the kidneys is diagnosed by assaying the biochemical parameters of renal function. Kidneys can be attacked if sufficient doses of toxic substances are administered, which can affect their excretory activity and make the body unable to get rid of these toxic products and their metabolites [72]. The nephrotoxicity may be due to decreased renal activity for the glomerular filtration rate; this is shown by elevated serum creatinine, urea, and uric acid in rats treated with n-hexane.

The elevation of urea and uric acid may be due to a decrease in renal reabsorption at the epithelial level [52, 73]. The n-hexane metabolite, 2,5-hexanedione, interact with cell membranes by increasing lipid peroxidation, which leads to the modification and reduction of renal function [40].

Histological and histochemical evaluation of the kidneys in our study reported the presence of renal tissue damage after exposure to n-hexane compared to control groups. Tissue damage was manifested by an exfoliation and degeneration of the proximal and distal renal tubules; this is a decrease in brush border space and a retraction of glomerular flocculus. An oral dose of 15 mmol/kg of n-hexane caused minimal damage to the renal tubules in male Sprague-Dawley rats [62]. Adedara et al. [40], showed that exposure to the n-hexane metabolite, 2,5-hexanedione, induced damage to

the proximal tubules with slight bleeding. Renal tissue changes and damage thus confirming the biochemical observations obtained in our work. The observed histological damage may be due to the absorption and storage in the renal tubules of n-hexane which has been filtered by the glomeruli [74,75].

An antioxidant defense system is established to fight the damaging effect of n-hexane. Adedara et al. [40,76], revealed a reduction in glutathione in the liver, kidneys, and testes after twenty-one (21) days of exposure of male rats to the n-hexane metabolite, 2,5-hexanedione. Also, Kamel and Shehata [77], recorded a decrease in Glutathione (GSH) in the liver, kidneys, and testes in rats exposed to toluene for a period of 15, 30, and 45 days. The observed reduction in GSH levels may suggest an increased demand or an overuse of GSH by tissues, to combat Reactive Oxygen Species (ROS) [40]. The oxidative stress caused by n-hexane boosts the antioxidants present in the cells of the organ to destroy Reactive Oxygen Species (ROS) [78].

Several previous scientific studies have shown that an increase in pollutants and environmental toxins can affect spermatogenesis and testicular cells, leading to infertility. However, there is limited data on the reprotoxic effect of n-hexane and its metabolites [79].

The results showed decreased sperm count and motility in all n-hexane treatment groups. These effects agree with those obtained by Adedara et al. [76], who demonstrated that exposure to the n-hexane metabolite 2,5-hexanedione in male rats for twenty-one (21) days induced a decrease in mobility and sperm count. The same results with the presence of Sertoli Cell Only Syndrome (SCOS), and destruction of seminiferous tubules with germ cell deficiency were observed by Kihaile et al. [80], after exposure of male rats to the metabolite of n-hexane at doses of 100, 200 and 400 mg/kg/day for twelve weeks by the dermal route. The reduction in the number of sperm and their mobility is associated with spermatotoxic effects, caused by exposure to the solvent n-hexane. This spermatotoxicity may be due to excessive production of Reactive Oxygen Species (ROS) [76].

Reactive Oxygen Species (ROS) are essential derivatives in the fertilization of spermatozoa, they participate in the process of chromatin condensation and the acrosomal reaction of spermatozoa. However, excessive production of these derivatives after exposure to foreign substances causes an imbalance in testicular activity [81-84]. Excess in reactive oxygen species induces oxidative stress by reducing antioxidant defenses in the testicles, and decreases sperm mobility, ultimately altering their DNA [85]. The metabolite of n-hexane could cause a dysfunction of the antioxidant mechanism which leads to an accumulation of Reactive Oxygen Species (ROS) [86,87].

The reported disruption of germ cell maturation in our study after exposure to n-hexane may be due to the decrease in the number and length of microtubules in sertoli cells that risk its activity [88], by disrupting the transportation, movement of spermatids to the epithelium and seminal fluid secretions to the seminiferous tubules all along the sertoli cells by microtubules [89]. This alteration of microtubules causes insufficient support and nourishment of germ cells [90].

Similar results with a drop in testosterone levels were observed by Djemil et al. [91], after exposure of male rabbits Cuniculus Lepus to two (02) solvents, toluene, and xylene at doses of 50, 100, and 150 ppm respectively for 24 days. Toluene and xylene induced a reprotoxic effect in male Cuniculus Lepus rabbits by attacking reproductive functions. The reduction in sperm count may also be due to decreased testosterone levels and germ cell apoptosis. Morphological changes in sperm were revealed in a study by Xiao et al. [92], in workers with short- and long-term exposure to benzene, toluene, and xylene. According to these researchers, changes in the morphology of sperm impede their passage through Sertoli cells, and as a result, a reduction in the number of sperm in the lumen of the seminiferous tubules has been recorded.

There were no recorded changes by Linder et al. [93], when it came to the mobility, concentration, and testicular tissues after exposure to male Sprague Dawley rats to undiluted n-hexane orally at doses of 10,000 mg/kg/d for five (05) days and 10,000 mg/kg in a single dose.

To determine whether the reprotoxicity of n-hexane was related to the harmful effect of the latter on the hypothalamic-pituitary-testicular axis, we measured the levels of two hormones, the Luteinizing Hormone (LH) and testosterone. The results revealed a non-significant disturbance in the levels of the hormones studied, where we observed a non-significant increase in LH with a non-significant decrease in the level of testosterone as well. These results are most remarkable in rats exposed to the highest dose of n-hexane. Exposure of female mice to n-hexane by inhalation at doses of 0.3, 15.1, and 75.8 ml/m^3 for five weeks did not cause a change in the levels of LH in all groups treated with n-hexane [94]. Whereas an increase in the level of LH was recorded in female rats exposed to the n-hexane metabolite at the highest dose 1% in water for twenty-one days [95]. LH stimulates the secretion of steroid hormones, a change in the level of this hormone can cause the reproductive system to malfunction.

Boekelheide and Hall [96], administered 1% of 2,5-hexanedione (2,5-HD) to male Charles River CD rats. After twenty-seven weeks, testosterone levels evaluation showed a non-significant decrease, while the luteinizing hormone and follicle-stimulating hormone levels showed a significant increase.

The explanation for the increase in luteinizing hormone levels following exposure to n-hexane or its metabolite, 2,5-hexanedione (2,5-HD) is not well defined. The exposure to n-hexane in our work revealed a non-significant reduction in testosterone levels, more remarkable at the 1200 mg/kg dose. The effect of n-hexane on testosterone concentration is unclear, few previous studies have investigated the effect of n-hexane on testosterone levels, moreover, no recent studies on this effect.

According to Setchell and Galil [97], a decrease in testicular blood flow occurs due to the loss of germ cells, the change in testicular hemodynamics reduces the level of testosterone leaving the testicles and the stimulation of the secretion of the luteinizing hormone increases. Also, the decrease in serum testosterone levels may be due to the decrease in serum levels of its precursors, cholesterol that participates in the synthesis of steroid hormones, the process of steroidogenesis [53].

CONCLUSION

This research has a scientific purpose to study the sub-chronic effect of low doses of n-hexane administered orally. As part of this research, we aimed to study the toxic effect of low doses of n-hexane in male Wistar rats after a sub-chronic oral exposure. The results reported in our research revealed that n-hexane causes harmful effects in the body, which were manifested by an increased relative weight of organs (liver and kidney); increase in biomarkers of oxidative stress,

Glutathione (GSH) in the liver, kidneys and testicles; increase in serum levels of hepatic and renal biochemical parameters (AST, ALT, ALP, creatinine, uric acid, urea) while serum lipid levels (cholesterol, triglycerides) and glucose decreased; decreased sperm concentration and mobility with insignificant disruption of the steroid hormone, testosterone and the gonadotropic hormone, LH; damage in liver, kidney and testicular tissues, despite other published studies, n-hexane only causes a reprotoxic effect in humans and male rats after exposure to high doses.

In conclusion, to respond to the question of this research, the results obtained in our research have shown and claimed that exposure to low doses of n-hexane orally induce hematologic toxicity, hepatotoxicity, nephrotoxicity, and mainly male reprotoxicity.

SUPPLEMENTARY MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

Not applicable.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The data are contained in this article and the doctoral thesis.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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