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Histopathological evaluation of selected organs of rabbit bucks exposed to dietary di-(2-ethylhexyl) Phthalate

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Abstract
This study used 45 crossbred rabbit bucks that had not yet reached puberty with an average weight of 1.37±0.24 kg to investigate the impact of di-(2-ethylhexyl) phthalate (DEHP) on certain internal organs of the rabbits that were divided into 5 different groups for their diets: T_1 (control), T_2, T_3, T_4, and T_5. DEHP was added to the diets at different levels: 0, 100, 200, 300 and 400 ppm for T_1, T_2, T_3, T_4 and T_5 respectively. There were 9 replicates for each treatment, with 1 rabbit assigned to each replicate, following a randomized design. After the 75-day feeding trial, histopathological evaluation was performed on 3 randomly chosen rabbits from each treatment. The rabbits were deprived of feed for a whole night and their live weights were taken. They were stunned, slaughtered and drained of blood before skinning. The liver, kidney, spleen, testis, and ileum were carefully extracted. Specific portions of these organs were sampled and immersed in 10% formalin fixative for 24 hours. After fixing, the tissue samples underwent histopathological procedures. The findings show that there was no noticeable liver damage in rabbits on T_1 and T_2. In T_3, 50% of the rabbits experienced mild congestion in liver central veins. Meanwhile, the other 50% had moderate congestion in their portal and central veins, with severe degeneration of liver cells. Half of the bucks' livers in T_4 displayed slight widespread swelling and degeneration of liver cells, while the other half showed a moderate blockage in the livers' portal system. At T_5, it was noted that all rabbits had moderate to severe infiltration of cells around the liver portal area. Furthermore, there were no notable abnormalities found in the kidneys of the rabbits on T_1. Rabbits on T_2 had a mild to moderate congestion in the interstitial region of their kidneys. In T_3, 50% of the rabbits experienced a slight blockage in the renal cortex, while the other 50% showed protein buildup in their renal tubules. Histological evaluation showed that bucks on T_4 had kidneys that exhibited widespread degeneration of glomeruli and tubular epithelial cells. However, kidneys of all bucks on T_5 experienced interstitial congestion of mild to moderate extent. Meanwhile, the bucks did not show any major abnormal conditions in their spleen, testis, and ileum. The findings indicate that the livers and kidneys of male rabbits are more vulnerable to the harmful impacts of DEHP in their diet.

Keywords: Di-(2-ethylhexy) Phthalate, Histopathological evaluation, organs, rabbit bucks, sinusoidal congestion, hydropic degeneration

Introduction
Phthalates, which are also called phthalic acid esters, are colourless, thick, lipid-loving substances without any smell. They typically exist in a liquid state between temperatures of 25 to 50 degrees Celsius. (Kashyap and Agarwal, 2018) [9]. Phthalates such as di-(2-ethylhexyl) phthalate (DEHP), di-buthyl phthalate (DBP) and di-isononyl phthalate (DiNP) are primarily utilized in the plastics sector as plasticizers to enhance the resilience of PVC materials (ang and Qian, 2021) Qian. These chemicals, recognized as endocrine-disrupting chemicals (EDCs), have been proven to disrupt normal hormonal functions (Wang and Qian, 2021) [21]. Many consumer items, such as nutritional supplements, medications, food packaging, cosmetics and clothing contain phthalates. (Schettler, 2006) [17]. Additionally, phthalates are found in medical devices like umbilical artery catheters, (Rowdhwal, and Chen, 2018) [16].

DEHP, similar to other phthalates, can easily move from consumer items to the environment due to its lack of chemical bonding in polymers (Tran *et al*., 2022) [20] and it has been detected in foods (Przybylińska and Wyszkowski, 2016) [15], meat and dairy products (Serrano *et al*., 2014) [18]. When ingested, DEHP is metabolized into different metabolites including di-n-butyl phthalate (DnBP), benzyl butyl phthalate (BBzP) and diethyl phthalate (DEP), (Koch *et al*., 2003) [10].

Reports have been made on the toxic effects of DEHP on the endocrine system of rats. Martinez-Arguelles *et al*. (2009) [12] discovered that when male adult rats were exposed to DEHP, their Leydig cells had reduced expression of mineralo-corticoid receptors, which resulted in a decrease in testosterone production. The studies conducted by Zhao *et al*. (2021) [23] and Liu *et al*. (2021) [11] have suggested that DEHP is associated with disorders in lipid metabolism and liver damage in rodents. In zebra fish, the exposure to DEHP has caused fetal death and other indications of harm such as tissue damage and abnormal tail shape (Rowdhwal and Chen, 2018) [16]. Furthermore, rats that were given a diet containing DEHP have experienced long-lasting alterations in their thyroid structure. These changes are marked by continued over activity caused by an increased number and size of lysosomes, as well as an enlargement of the Golgi apparatus (Price *et al*., 1988) [14]. Jia *et al*. (2016) [8] also observed a disruption in the thyroid endocrine system of zebra fish when exposed to DEHP. Based on the findings of Herreros *et al*., in the year 2013 [5], ewes that were cycling and were exposed to DEHP had a shorter ovulatory cycle due to a reduction in the size and lifespan of corpora lutea. However, the ewes showed an increase in progesterone levels in their bloodstream, suggesting that DEHP influenced metabolism of steroid. According to Olatundun and Ogunlade (2020) [13], there was a notable decrease in red blood cells (RBC), platelets, albumin, thyroid stimulating hormone (TSH), and testosterone, as well as a notable increase in aspartate aminotransferase (AST) and creatinine, in male rabbits exposed to 400 ppm DEHP compared to those on the control diet. Histopathological findings in the spleen tissue of quail exposed to DEHP revealed an increase in cell gap and changes in the corpuscular border of spleen (Yu *et al*., 2019) [22]. Testicular toxicity of DEHP in mice and rats has been documented. According to David (2006) [3], DEHP has been discovered to disrupt the regular process of sexual development in male rodents due to a decrease in testosterone production.

However, the current information regarding the impact of DEHP on certain organs such as the liver, kidney, spleen, testis, and ileum of rabbits is not extensive. Hence, this research was conducted to evaluate the influence of a diet containing DEHP on specific organs of male rabbit.

Materials and Methods

Experimental site

The research was conducted at the Rabbitry Unit located in the Teaching and Research Farm of Ekiti State University in Ado Ekiti, Nigeria. The site can be found at a latitude of 7º 37^1 15^1N and Longitude 05º 13º 28º E. The temperature at the site typically varies between 21 and 28 degrees Celsius.

Formulation of experimental diets

A high-quality analytical standard with a purity of more than 99.5% of Di-(2-ethylhexyl) phthalate was purchased from Sigma-Aldrich, based in the USA, through Bristol Scientific Company located in Lagos, Nigeria. This standard was utilized in the study at specific levels of inclusion. Treatment 1 was the control diet, containing no DEHP, while Treatments 2, 3, 4 and 5 contained 100, 200, 300 and 400 ppm DEHP respectively.

Experimental rabbits and design

A total of 45 young male rabbits that had not yet reached puberty were bought from trusted rabbit breeders in the Nigerian States of Oyo and Ekiti. These rabbits had an average weight of 1.37±0.24 kg when they were initially acquired. During the acclimatization period, which lasted for 2 weeks, all the rabbits were given the control diet and had unrestricted access to water. After this period, the rabbits were weighed and randomly assigned to 5 different treatments.

Five diets were created, one of which was the control diet (T_1). This diet had a crude protein content of 17.99%, crude fiber content of 11.33%, and a digestible energy content of 2,789 kcal/kg (Table 1). All diets contained the same amount of nitrogen and calories. The dietary treatments T_2, T_3, T_4, and T_5 had the exact same ingredients as the control diet, but they also had DEHP added at levels of 100, 200, 300, and 400 ppm respectively. There were 9 replicates for each treatment, consisting of one rabbit per replicate, in a design that was completely randomized. At the conclusion of the 75-day feeding trial, three male rabbits were chosen at random from each group to undergo Histopathological evaluation. The rabbits were deprived of food for one night and their weights were measured. After that, they were then stunned, slaughtered and bled properly before skinning. The liver, kidney, spleen, testis, and ileum were collected. Then, small sections of these organs were extracted and preserved in 10% formalin fixative, which was about ten times the volume of the samples. The preservation process lasted for 24 hours. After fixing the tissue samples, they underwent additional histopathological procedures as outlined by Slaoui and Fiette in 2011 [19].

Table 1: Experimental Diet (g/100g)

Ingredients	Composition
Maize	20
Soyabean meal	20
Fish meal	1
Palm kernel meal	10.5
Rice husk	20
Wheat offal	25
Di-calcium phosphate	2
Limestone	1
*Vitamin-mineral premix	0.25
Common Salt	0.25
Total	100
Calculated analysis	
Crude protein (%)	17.99
D.E (kcal/kg)	2,789.10
Crude fiber (%)	11.33

***Supplies per kg diet:** 2 000 000 iu vit. A; 400 000 iu D_3; 8.0 g vit. E; 4 g vit. k; 0.3 g Vit B_1; 1.0 g vit. B_2; 0.6 g vit.; 4.0 mg vit. B_{12}; 24.0 g Niacin; 0.2 g Folic acid; 8.0 g Biotin; 48.0 g

Results

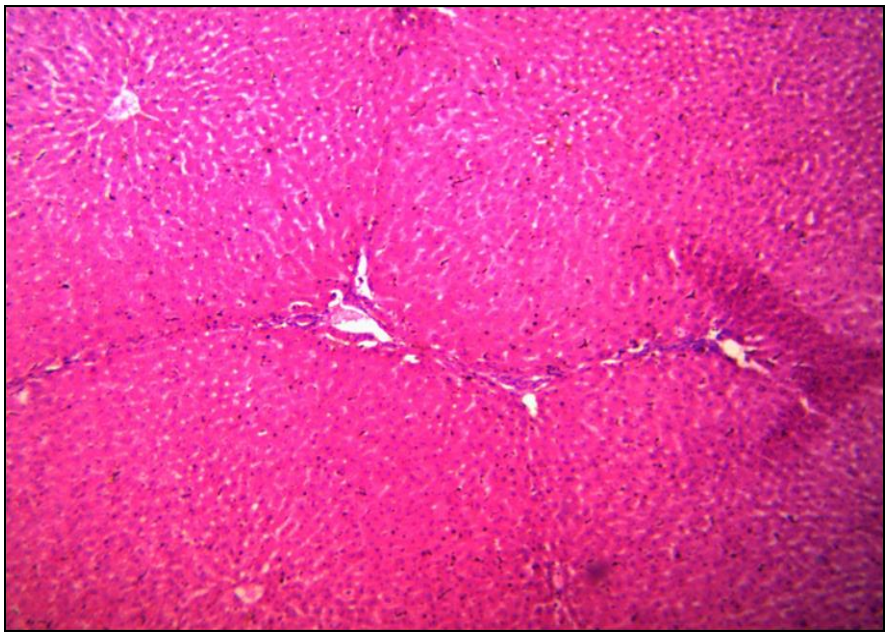

Plate 1: Photomicrograph of Liver (Control) showing no visible lesions

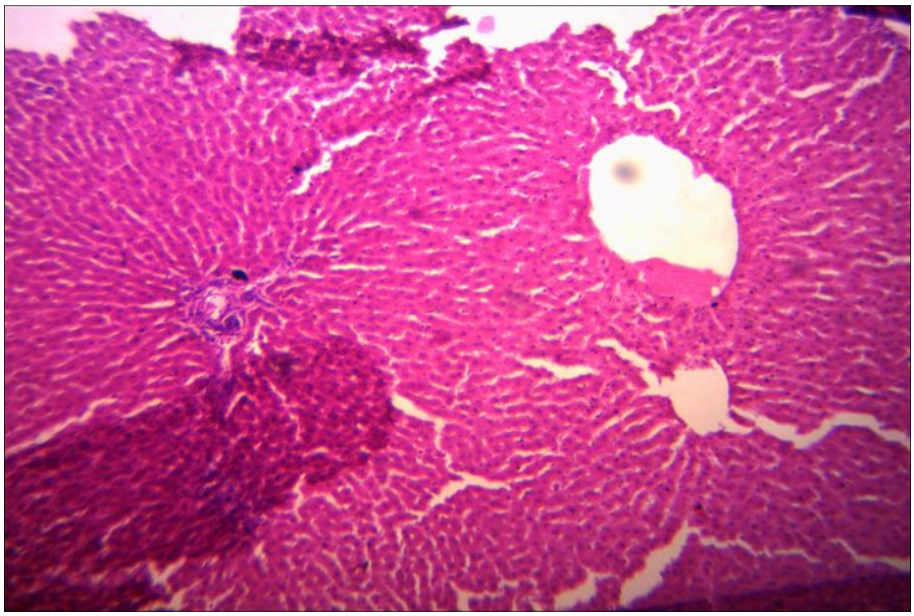

Plate 2: Photomicrograph of Liver (100 ppm) showing no visible lesions

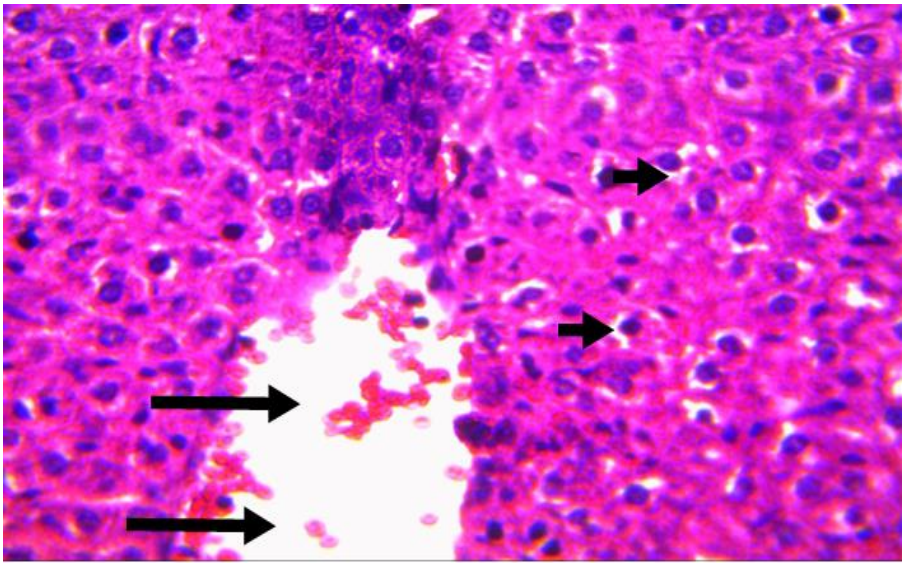

Plate 3: Photomicrograph of Liver (200 ppm) showing mild to moderate portal and central venous congestion (long arrows). There is also a severe diffuse vacuolar degeneration of hepatocytes (short arrows)

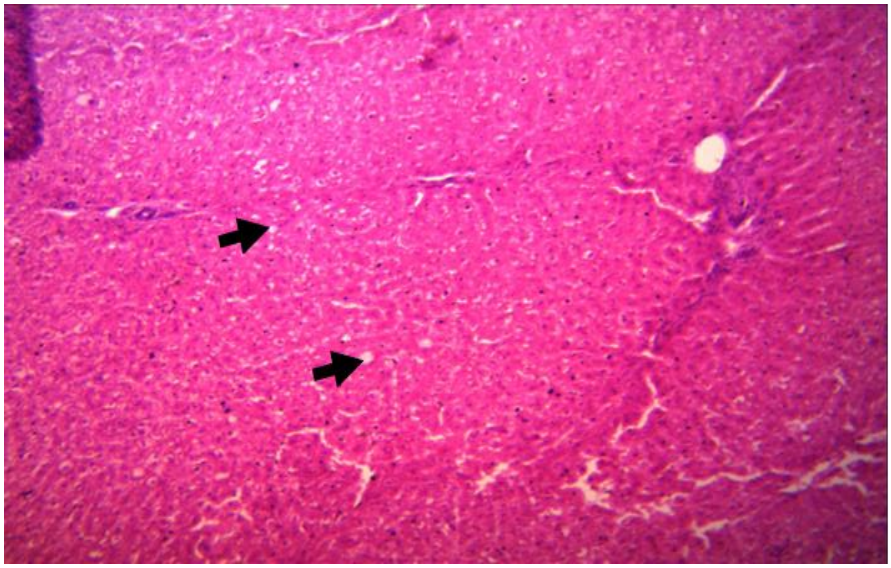

Plate 4: Photomicrograph of Liver (300 ppm) showing mild diffuse hydropic degeneration of hepatocytes (arrows)

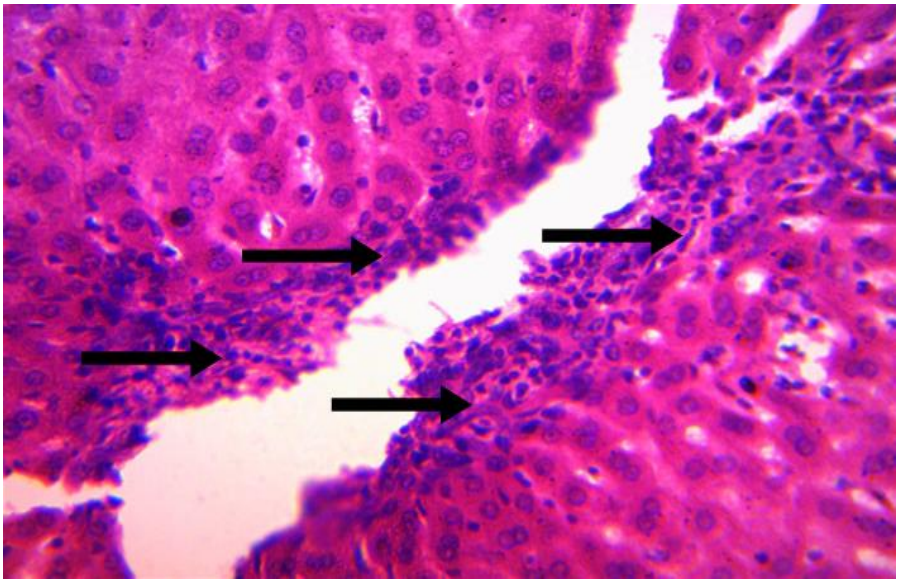

Plate 5: Photomicrograph of Liver (400 ppm) showing a severe periportal cellular infiltration (arrows)

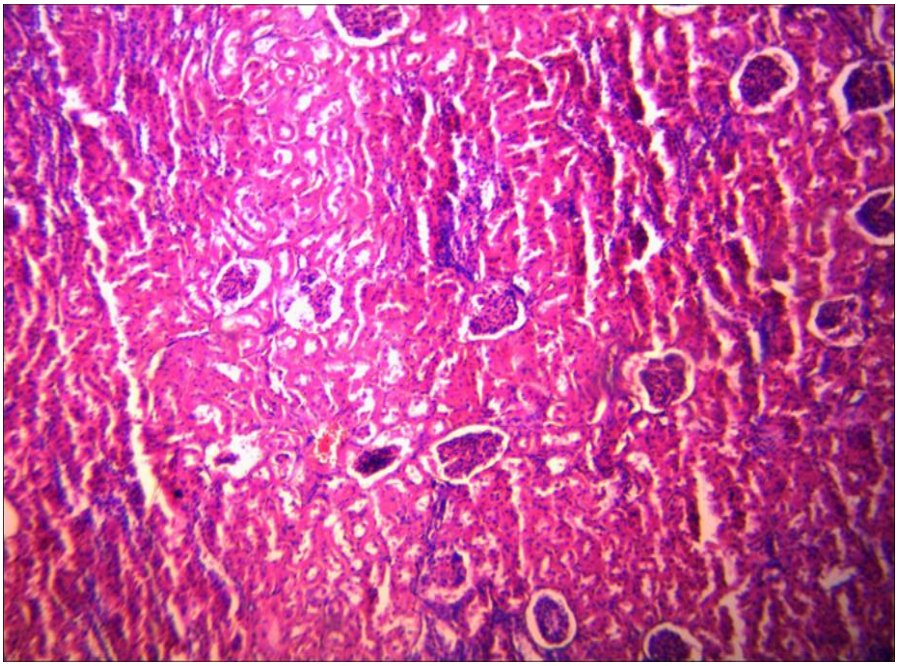

Plate 6: Photomicrograph of Kidney (Control) showing no visible lesions

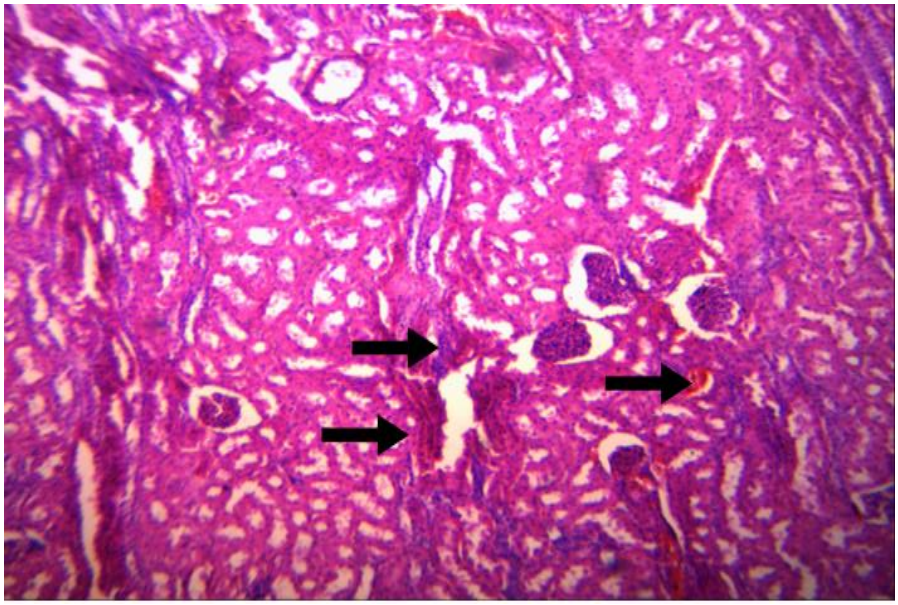

Plate 7: Photomicrograph of Kidney (100 ppm) showing a mild to moderate interstitial congestion (arrows)

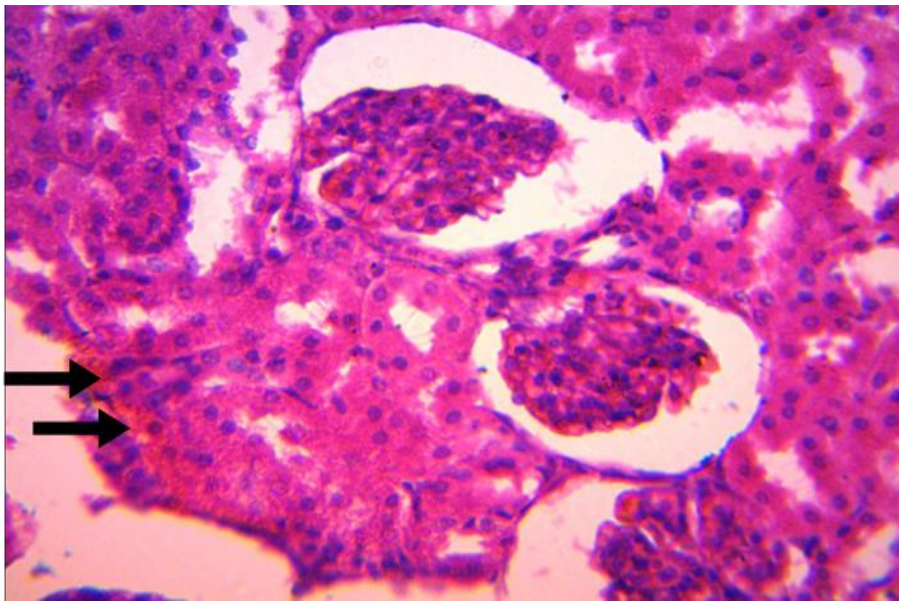

Plate 8: Photomicrograph of Kidney (200 ppm) showing a very mild interstitial congestion at the renal cortex (arrows)

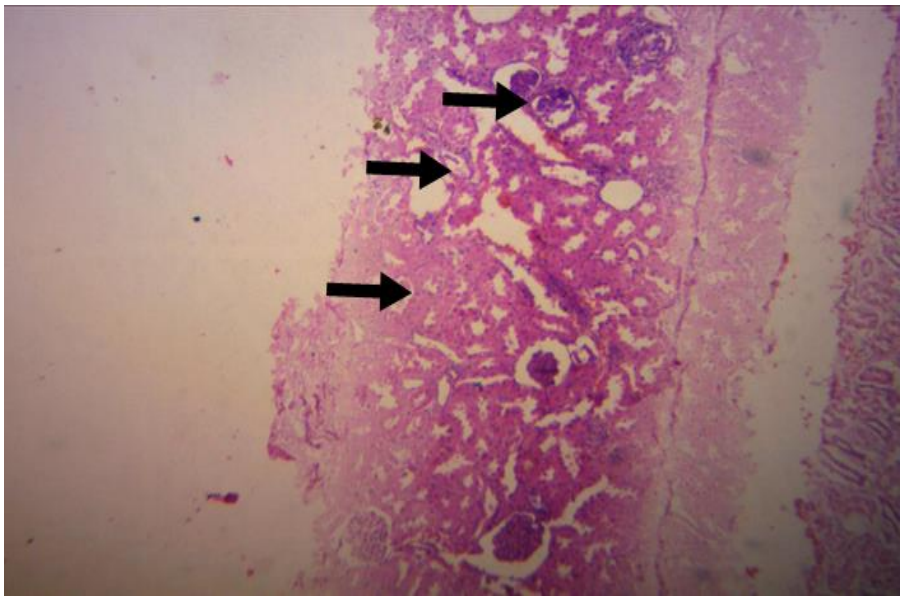

Plate 9: Photomicrograph of Kidney (300 ppm) showing moderate diffuse degeneration of glomeruli and tubular epithelial cells (arrows)

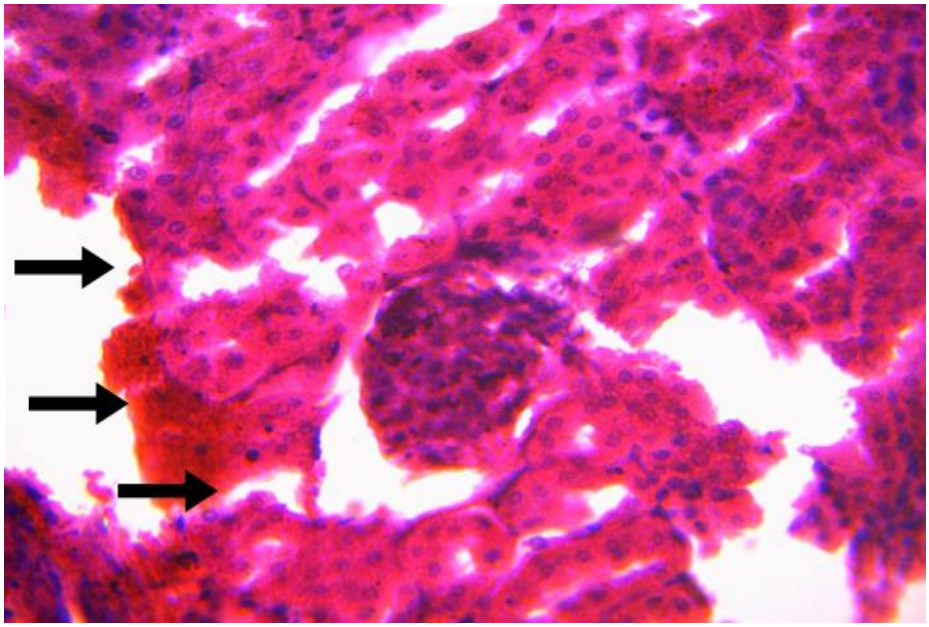

Plate 10: Photomicrograph of Kidney (400 ppm) showing moderate renal interstitial congestion

Table 2: Histopathological examination of selected organs of rabbit bucks fed varied levels of dietary DEHP

	Dietary Treatments				
	T_1	**T_2**	**T_3**	**T_4**	**T_5**
Organs	**Control**	**100 ppm**	**200 ppm**	**300 ppm**	**400 ppm**
Liver	No significant lesion	No significant lesion	Mild venous congestion	Mild hydropic hepatocytes degeneration	Severe periportal cellular infiltration
Kidney	No significant lesion	Mild interstitial congestion	Mild interstitial congestion	Moderate degeneration of glomeruli and tubular epithelial cells	Moderate renal interstitial congestion
Spleen	No significant lesion	No significant lesion	No significant lesion	No significant lesion	No significant lesion
Testis	No significant lesion	No significant lesion	No significant lesion	No significant lesion	No significant lesion
Ileum	No significant lesion	No significant lesion	No significant lesion	No significant lesion	No significant lesion

ppm = part per million (equivalent of mg/kg)

No significant lesion was seen in the liver of experimental rabbit bucks on the control diet (Plate 1) and those in 100 ppm diet (Table 2) (Plate 2). In 200 ppm diet (Plate 3), 50% of the bucks had mild central venous and sinusoidal congestion, while in the remaining 50% there was a combination of mild to moderate blockage in the portal and central veins, along with a severe widespread deterioration of liver cells in the form of vacuolar degeneration.

The liver of half of the rabbit bucks on 300 ppm diet (Plate 4) had a mild widespread swelling and damage to liver cells, while the liver of the remaining half had a slight congestion in the portal, along with a mild infiltration of cells surrounding the portal. In the 400 ppm diet, it was noticed that all of the male rabbits had a significant and noticeable infiltration of cells in the liver, specifically around the portal areas (Plate 5).

No significant lesion was seen in the kidney of experimental rabbit bucks on the control diet (Plate 6). In 100 ppm diet, it was also discovered that 100% of the bucks had mild to moderate interstitial congestion in their kidneys (Plate 7). In 200 ppm diet, 50% showed a very mild interstitial congestion at the renal cortex, while 50% had many of their renal tubules showing small sized protein casts in the lumen (Plate 8). In 300 ppm diet, histological examination revealed that 100% of the bucks had moderate diffuse degeneration of glomeruli and tubular epithelial cells in their kidneys (Plate 9) while 400 ppm DEHP in the diet of the bucks induced 100% mild to moderate interstitial congestion in their kidneys (Plate 10).

The various dietary DEHP levels failed to induce significant pathological lesions in the spleen, testis and ileum of the experimental rabbit bucks

Discussion

The findings from the histological evaluation of tissue samples of certain organs in rabbits involved in the experiment showed that there were no notable lesions found in the liver of male rabbits in the control group and those given a diet with 100 ppm of DEHP. However, rabbits given diets with 200, 300, and 400 ppm of DEHP exhibited mild to severe pathological abnormalities in the liver.

Based on these findings, it appears there is evidence to support the rise in serum liver enzyme levels and the relative weight of the liver in rabbits exposed to DEHP-contaminated diets (Olatundun and Ogunlade, 2020) [13]. The outcomes align with the discoveries made by Jarosova *et al.* (2009) [7], that the liver is the organ most frequently affected by acute toxicity from DBP and DEHP. George *et al.* (2017) [4] noted that when fish were exposed to DEP, they displayed signs of liver cell death, the presence of small fluid-filled sacs in their cells, and a densely stained nucleus. Ikele *et al.* (2011) [6] found that the liver of *Clarias gariepinus*, when exposed to DEP, exhibited congested (cirrhotic) conditions, reduction in filament, enlarged sinusoids, and necrotic areas.

Dietary DEHP elicited various degrees of pathological abnormalities on the kidney of the rabbits across the treatment groups. The nephrotoxicity and nephropathy effects of DEHP and other phthalates have been documented (George *et al.*, 2017) [4]. Elevated levels of urea and creatinine in the bloodstream, as observed in a study conducted by Olatundun and Ogunlade (2020) [13], indicate decline in kidney function caused by DEHP exposure.

Conclusion
DEHP is both hepatotoxic and nephrotoxic because of histologic lesions found in the liver and kidney of the rabbits at inclusion level above 100 ppm

Conflict of Interest
Not available

Financial Support
Not available

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