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Research Article

Haematology, Serum Biochemistry and Hormone Profile of Rabbit Bucks Fed Dietary Di (2-Ethylhexyl) Phthalate

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Abstract

This study was conducted with forty-five pre-pubertal crossbred rabbit bucks to evaluate the effects of di (2-ethylhexyl) phthalate (DEHP) on their haematology, serum biochemistry and hormone profile. The rabbits were weighed and randomly allotted to 5 dietary treatments containing DEHP at inclusion levels of 100, 200, 300 and 400 ppm constituting diets 2 (T_2), 3 (T_3), 4 (T_4) and 5 (T_5) respectively while the control diet 1 (T_1) had no DEHP. The rabbits were housed individually and fed their respective diets for a period of 75 days. At the end of the feeding trial, 3 rabbits were randomly selected from each treatment for blood collection and analyses. Blood samples were collected using 2 ml needle syringe from the ear vein of each rabbit for haematological and serum biochemical analyses. The mean values of the red blood cells (7.04 x 10^3/µl), white blood cells (4.18 x 10^6/µl) and platelets (69.67 x 10^3/µl)of rabbits fed the control diet were significantly ($p > 0.05$) superior to those on diet 4 (6.69 x 10^3/µl, 2.92 x 10^6 and 61.33 x 10^3/µl for RBC, WBC and platelets respectively) and diet 5 (6.29 x 10^3/µl, 2.23 x 10^6/µl and 61.00 x 10^3/µl for RBC, WBC and platelets respectively). Albumin level of rabbits fed the control diet (3.74 g/dl) was significantly ($p > 0.05$) higher than those on diets 3 (3.22 g/dl), 4 (3.11 g/dl) and 5 (2.42 g/dl). Highest creatinine value (1.57 mg/dl) was recorded in rabbits fed diet 5. Aspartate amino transferase (AST) value was lowest in the rabbits fed the control diet (42.51 I.U/L) while AST values were significantly ($p > 0.05$) higher in the other treated groups. Alkaline phosphatase (ALP) of rabbits on diet 5 was significantly lower than those on other diets. The serum testosterone levels of rabbit bucks fed control diet (3.25 I.U/L), diet 2 (3.20 I.U/L), diet 3 (2.60 I.U/L) and diet 4 (2.65 I.U/L) were statistically similar ($p < 0.05$) while bucks on diet 5 had the lowest serum testosterone value of 2.20 I.U/L. Thyroid Stimulating Hormone (TSH) levels of rabbit bucks on control diet (2.30 I.U/L), T_2 (1.15 I.U/L) and T_3 (1.05 I.U/L) were statistically similar ($p < 0.05$) while TSH values in T_4 (0.85 I.U/L) and T_5 (0.75 I.U/L) were significantly lower ($p < 0.05$) than the control. The study revealed that exposure of rabbits to DEHP up to 400 ppm compromised their health status and could be deleterious to their growth and reproductive performance.

Keywords: *Di (2-Ethylhexyl) Phthalate; Rabbit Bucks; Haematology; Serum; Aspartate Amino Transferase; Alkaline Phosphatase; Testosterone*

Introduction

Plasticisers are substances used in the making of plastics in order to improve their flexibility or elasticity. This is achieved by reducing the temperature glass transition of the respective polymer. They are mostly esters with low vapour pressure that do not form covalent

bond with the polymer but interact with it only physically in order to produce a homogeneous compound. They are liquid or solid, odourless and colourless. Plasticisers are also used in other products like rubbers, varnishes, adhesives, coatings, pesticides and sealants [1].

Phthalates are chemical compounds commonly used as plasticisers and because of their volatility, they have been reportedly found to be environmental contaminants. Their presence in water, soil and animal feedstuffs has been reported [2-4]. They are endocrine disrupting substances and their effects on many animals have been reported [5]. The term phthalate encompasses many chemical compounds including dimethyl phthalate (DMP), Diethyl phthalate (DEP), Dibutyl phthalate (DBP), Dicyclohexyl phthalate (DCHP), Di-n-octyl phthalate (DOP), Di- (2-ethylhexyl) phthalate (DEHP), Di-isononyl phthalate (DiNP), and Benzylbutyl phthalate (BzBP), which is commonly called butyl benzyl phthalate (BBP) [6].

Phthalates like DBP, DiNP and DEHP have been reported to induce alterations in blood parameters in fish [7,8], rats [9-11] and monkeys [12]. However, little is known about the effect of DEHP on blood parameters of rabbits. Therefore, the present investigation was designed with the aim of evaluating the effects of graded levels of dietary DEHP on haematology, serum biochemistry and hormone production of rabbit bucks.

Materials and Methods

Experimental site

The experiment was carried out at the rabbitry unit of the Teaching and Research Farm, Ekiti State University, Ado Ekiti, Nigeria. The site is located on Latitude $7^{o}37^{1}15^{11}$N and Longitude $05^{o}13^{o}28^{o}$E with the temperature range of 21 to 28^{o}C.

Formulation of experimental diets

Analytical standard on purity of over 99.5% Di (2-ethylhexyl) phthalate was purchased from Sigma-Aldrich, USA through Bristol Scientific Company, Lagos, Nigeria and was used for this study at the following inclusion levels:

- **Treatment 1:** Control diet.
- **Treatment 2:** Control diet + 100ppm DEHP.
- **Treatment 3:** Control diet + 200ppm DEHP.
- **Treatment 4:** Control diet + 300ppm DEHP.
- **Treatment 5:** Control diet + 400ppm DEHP.

The gross composition of experimental diet is shown on table 1.

Ingredient	Composition
Maize	20
Soybean meal	20
Fish meal	1
Palm kernel meal	10.5
Rice Husk	20
Wheat offal	25
DCP*	2
Limestone	1
Vit. mineral premix	0.25
Salt	0.25
Total	100
Calculated analysis	
Crude protein (%)	17.99
D.E (kcal/kg)	2,789.10
Crude fiber (%)	11.33

Table 1: *Gross composition of experimental diet (g/100g).*

**: Dicalcium Phosphate.*

Experimental rabbits and design

Forty-five pre-pubertal rabbit bucks (crosses) were purchased from reputable rabbit farmers in Oyo and Ekiti States, Nigeria. All the rabbits were fed the control diet and provided water *ad libitum* during acclimatization period which lasted for 2 weeks after which they were weighed and randomly allotted to 5 dietary treatments as described above. Each treatment had 9 replicates with 1 rabbit per replicate in a completely randomized design. At the end of the feeding trial, which lasted for 75 days, three (3) rabbit bucks were randomly selected from each treatment for blood collection and analyses.

Collection of blood samples

Blood samples were collected using 2 ml needle syringe from the marginal ear veins of each rabbit into vacutainer bottles containing ethylene diamine tetra acetic acid (EDTA) and another vacutainer bottles without EDTA. The vacutainer bottles with EDTA were immediately capped and content mixed gently for about a minute by repeated inversion and taken to the laboratory for haematological analyses while vacutainer bottles without EDTA were allowed to stand in a slanting position for about 6 hours and serum decanted for biochemical studies. The haematological and biochemical studies were carried out as described by Jain [10,13,14].

Hormonal assay

The test for hormonal parameters in the blood serum was carried out with the aid of the tube-based enzyme immunoassay (EIA) method. The procedure used for the hormonal assay was according to the method of Micaleft., *et al*. [15] as described for the Kit (BioCheck ELISA Assay, USA).

Statistical analysis

Data obtained were subjected to standard statistical analysis using ANOVA procedure of Statistical Analysis Systems Institute (SAS, 1999) while the treatment means were separated using the Duncan's Multiple Range Test of SAS [16].

Results

Table 2 shows the haematological indices of rabbit bucks fed varied levels of dietary DEHP. Statistical analysis of the results showed that the mean values of the red blood cells (7.04 x 10^3/µl), white blood cells (4.18 x 10^6/µl) and platelets (69.67 x 10^3/µl) of rabbits fed the control diet were significantly ($p > 0.05$) superior to those on diet 4 (6.69 x 10^3/µl, 2.92 x 10^6 and 61.33 x 10^3/µl for RBC, WBC and platelets respectively) and diet 5 (6.29 x 10^3/µl, 2.23 x 10^6/µl and 61.00 x 10^3/µl for RBC, WBC and platelets respectively).

Dietary Treatments	T_1	T_2	T_3	T_4	T_5	
Parameters	**Control**	**100 ppm**	**200 ppm**	**300 ppm**	**400 ppm**	**SEM**
PCV (%)	38.67	41.67	40.33	42.33	43.00	0.31
Haemoglobin (g/dl)	12.77	12.73	11.77	11.63	11.53	0.07
RBC (x10^3/µl)	7.04^a	6.98^{ab}	6.91^{ab}	6.69^b	6.29^b	0.06
WBC (x10^6/µl)	4.18^a	3.33^{ab}	3.30^{ab}	2.92^b	2.23^b	0.15
Platelets (x10^3/µl)	69.67^a	64.00^{ab}	63.33^{ab}	61.33^b	61.00^b	1.64
MCHC (g/dl)	33.04	33.63	33.71	33.25	32.96	0.03
MCH (pg)	20.30	19.94	20.35	20.18	20.51	0.04
MCV (**µ**^{3})	61.47	59.28	60.39	60.67	62.25	0.12
Lymphocytes (%)	71.00	70.68	70.67	70.00	69.94	0.36
Neutrophil (%)	26.33	25.68	25.67	25.10	27.86	0.37
Monocytes (%)	1.00	2.24s	2.33	2.99	1.00	0.08
Eosinophil (%)	1.67	1.40	1.33	2.00	1.20	0.01

Table 2: *Haematological indices of rabbit bucks fed varied levels of dietary DEHP.*
Values shown on the table are means.
SEM: Standard Error of Means; PCV: Packed Cell Volume; RBC: Red Blood Cell; WBC: White Blood Cell; MCHC: Mean Cell Haemoglobin Concentration; MCH: Mean Cell Haemoglobin; MCV: Mean Cell Volume; ppm: Part Per Million (Equivalent of mg/kg).

Data on serum protein quality, enzyme activities and some chemical components in the serum of rabbit bucks fed diets contaminated with varied levels of DEHP are presented in table 3. Exposure of bucks to dietary DEHP at the various inclusion levels did not significantly alter some serum biochemical parameters like total protein, globulin, urea, ALT, cholesterol, HDL and LDL. However, albumin level of rabbits fed the control diet (3.74 g/dl) was significantly ($p > 0.05$) higher than those on diets 3 (3.22 g/dl), 4 (3.11 g/dl) and 5 (2.42 g/dl). Highest creatinine value (1.57 mg/dl) was recorded in rabbits fed diet 5. Aspartate amino transferase (AST) value was lowest in the rabbits fed the control diet (42.51 I.U/L) while AST values were significantly ($p > 0.05$) higher in the other treated groups. Alkaline phosphatase (ALP) of rabbits on diet 5 was significantly lower than those on other diets. From Table 4, the serum testosterone levels of rabbit bucks fed control diet (3.25 I.U/L), diet 2 (3.20 I.U/L), diet 3 (2.60 I.U/L) and diet 4 (2.65 I.U/L) were statistically similar ($p < 0.05$) while bucks on diet 5 had the lowest serum testosterone value of 2.20 I.U/L. Thyroid Stimulating Hormone (TSH) levels of rabbit bucks on control diet (2.30 I.U/L), T_2 (1.15 I.U/L) and T_3 (1.05 I.U/L) were statistically similar ($p < 0.05$) while TSH values in T_4 (0.85 I.U/L) and T_5 (0.75 I.U/L) were significantly lower ($p < 0.05$) than the control.

Dietary Treatments	T_1	T_2	T_3	T_4	T_5	
Parameters	**Control**	**100 ppm**	**200 ppm**	**300 ppm**	**400 ppm**	**SEM**
Total Protein (g/dl)	9.22	9.27	8.79	8.47	7.43	0.18
Albumin (g/dl)	3.74^{a}	3.60^{ab}	3.22^{b}	3.11^{b}	2.42^{c}	0.08
Globulin (g/dl)	5.48	5.67	5.57	5.36	5.01	0.20
Creatinine (mg/dl)	1.00^{b}	1.07^{b}	1.07^{b}	1.23^{ab}	1.57^{a}	0.01
Urea (mg/dl)	20.26	19.97	22.80	20.28	21.62	0.17
AST (I.U/L)	42.51^{c}	53.61^{b}	62.66^{abc}	78.64^{ab}	85.54^{a}	2.63
ALT (I.U/L)	30.99	35.31	40.30	41.15	42.47	1.32
ALP (I.U/L)	119.96a	115.61ab	114.78ab	107.59b	96.04c	2.23
CHOL (mg/dl)	103.00	104.34	104.85	105.27	105.34	1.17
HDL (mg/dl)	81.09	94.75	78.69	82.32	64.96	1.53
LDL (mg/dl)	30.18	36.23	33.11	30.72	27.63	0.41

Table 3: *Serum biochemical indices of rabbit bucks fed varied levels of dietary DEHP.*
a,b,c, means on the same row with different superscripts are statistically different ($p < 0.05$).
SEM: Standard Error of Means; AST: Aspartate Amino Transferase; ALT: Alanine Amino Transferase; ALP: Alkaline Phosphatase; CHOL: Cholesterol; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; ppm: Part Per Million (Equivalent of mg/kg).

Data on hormone production of rabbit bucks fed varied levels of DEHP are presented in table 4. The DEHP failed to elicit any significant changes in the LH and FSH of bucks. However, significant variations ($p < 0.05$) were obtained in the testosterone and thyroid stimulating hormone levels.

Dietary Treatments	T_1	T_2	T_3	T_4	T_5	
Hormone Level (I.U/L)	**Control**	**100 ppm**	**200 ppm**	**300 ppm**	**400 ppm**	**SEM**
LH	11.00	12.00	12.50	9.50	9.50	0.11
FSH	8.50	9.50	9.50	7.50	7.00	0.09
Testosterone	3.25^{a}	3.20^{a}	2.60^{ab}	2.65^{ab}	2.20^{b}	0.06
TSH	2.30^{a}	1.15^{ab}	1.05^{ab}	0.85^{b}	0.75^{b}	0.01

Table 4: *Serum hormones of rabbit bucks fed varied levels of dietary DEHP.*
a,b,c, means on the same row with different superscripts are statistically different ($p < 0.05$).
SEM: Standard Error of Means; LH: Luteinizing Hormone; FSH: Follicle Stimulating Hormone; TSH: Thyroid Stimulating Hormone; ppm: Part Per Million (Equivalent of mg/kg).

Discussion

The haematological parameters of the experimental bucks suggest that their health status was compromised by incorporation of DEHP. The data obtained shows that DEHP caused a significant decrease in the red blood cells, white blood cells and platelets of the bucks. Similar observation was reported by Karabulut and Barlas [17] and George., *et al*. [10] and this reduction in the RBCs of rabbit bucks exposed to DEHP most likely depicts the physiological execution of haemopoietic system, which is considered as the most susceptible marker with respect to environmental contaminants. Erythrocyte count reduction produced erythropenia which could be ascribed to an amplified instability leading to reduction in the existence of the erythrocytes [10].

The decrease in white blood cells can be attributed to reasons reported in the case of other plasticizers. Plasticizers like Bisphenol A (BPA) have been shown to inhibit lymphocyte mitogenesis and macrophage adhesion [10]. Lymphopenia and granulocytosis have been observed after exposure to numerous toxins [18]. These changes in differential white blood cell count also give evidence for decreased level of nonspecific immunity in rabbits after exposure to toxic substances like DEHP [18].

Platelet as one of the main components in the hematological system plays an irreplaceable role in coagulation function. The platelet counts of rabbit bucks treated with varied levels of DEHP significantly reduced compared with the control. This result is similar to the findings of Pugh., *et al* [12]. Decrease in the level of platelets circulating in the blood may result in blood coagulation problem leading to serious haemorrhage [19]. It therefore implies rabbits fed DEHP contaminated diets could be said to be prone to haemorrhage, although physical sign of this condition was not observed in the bucks.

The significant decrease in albumin level is consistent with the findings of Walseth., *et al.* [20] for rats after treatment with DBP. Also, Crocker., *et al.* [21], Pugh., *et al.* [12] and Kobroob., *et al*. [22] reported a significant decrease in kidney function as evidenced by increased serum urea and creatinine and decreased creatinine clearance. These changes were associated with increased lipid peroxidation and decreased antioxidant glutathione and superoxide dismutase [22].

Significant increase in liver enzyme aspartate aminotransferase (AST) is similar to those reported by Pereira., *et al*. [11] and Milošević., *et al* [23]. ALT and AST activities were analyzed as a biochemical marker which may refer to liver damage. Serum aminotransferases are sensitive markers of hepatocellular injury and increased levels of AST and ALT enzymes in the serum allows for the detection of acute and chronic liver injury before the onset of symptoms [24,25]. Thus, it is suspected that administration of DEHP to the experimental rabbit bucks might have caused liver damage.

Furthermore, alkaline phosphatase (ALP) is also an enzyme found in many tissues all through the body, although, the highest elevated levels of ALP are found in the cells of bone and the liver. ALP is a membrane-bound enzyme, which plays an essential role in osteoid mineralization through increased hydrolysis of pyrophosphate, the inhibitor of mineralization [26]. The result from this study shows a significant decrease in ALP levels compared with the control. The result is in agreement with the findings of Bhat., *et al*. [26] who reported that there was a drastic decrease in the ALP level of neonatal rats exposed to varied levels of DEHP. They observed impaired osteoblast differentiation and concluded that DEHP can decrease bone formation by decreasing collagen and ALP expression.

Although not physically expressed, it could be implied that bucks exposed to varied levels of DEHP in this study could have suffered depressed bone formation as a result of the significant decrease in serum ALP levels which could have been brought about by inhibited mineralization occasioned by exposure to DEHP. The result however contradicts the findings of Kwack., *et al.* [27] who reported a significant increase in the serum ALP values of rats exposed to 500 mg/kg/day of phthalate esters when compared with those in the control.

Among the serum reproductive hormones investigated, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were not statistically different for all the treatments while significant decrease was observed in the testosterone concentration across the treatments. The observed decrease in the concentration of testosterone agrees with those reported previously [28-30].

Phthalate exposure affects the male reproductive system in multiple ways including reduced anogenital distance, cryptorchidism, hypospadias, testosterone suppression, impaired sperm production and testicular cancer [31,32]. Both high-dose [33] and low-dose [34] exposure to DEHP reduce circulating testosterone concentrations in adult animal testes. DEHP exposure is associated with Leydig cell tumor induction in the rat [17].

The Leydig cell's primary function is the production of testosterone, and that production is stimulated by luteinizing hormone (LH). Estradiol is synthesized from testosterone and provides negative feedback on the production of LH, as does testosterone. Hence, decreased levels of testosterone or estrogen can stimulate LH production and stimulate Leydig cell tumor induction [17]. As long as Leydig cells tumor is produced, testosterone level will reduce since it is produced by the Leydig cells. Testosterone, estradiol, and LH are regulated through the hypothalamic-pituitary-testis (HPT) axis. This suggests that DEHP caused a disruption in HPT axis. The decrease in testosterone concentration explains the decrease in daily sperm production of the experimental rabbits [35] since testosterone is a steroid hormone known to be critically involved in the initiation and maintenance of spermatogenesis [36].

Thyroid stimulating hormone (TSH) level also decreased as DEHP inclusion levels increased. The result agrees with the findings of Liu., *et al*. [37] and Sekiguchi., *et al*. [38] for rats. Phthalates can interfere with thyroid function, such as the central regulatory system in the hypothalamus and pituitary, the secretion and transport of thyroid hormone (TH) in the thyroid, and the metabolism of TH [39,40]. The DEHP can also disturb TH signaling by reducing circulating TH levels in serum [41]. Ye., *et al*. [42] also reported that after DEHP treatment of rats there was development of oxidative stress, as characterized by reactive oxidation species production in thyrocytes.

Conclusion

This study has shown that feeding diets contaminated with DEHP as high as 400 ppm will compromise the health status of rabbit bucks as revealed by the decline in the RBC, WBC platelets and ALP as well as increases in creatinine and AST. The decrease in testosterone level could also limit their growth and reproductive potentials.

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