

Response of *Rattus norvegicus* to Bitumen Leachate Toxicity

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Abstract

This study investigated the response of *Rattus norvegicus* to bitumen leachate to evaluate its toxicity in a terrestrial animal model following previous aquatic studies on the environmental impacts of Nigerian bitumen exploration. Adult rats were administered different concentrations (20 to 100%) of bitumen leachate for 30 days before analyses. Fourteen blood plasma clinical-chemical parameters (BCCPs), seven hematological parameters as well as histological changes in organs of exposed animals were studied. The analyses showed that all values for the BCCPs and the hematological parameters are significantly different ($P < 0.05$) from the control values. Concentrations of liver enzymes, Alanine aminotransferase (ALT), Gamma glutamyltransferase (GGT) and Alkaline phosphatase (ALP) increased with increasing concentrations of bitumen leachate but were not dose-dependent. In the same vein, counts for Packed cell volume (PCV), White blood cell (WBC), Red blood cell (RBC) and hemoglobin (Hb) all decreased with increasing concentration of toxicants but was not so for differential counts (Neutrophils, Lymphocytes and Eosinophils). Results of histological study revealed several changes ranging from mild to severe lesions in organs of exposed rats. The very pronounced changes include irregularly arranged cardiac muscle fibres (Heart), pronounced inflammation (Spleen), hyperchromic nuclei and degenerated flattened squamous epithelial cells lining the Bowman's capsule (Kidney), pronounced reduction of Graafian follicle (Ovary) and cellular hypertrophy with severe congestion of the central vein (Liver). Based on these results, important organ functions could be negatively affected by continuous exposure to bitumen leachate which reflects health effects having an overall impact on both animal and human populations.

Keywords: Biochemistry; Bitumen; Environment; Hematology; Histology; Rat

Introduction

Toxic/harmful substances may be introduced deliberately or accidentally into the environment, impairing its quality and making it unsuitable for life forms. When the concentration of toxicants exceeds the homeostasis of the organisms, it can lead to death or organ damage [1,2]. Few of the well-known pollutants are herbicides, pesticides, industrial compounds/wastes, etc. [3]. Persistent organic pollutants (POPs) have been widely reported to induce environmental stress due to their inefficient biochemical and transport properties [4,5]. This makes them to be retained within the body of organisms where they biomagnify in food webs especially found in top dwellers. Such induced stress could be the precursor of various health defects, such as neuro-endocrine disruption, immune suppression and tissue/organ disruption in animals [4-8].

Blood plasma clinical-chemical parameters (BCCPs) are known to be qualitative biochemical indicators of health disorders such as organ dysfunctions, bone diseases, metabolic/hormonal imbalances, etc. [9]. Series of factors are known to influence BCCPs including infectious diseases, genetic aberrations, starvation, dehydration and pollution [10-12] and they are therefore being used as biomarkers for pollution studies involving different animal models [10-14]. It has equally been enormously reported that biological markers like hematological and biochemical indices are useful tools for monitoring environmental quality and the health conditions of organisms. In fact, previous researches [15-22] have reported that Hematological and Biochemical indices such as hemoglobin (Hb), hematocrit (Ht), red blood cell (RBC) and white blood cell (WBC) counts, glucose, protein, and enzymes activities are currently used as indicators of the general wellbeing and early signals of stress in organisms.

Blood often shows pathological changes before the morphological symptoms usually seen in animal which are exposed to toxicants [23]. Over the decades, many researches have documented changes in blood indices caused by exposures to environmental pollutants or other

unfavorable conditions [24-29]. In the same vein, the activities of serum/plasma enzymes have severally been used as sensitive markers of stress in animals exposed to different pollutants and these are commonly used as pointers of tissue and cell disruption [20,21].

Bitumen was first spotted in Nigeria in 1910 after which two bitumen observatory wells were dug in the Ondo State in the 60s during the early explorative activity of Nigerian natural bitumen. Currently, a large deposit of natural bitumen occurs in the so called bitumen belt of South-western Nigeria. The seepage of the bitumen material exists especially during the dry season when temperature is above 37°C during when it occurs as a free flowing liquid into the environment (aquatic and terrestrial) and serving as the major environmental pollutants in the communities neighboring the bitumen exploration. Previous researches [30,31] have identified bitumen as the major pollutant/toxicant to aquatic life forms in the neighborhoods hence, the need to validate its toxicity level in terrestrial animal in order to evaluate its possible public health effects on humans.

Materials and Methods

All chemicals, reagents and solvents used in the present study were analytical or high-pressure liquid chromatography (HPLC) Grade.

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Received June 13, 2017; **Accepted** June 22, 2017; **Published** June 30, 2017

Citation: Ayandiran TA, Fawole OO, Dahunsi SO, Ogundiran MA (2017) Response of *Rattus norvegicus* to Bitumen Leachate Toxicity. Biochem Physiol 6: 221. doi: 10.4172/2168-9652.1000221

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Bitumen stock used was obtained from the bitumen observatory well in Agbabu, Ondo State, Nigeria where the bitumen flows out continuously thereby polluting the environment.

Experimental animal

Adult male and female *Rattus norvegicus* var. *albinus*, weighing 200-250 g with specific pathogen-free certified status were procured from the animal center, Physiology Department (Faculty of Basic Medical Sciences), Ladoke Akintola University of Technology, Nigeria. Rats were maintained on a 12 h light/dark cycle and at a temperature of $23 \pm 2^\circ\text{C}$ with unlimited access to food and water and these conditions persist throughout the experimental period. All experimental animals were given humane treatment according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences, USA and experiments were carried out in accordance to Ladoke Akintola University of Technology Ethical Committee Acts.

Experimental design

Rats were randomly divided into six groups: A=Control group (Distilled water only); B=20% bitumen leachate treated group; C=40% bitumen leachate treated group; D=60% bitumen leachate treated group; E= 0% bitumen leachate treated group and F=100% bitumen leachate treated group. The toxicants (each bitumen leachate group) were previously prepared by dilution of the bitumen stock with distilled water in appropriate proportions and with serious agitation (for dissolution of soluble portions) for a period of 4 weeks before commencement of administration. Each stock solution was then refrigerated under hygienic condition prior to administration. Each experimental group consisted of six rats (3 each of male and female). 100 mg/kg/day of each bitumen leachate stock composition were administered orally once daily in each group for 30 days. All experimental animals were sacrificed 24 h after the final administration and blood samples were obtained from the femoral arteries by intracardiac puncture.

Plasma biochemical parameters evaluation

The biochemical analyses were carried out using the methods of Sonne et al. [10-12,32]. Indices analyzed include the following: Albumin (Alb, gL^{-1}); Glucose (Glu, mmolL^{-1}); Total protein (TP, gL^{-1}); Alkaline phosphatase (ALKP, UL^{-1}); Alanine aminotransferase (ALAT, UL^{-1}); Gamma glutamyltransferase (GGT, UL^{-1}); Total bilirubin (TB, mmolL^{-1}); Total cholesterol (TC, mmolL^{-1}); Bile acids (BA, mmolL^{-1}); Amylase (Amy, UL^{-1}); Urea (Urea, mmolL^{-1}); Calcium (Ca, mmolL^{-1}); Sodium (Na, mmolL^{-1}) and Potassium (K, mmolL^{-1}). All analyses were routinely conducted in the laboratory using an automated spectrophotometric analyzer also containing ion-selective electrodes (ADVIA1800, Siemens) and assays were subjected to daily internal and quarterly external quality control.

Evaluation of hematological parameters

Hematological values were measured following standard methods [33,34]. Packed cell volume (PCV) was evaluated using hematocrit method and hemoglobin (Hb) concentration using cyanmethaemoglobin method) was analyzed within two hours after collection. Red blood cells (RBC) and White blood cells (WBC) were counted by Neubauer's improved hemacytometer using Hyem's and Turk's solution as a diluting fluid respectively. Differential white cell and thrombocyte counts were done on blood films stained with Giemsa. For every 1,200 erythrocytes counted at random, the number

of thrombocytes and the different types of leucocytes was determined on each blood smear and a mean relative percent was calculated. The absolute value was then obtained and was then multiplied by the WBC+thrombocytes from the hemocytometer. Thrombocyte numbers were subtracted from the WBC+thrombocytes count to obtain a total WBC [26]. Replicate counts were made for each blood sample.

Histological analyses

At the end of the toxicant administration, organs (liver, kidney, spleen and heart from both animal sexes and ovaries from female animals) were obtained. Each organ was dissected out, trimmed of excess fat and then fixed in 10% buffered formalin and was processed for paraffin sectioning by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks. Sections of about 5 m thickness were stained with Harris hematoxylin and eosin (H&E) for histological study following the methods of Delafield [35] and Bancroft and Gamble [36].

Statistical analysis

All the results were subjected to analysis of variance (ANOVA). Duncan multiple range test was further used to evaluate the mean differences at 0.05 significant levels.

Results

Blood plasma clinical-chemical parameters (BCCPs)

Fourteen (14) blood plasma clinical-chemical parameters were evaluated in all treatment groups of rats exposed to bitumen leachate plus the control (Table 1). These comprised of three liver enzymes (ALAT, ALKP and GGT), one digestive enzyme (amylase), two protein groups (albumin and total protein), two liver/erythrocyte metabolism products (bile acid and bilirubin), cholesterol, a carbohydrates (glucose), urea (muscle and protein metabolism) and three electrolytes/minerals (calcium, sodium and potassium). Values obtained from each treatment groups significantly different from the control. In all, values for albumin, total bilirubin, potassium and glucose followed the same pattern of increase with increasing concentration of toxicant while amylase recorded a low value in the 100% bitumen leachate treated group. With regard to ALAT, ALKP, GGT, total protein, bile acids, cholesterol, urea, sodium and calcium, significantly low values were obtained in some of the treatment groups compared to the others.

Hematological parameters

Also, seven (7) different hematological parameters were measured in all the treatment groups plus the control (Table 2). Indices evaluated include four blood parameters (PCV, WBC, RBC and Hb counts) and three differential blood counts (Eosinophil, Neutrophils and Lymphocytes). All values obtained for the treated groups are significantly different from the control. Values obtained for PCV, Hb and Eosinophil count are all concentration dependent, i.e., values increase with increase in toxicity while values for WBC, RBC, Neutrophils and Lymphocytes counts were significantly low in some of the treated groups.

Histological analysis

Histological changes in tissues of different organs (heart, spleen, kidney, ovary and liver) from all treated groups and the control are incident and rarely severe as shown in Figures 1 to 5. All histological findings were normal in the organs from control group. Histology of the heart showed normal muscle fibres in the control group, while changes seen in the treated groups are mild and pronounced morphological

Parameter	Control	20%	40%	60%	80%	100%	Clinical implications (Sonne et al., 2013)
Alanine aminotransferase (ALAT; UL ⁻¹)	59 ± 3.2	65 ± 4.10*	64 ± 5.30*	73 ± 5.31*	100 ± 10.20*	115 ± 12.11*	Increase during liver disease
Gamma glutamyltransferase (GGT; UL ⁻¹)	0.5 ± 0.1	0.6 ± 0.01*	0.5 ± 0.01	1.2 ± 0.01*	1.4 ± 0.11*	1.5 ± 0.20*	Increase during liver disease
Alkaline phosphatase (ALKP;UL ⁻¹)	120 ± 10.21	117 ± 9.10*	131 ± 12.00*	140 ± 12.00*	156 ± 14.10*	148 ± 13.20*	Increase during liver and bone diseases
Total protein	7.6 ± 0.4	7.8 ± 1.00*	7.9 ± 1.00*	7.7 ± 0.20*	8.6 ± 0.20*	9.1 ± 1.30*	Increase during liver disease
Amylase	309 ± 17.1	407 ± 21.30*	421 ± 18.00*	500 ± 30.12*	540 ± 31.30*	530 ± 25.10*	Increase during liver pancreatitis
Albumin	4.6 ± 0.2	4.7 ± 0.21*	4.9 ± 0.10*	4.9 ± 0.10*	5.6 ± 0.40*	8.2 ± 1.00*	Increase during liver disease and dehydration
Total cholesterol	77 ± 4.1	86 ± 5.30*	82 ± 7.20*	92 ± 10.01*	93 ± 9.10*	89 ± 5.21*	Increase during liver disease
Urea	40 ± 2.3	41 ± 2.30*	47 ± 3.30*	64 ± 5.30*	57 ± 6.10*	64 ± 5.00*	Increase during dehydration and kidney disease
Total bilirubin	17 ± 1.4	19 ± 1.11*	22 ± 2.10*	23 ± 3.00*	23 ± 3.00*	25 ± 2.20*	Increase during liver disease
Bile acids	89 ± 5.3	93 ± 5.10*	100 ± 10.10*	95 ± 8.30*	103 ± 10.10*	97 ± 4.22*	Increase during liver disease
Sodium	111 ± 7.22	116 ± 8.60*	117 ± 11.20*	123 ± 13.10*	120 ± 9.11*	124 ± 6.10*	Increase during dehydration
Potassium	6.7 ± 1.5	9.1 ± 1.21*	10.2 ± 1.31*	12.3 ± 1.80*	12.8 ± 1.41*	13.0 ± 1.20*	Increase during renal disease
Calcium	6.2 ± 0.2	6.3 ± 0.20*	6.8 ± 0.40*	8.1 ± 0.30*	7.7 ± 0.50*	7.6 ± 0.60*	Elevated levels largely linked to albumin levels and bone disease
Glucose	143.8 ± 11.2	124 ± 10.20*	101 ± 10.11*	82.6 ± 6.10*	36 ± 3.30*	27 ± 2.50*	Increase during stress, digestion and diabetes mellitus

Table 1: Statistical summary of 14 blood plasma clinical–biochemical parameters of *Rattus norvegicus* exposed to bitumen leachate.

Parameter	Control	20%	40%	60%	80%	100%
PCV (%)	75 ± 5.22	74 ± 4.20*	63 ± 3.02*	58 ± 2.12*	49 ± 2.04*	46 ± 2.02*
WBC (mm ³ of blood)	5000 ± 10.02	7800 ± 9.21*	13600 ± 10.22*	11000 ± 12.02*	17600 ± 10.01*	24000 ± 13.22*
RBC (millions/ml)	6550000 ± 15.20	6200000 ± 20.12*	5050000 ± 19.02*	5300000 ± 31.02*	4950000 ± 17.40*	4500000 ± 23.02*
Hb (g/dl)	23 ± 0.02	23 ± 1.03*	22.6 ± 1.01*	17.7 ± 1.20*	16.1 ± 1.22*	14.8 ± 1.20*
Neutrophils (%)	08 ± 1.01	16 ± 1.02*	18 ± 1.02*	28 ± 1.01*	42 ± 3.02*	41 ± 2.03*
Lymphocytes (%)	54 ± 2.12	58 ± 3.32*	63 ± 2.02*	84 ± 3.02*	77 ± 4.02*	82 ± 4.02*
Eosinophils (%)	01 ± 0.01	01 ± 0.01	02 ± 0.01*	04 ± 0.02*	03 ± 0.02*	03 ± 0.02*

Each value is an average of three (3) measurement; **statistically significant

Table 2: Statistical summary of 7 haematological parameters of *Rattus norvegicus* exposed to bitumen leachate.

distortion, narrowing of the interfibre space and flattened nuclei and irregularly arranged cardiac muscle fibres. Histology of the spleen in all treated animal groups reveal mild congestion and pronounced inflammatory changes. In the kidney, histological changes seen after the exposure to different concentrations of bitumen leachate includes wide Bowman's space, dilated convoluted tubules and gradual loss of flattened squamous epithelial cells lining the Bowman's capsule, hyperchronic nuclei and degenerated flattened squamous epithelial cells lining the Bowman's capsule. In the ovary, histological changes in the treated groups includes slight reduction in size of Graffian follicle, slight distortion of cellular appearance with further reduction of Graffian follicle, pronounced cellular distortion and reduced Graffian follicles. In the liver tissues, the histological changes seen are mild congestion of the central vein, dilated sinusoids, cellular hypertrophy and severe congestion of the central vein.

Discussion

Evaluation of BCCPs is a common practice in the clinical diagnosis of animal's physiology and this gives an indication of the general health status [18,37]. The significant decrease in blood glucose during exposure can be attributed to high utilization of glucose for oxidation or hypoxic conditions leading to an excess utilization of stored carbohydrates which are the main source of energy in organisms. Alterations of enzyme activity, e.g. the transaminase enzymes in animals under exposed conditions have been documented as a strong biochemical parameter most useful in diagnosis [38,39]. The transaminase enzymes in this study were found to increase significantly as the concentration

of toxicant increased and when this is found in blood plasma; it can be linked to organ dysfunction in organisms during stress condition as earlier reported by Gabriel and George [40]. Several other researchers have reported elevated levels of plasma enzymes in animals exposed to different toxicants [39,41,42]. All the fourteen BCCPs were significantly different between the various treatment groups. Such differences could be linked to the variation in toxicant concentration which they were exposed to and may not be dietary since all animals were fed the same diet all through the experimental period. Another important factor to note here is the individual physiology of animals which usually shows different response to toxicants. Most of the indices evaluated in the experimental groups recorded increase values as toxicant concentration increased, indicating that the toxicant load is a major factor in the resultant responses (damages and lesions) found in the animal. This is supported by many studies who have reported on several organochlorines inducing toxicity which was evident in the increased values for blood plasma parameters especially those of ALAT, GGT, bile acid, total bilirubin, albumin, total protein and cholesterol [14,43].

Different substances have been found to induce various toxicological responses in animals in which blood parameters followed a regular pattern of increase with concentration of toxicant or variation in location of animal samples but with significantly reduced values at some points during the exposure [9-13,44]. For the electrolytes calcium, potassium and sodium, the relationships may be dietary and/or bitumen related, which also have a strong linkage to renal disorders or bone metabolism [9,45,46].

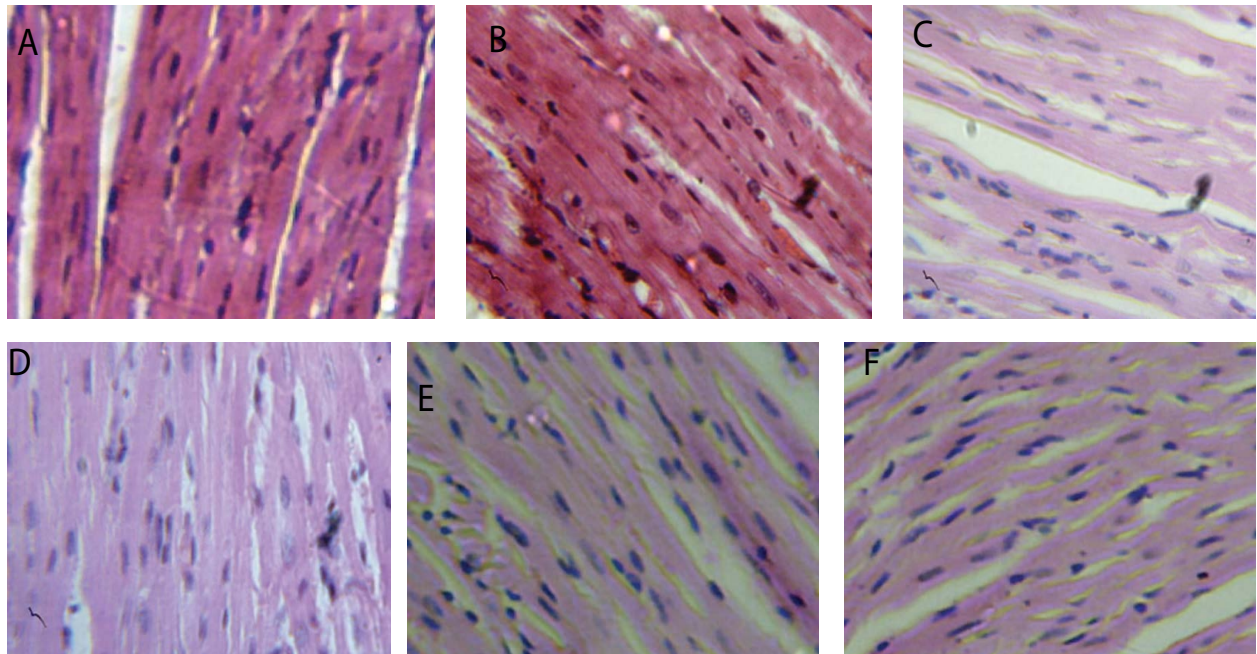


Figure 1a: A photomicrograph of the heart showing normal histology of the muscle fibres (Group A). H & E Stain, X400.
b: A photomicrograph of the heart showing normal appearance of the cardiac muscle fibres of the heart. (Group B). H & E Stain, X400.
c: A photomicrograph of the heart showing mild morphological distortion (Group C). H & E Stain, X400.
d: A photomicrograph of the heart showing further morphological distortion with the narrowing of the interfibre space and flattened nuclei. (Group D). H & E Stain, X400.
e: A photomicrograph of the heart showing pronounced morphological distortion with the narrowing of the interfibre space, flattened nuclei and irregularly arranged cardiac muscle fibres. (Group E). H & E Stain, X400.
f: A photomicrograph of the heart showing pronounced morphological distortion with the narrowing of the interfibre space, flattened nuclei and irregularly arranged cardiac muscle fibres. (Group F). H & E Stain, X400.

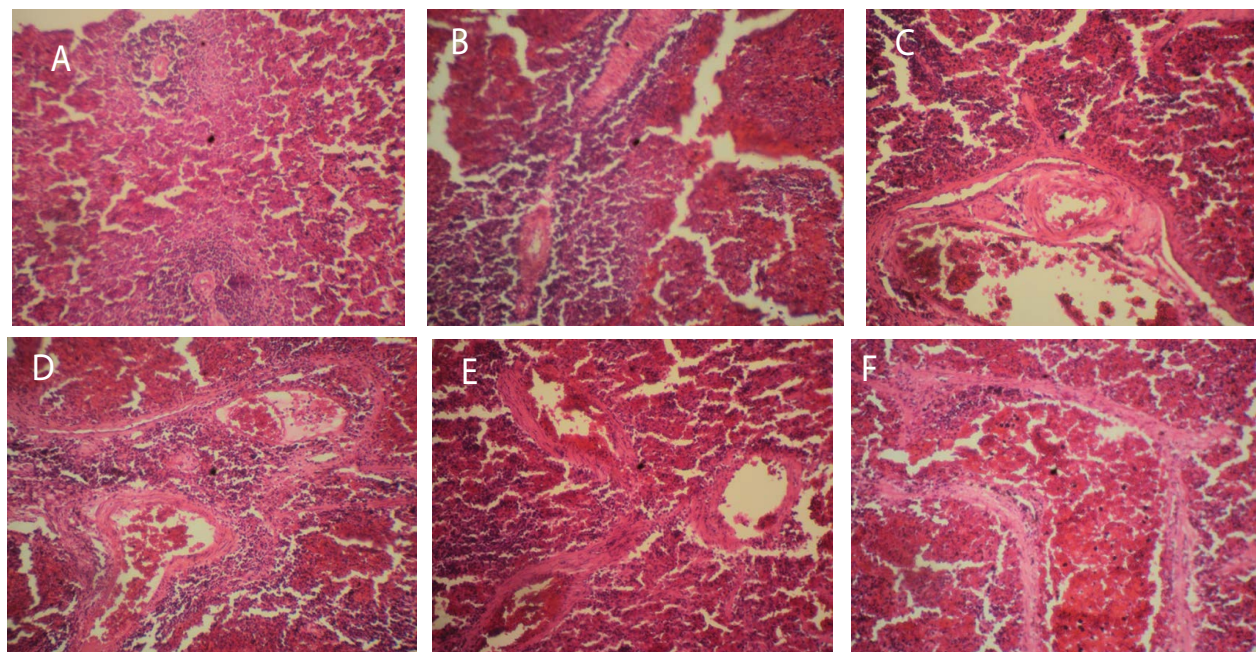


Figure 2a: A photomicrograph of the spleen showing normal morphological appearance (Group A). H & E Stain, X400.
b: A photomicrograph of the spleen showing normal morphological appearance (Group B). H & E Stain, X400.
c: A photomicrograph of the spleen showing mild congestion (Group C). H & E Stain, X400.
d: A photomicrograph of the spleen showing mild areas of inflammatory changes (Group D). H & E Stain, X400.
e: A photomicrograph of the spleen showing mild areas of inflammatory changes (Group E). H & E Stain, X400.
f: A photomicrograph of the spleen showing pronounced areas of inflammatory changes (Group F). H & E Stain, X400.

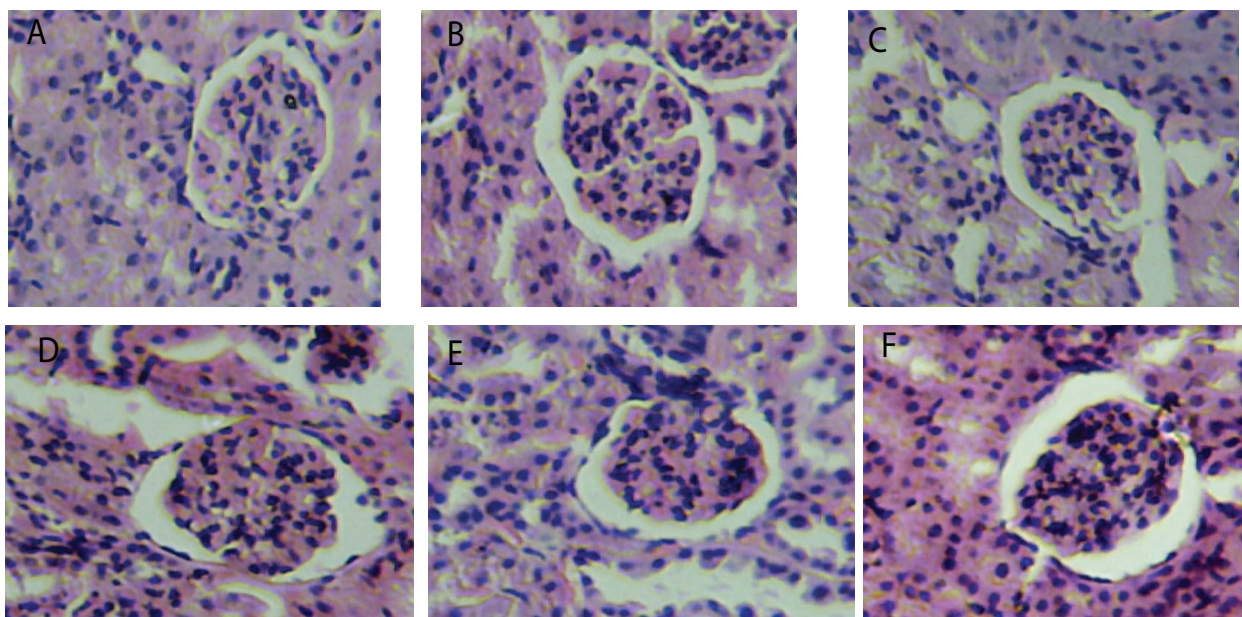


Figure 3a: A photomicrograph of the kidney showing normal morphological appearance (Group A). H & E Stain, X400.
b: A photomicrograph of the kidney showing normal morphological appearance but with a wide Bowman's space (Group B). H & E stain, (X400).
c: A photomicrograph of the kidney showing a wide Bowman's space, dilated convoluted tubules and gradual loss of flattened squamous epithelial cells lining the Bowman's capsule (Group C). H & E stain, (X400).
d: A photomicrograph of the kidney showing a wide Bowman's space, hyperchromic nuclei and gradual loss of flattened squamous epithelial cells lining the Bowman's capsule (Group D). H & E stain, (X400).
e: A photomicrograph of the kidney showing a wide Bowman's space and degenerated flattened squamous epithelial cells lining the Bowman's capsule (Group E). H & E stain, (X400).
f: A photomicrograph of the kidney showing a wide Bowman's space, hyperchromatic nuclei and degenerated flattened squamous epithelial cells lining the Bowman's capsule (Group F). H & E stain, (X400).

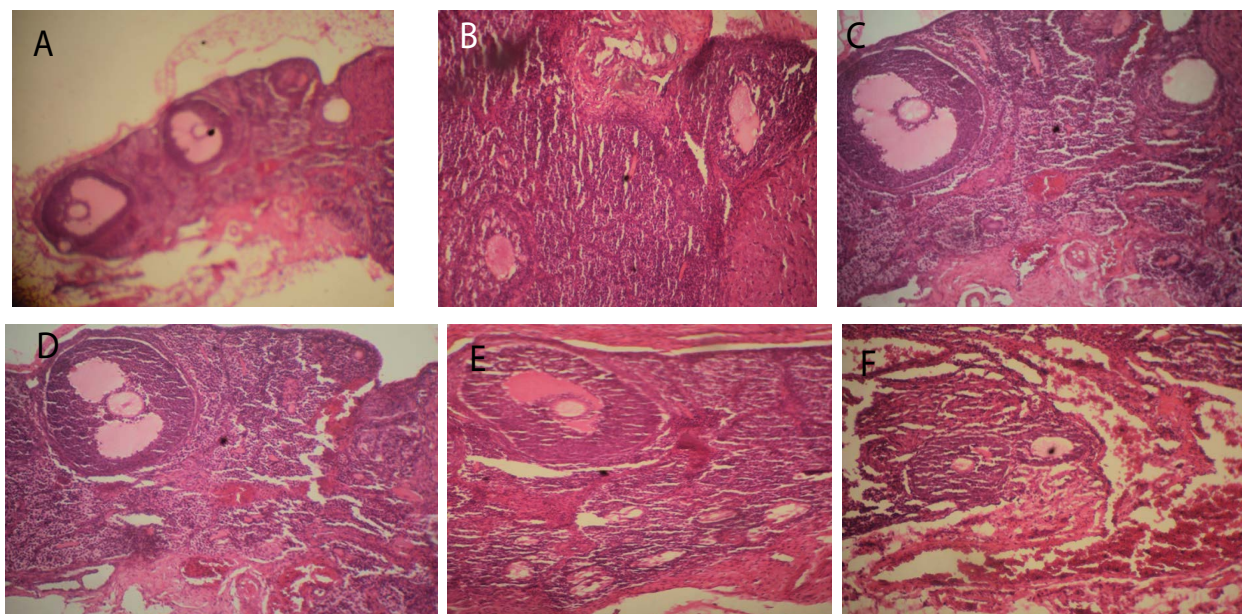


Figure 4a: A photomicrograph of the ovary showing normal well developed Graafian follicles (Group A). H & E stain, (X400).
b: A photomicrograph of the ovary showing normal well developed Graafian follicles (Group B). H & E stain, (X400).
c: A photomicrograph of the ovary showing normal cellular architecture but with a slight reduction in size of Graafian follicle (Group C). H & E stain, (X400).
d: A photomicrograph of the ovary showing slight distortion of cellular appearance with further reduction of Graafian follicle (Group D). H & E stain, (X400).
e: A photomicrograph of the ovary showing pronounced cellular distortion and reduced Graafian follicle (Group E). H & E stain, (X400).
f: A photomicrograph of the ovary showing pronounced cellular distortion and reduction of Graafian follicle (Group F). H & E stain, (X400).

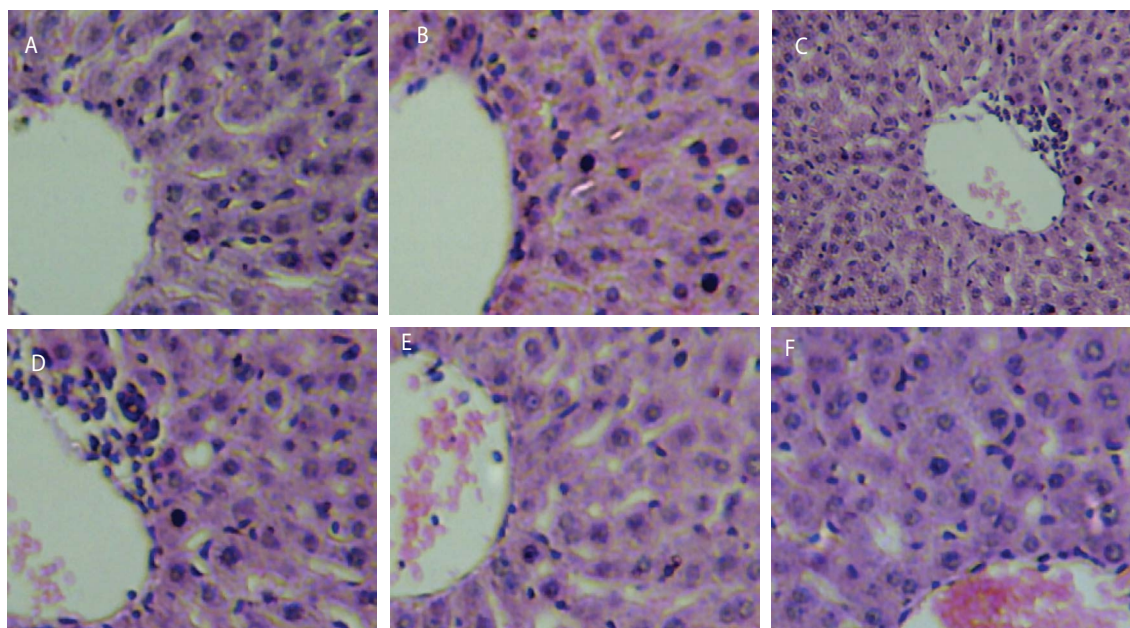


Figure 5a: A photomicrograph of the liver showing normal morphological appearance (Group A). H & E stain, (X400).
b: A photomicrograph of the liver showing normal morphological appearance (Group B). H & E stain, (X400).
c: A photomicrograph of the liver showing normal morphological appearance with mild congestion of the central vein (Group C). H & E stain, (X400).
d: A photomicrograph of the liver showing dilated sinusoids, cellular hypertrophy with mild congestion of the central vein (Group D). H & E stain, (X400).
e: A photomicrograph of the liver showing dilated sinusoids, cellular hypertrophy with mild congestion of the central vein (Group E). H & E stain (X400).
f: A photomicrograph of the liver showing dilated sinusoids, cellular hypertrophy with severe congestion of the central vein (Group F). H & E stain (X400).

In this study, the decreased level of RBC, hemoglobin and PCV counts in rats treated with bitumen leachate might have resulted from hemolysis caused by this toxicant. Previous studies, [47,48] have reported similar observations in experimental animals exposed to different toxicants while other reported a decrease in the same parameters on exposure to other toxicants [49]. The observed increase in WBC during treatment may be due to stimulated lymphopoiesis and/or enhanced release of lymphocytes from lymphomyeloid tissue as a defense mechanism of the rats to withstand the toxic effect of the bitumen leachate and this agrees with the submission of Kavitha et al. [20]. The increase in leucocyte count indicates the stimulatory effect of the toxicant on immune system and also depends on the toxicant stress and this is confirmatory to the findings of Ates et al. [50].

Several histological responses were shown in organs of exposed animal specimens with the most prevalent one being pronounced distortion of cardiac muscle, degeneration of epithelial lining of kidneys Bowman capsule, congestion of blood vessels, and pronounced cellular swelling of ovarian Graffian follicle. All these could be associated to the response of organs to bitumen leachate toxicity. Previous studies [51,52] have reported similar submissions. Cellular swelling is known to occur either directly by denaturation of volume-regulating ATPases or indirectly by disruption of the cellular energy transfer pathway required for ionic regulation [51]. The present study showed that induced morphological damages are associated with increased oxidative stress and apoptosis in tissues. It has been proved that chronic exposure to toxicants inhibits cholinesterase enzyme and introduces oxidative stress damage as well as free radicals production in different organs such as cardiovascular system [53-58]. Such histological findings also indicated inducement of congestion, infiltration of inflammatory cells and multifocal necrosis in cardiac tissue and there has been evidence that oxidative stress is a major apoptotic stimulus in various diseases

[59]. Several studies also revealed that apoptosis could be a possible mechanism of toxicity in low-dose exposure to some toxicants [60-62].

Conclusion

Judging by the level of blood and histological alterations found in this study, our conclusion is that the indiscriminate release of bitumen into the environment should be discouraged. The exploration activities in the bitumen belt of Nigeria are causing lots of environmental pollution with the continuous discharge of bitumen runoff into rivers and land. Though, the effects are chronic as seen here, the continuous exposure of animals (aquatic and terrestrial) to bitumen leachate could in the long run pose serious health dangers to humans who are at the peak of the food chain. Moreover, fishes from bitumen polluted rivers in Nigeria constitute good sources of food and income for the inhabiting human population and they alongside the water from the river has been implicated to be polluted [31,63].

Conflict of Interest

Authors declare that there has been no conflict of any sort throughout the period of this research till now. Both of us approves of this submission.

Highlights

1. The toxicological effect of bitumen leachate was established.
2. Biochemical analyses showed multiple abnormalities.
3. Hematological analysis also revealed multiple abnormalities.
4. Histological changes were pronounced in exposed rat.
5. Observed aberrations were all concentration dependent.

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Citation: Ayandiran TA, Fawole OO, Dahunsi SO, Ogundiran MA (2017) Response of *Rattus norvegicus* to Bitumen Leachate Toxicity. Biochem Physiol 6: 221. doi: [10.4172/2168-9652.1000221](https://doi.org/10.4172/2168-9652.1000221)

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