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Attenuating Effect of *Polyalthia Longifolia* in Cadmium Sulfate-Induced Testicular Toxicity

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Abstract

Polyalthia longifolia extract has numerous beneficial effects on human health. They have been reported to have antiviral, anti-allergic, anti-inflammatory, anti-tumor and antioxidant activities. Cadmium is used in the production of nickel-cadmium batteries, pigments, ceramics, plastic stabilizers, and fertilizers. Cigarette smoke is one of the most important sources of cadmium exposure in the general non-occupationally exposed population. In nonsmokers, the main source of cadmium is food, particularly cereals such as rice and wheat, green leafy vegetables, potatoes, and offal products such as liver and kidney. Twenty male Wistar rats were divided into four groups. Group A was orally treated with distilled water for twenty-eight days, Group B was intraperitoneally treated with cadmium sulfate for three days and sacrificed twenty-four hours later, Group C received oral administration of *Polyalthia longifolia* for twenty-eight days and Group D received cadmium sulfate intraperitoneally for three days and post-treated orally with *Polyalthia longifolia* for twenty-five days. On the 29th day the animals were sacrificed through cervical dislocation, the right testis was excised and processed histologically, while the left testis was processed for semen analysis. The histological observation showed that *Polyalthia longifolia* administration improved the histoarchitectural distortion of the testis in the cadmium sulphate treated group. *Polyalthia longifolia* administration restored the alteration on the sperm parameters of the damaged testes. The result suggested that *Polyalthia longifolia* restored testicular toxicity induced by cadmium sulfate and may be used as sub fertility drug.

Keywords: Cadmium Sulfate, Testis, Histology, Immunohistochemistry, Semen analysis

Introduction

Infertility is a problem affecting one in six couples (1). Infertility affects approximately 15% of all couples trying to conceive and male factor infertility is implicated in almost half of these cases (2). A recent metaanalysis by Levine et al. identified a 50% decline in sperm counts of Western men over the last 40 years (3), which may be due to exposures to toxins from industrial, agricultural, and by-products of other technological advancements, as spermatogenesis is more sensitive to environmental contaminants when compared to their female counterparts (4). Environmental and occupational exposure to toxic metals has long been suggested to have contributed to at least half of the cases of human male infertility of unknown etiology (5). Cadmium is ranked seventh in the "Top 20 Hazardous Substances Priority List" by the Agency for Toxic Substances and Disease Registry and the U.S. Environmental Protection Agency (6). Principal uses of cadmium include nickel-cadmium batteries, pigments and plastic stabilizers. Major occupational exposures to cadmium occur in nonferrous metal smelters, production and processing of cadmium alloys and compounds and, increasingly, in the recycling of electronics (7). Heavy metal exposures increase the formation of reactive oxygen species, leading to oxidative stress (8), inducing DNA damage, and disrupting the blood-testis barrier causing apoptosis of spermatozoa (9). Cadmium is known as endocrine disruptor and is able to exert reproductive toxicity in males even at a low level of exposure. While it is found in cereals, grains, and green leafy vegetables, cadmium exposure can occur from contact with dyes, ceramics, plastics, fertilizers, and cigarettes (4). Cadmium-induced reproductive toxicity is mediated by multiple mechanisms, including structural damage to the testis vasculature and blood-testis barrier, inflammation, cytotoxicity on Sertoli and Leydig cells, oxidative stress (mainly by means of mimicry and interference with essential ions), apoptosis, interference with selected cell signaling pathways, epigenetic regulation of genes involved in the regulation of reproductive function, and disturbance of the hypothalamic-pituitary-testicular axis (10, 11).

Polyalthia longifolia has been used in traditional medicine for the treatment of fever, skin diseases, diabetes, hypertension, ulcer and helminthiasis (12-15). The plant extract and its various isolated compounds have been studied for biological activities like antibacterial activity, cytotoxicity, antifungal activity, acute toxicity, anti-inflammatory and hepatoprotective activity (16-18). This study is therefore designed to evaluate the effect(s) of oral

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administration of aqueous extract of *Polyalthia longifolia* leaf on testes adversely affected by Cadmium-induced testicular toxicity.

Materials and methods

Animal model and experimental design

Twenty adult male Wistar rats, average weight of 180g were obtained and maintained in the animal house of the College of Health Sciences, University of Ilorin. They were housed in cages with adequate space to encourage free movement. They were housed under natural light and dark cycles (12hr light and 12hr dark) at room temperature and were bred on conventional commercial standard rat pellet diet obtained in piece meal fashions from a commercial rat feed shop and were also supplied with water ad libitum and allowed to acclimatize for two weeks. Interval weighing of animals was done throughout. Strict hygiene was maintained by clearing their wastes twice daily and changing their food and water twice daily. At the commencement of the experiment, the animals were assigned to four groups, each group consisted five (5) animals each as shown in the table 1. Cadmium Sulfate was administered intraperitoneally with an insulin syringe while *Polyalthia longifolia* leaf extract was administered orally to the male rats using oral cannula. All the experimental procedures were done following the experimental guidelines of Institutional Ethical Review Committee of the University of Ilorin, Kwara State.

Table 1. Grouping of the animals with their corresponding treatments, duration of administration and number of animals in each sub-group

Group	Treatment	Dosage	Duration	No of Animals
A	Distilled water	2ml/kg body weight	28 days	5
B	Cadmium Sulfate	0.3mg/kg body weight	3 days	5
C	*Polyalthia longifolia*	500mg/kg body weight	28 days	5
D	Cadmium Sulfate and *Polyalthia longifolia*	0.3mg/kg body weight and 500mg/kg body weight	28 days	5

Cadmium sulphate

Commercially available formulation of cadmium sulfate was purchased from Momrota Pharmacy, Ilorin, Kwara State.

Procurement and preparation of extract

Polyalthia longifolia leaves were procured from Ado-Ekiti, Ekiti state and prepared according to Ukwenyaet al.(19). The Polyalthia longifolia leaves were pulverized into powder with an electrical blending machine. Fifty grams (50g) of the powder was poured into 1000ml of distilled water and allowed to boil for 45 minutes; the mixture was sieved and filtered with Whatmann No.1 filter paper. After sieving the process was repeated three times to get the concentrate. The animals treated with Polyalthia longifolia leaf extract were given 500mg/kg body weight of the extract.

Animal sacrifice and sample collection

Twenty-four hours after 28th day of treatment, the animals were sacrificed by cervical dislocation. Blood samples were collected from the heart into lithium heparinized bottles for hormonal assay. The right testis was excised and quickly placed in Bouin's fluid and processed for light microscopic examination using haematoxylin and eosin (20), Feulgen's reaction and immunohistochemical examination using Ki67 marker.

Histological examination for hematoxylin and eosin stain and feulgen reaction

The excised testes were fixed in Bouin's fluid, dehydrated in ascending grades of alcohol, cleared in xylene, quickly dipped into molten paraffin wax before finally embedding into molten paraffin wax in order to form paraffin block. The paraffin block containing the tissue was then sectioned by a rotary microtome at 4µm thickness. Two sections were taken from the microtome, the first section was stained with Hematoxylin and Eosin stain and the other paraffin section used for DNA demonstration uses the Feulgen reaction. In which sections were placed in normal hydrochloric acid (N-HCl) at room temperature for one minute, then treated with preheated (NHCl) at 60°C for ten minutes, again sections were rinsed in (NHCl) at room temperature for one minute, after that sections transferred to Schiff's reagent for 45 minutes, then rinsed in three changes of bisulphite solution two minutes each, then rinsed in water and counter stained in 1% light green SF for one minute, finally sections were dehydrated in alcohol cleared in xylene and mounted in D.P.X.

Johnsen's score analysis

For densitometry evaluation, Johnsen's score which are known to have good reproducibility was implored. The Johnsen criteria are into a ten-point scoring system for quantifying spermatogenesis according to the profile of the cells encountered along the seminiferous tubules. A Johnsen score of 10 indicates maximum spermatogenesis activity, whereas a score of 1 indicates complete absence of germ cells. To obtain the Johnsen score, slides were examined under an optical microscope (magnification, ×100). A score was assigned for each tubule counted. The number of tubules with a given score was multiplied by the score. The result was summed across different scores and then divided by the number of evaluated tubules, giving the final Johnsen score.

Immunohistochemical examination

The testes fixed in Bouin's fluid were dehydrated in ascending grades of alcohol, cleared in xylene, quickly dipped into molten paraffin wax before finally embedded in molten paraffin wax in order to form paraffin block. The paraffin block containing the tissue was then sectioned by the rotary microtome at 3 µm thickness. The sections were mounted onto microscope slides. The section was taken down to distilled water and the slides were rinsed with buffer. Endogenous peroxidase was then blocked with hydrogen peroxide for 10 minutes and rinsed with buffer for 1 minute and later digested with proteinase K and rinsed with buffer for two minutes. Protein block was added for 10 minutes and protein block was shake off. Anti-Ki-67 was added and rinsed

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with buffer for 2 minutes and later antibody enhancer was added for 10 minutes and rinsed with buffer for one minutes. Polymer was added for 15 minutes and rinsed with buffer for two minutes while DAB was added for ten minutes and rinsed with buffer for two. The tissue was counter-stain with Hematoxylin and rinsed with buffer for two minutes and finally washed in water, dehydrated, cleared and mounted.

Sperm count

The spermatozoa were counted by hemocytometer using the improved Neubauer (Deep 1/10 mm, LABART, Germany) chamber as described by Rouge & Bowen (20).

Sperm morphology and motility analysis

Sperm live/dead ratio and motility were determined using 1% Eosin and 5% Nigrosin in 3% sodium-citrate dehydrate solution according to the method described by Rouge & Bowen (20).

Hormone measuring assay

Blood were collected by cardiac puncture and the serum was centrifuged (4000 rpm at 4°C) and stored at -20°C for further analysis. Serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were determined using ELISA kits (from Monobind Inc. Lake forest, CA, U.S.A) according to the manufacturer's instructions and all samples were tested in triplicate.

Statistical analysis

GraphPad Prism version 7.0 was used for all statistical analyses. All data were expressed as Mean±SEM. Differences among the groups were analyzed by one-way ANOVA while Tukey's correction was used to adjust for multiple comparisons. P value <0.05 was considered to be statistically significant.

Results

Histological and histochemical observations

The control (A) group showed well defined basement membrane with numerous spermatogonia, primary spermatocytes and spermatids. The interstitial cells are also well defined, the lumen is well spaced with numerous spermatozoa present. The cadmium sulfate only (B) group showed degenerated basement membrane, the lumen is clustered with variable cells and spermatozoa cannot be observed. The *Polyalthia longifolia* (C) cadmium sulfate and *Polyalthia longifolia* (D) group showed normal basement membrane, lumen and interstitial cell when compared with cadmium sulfate treated testis (B) (Figure 1).

Histopathological ranking

Table 2 shows the Johnsen's Scoring System of the histology of the testes across the groups. The Control (A) and *Polyalthia longifolia* (C) had high Johnsen's score as compared to the Cadmium sulfate group. The higher the score, the more preserved is the histology of the testes and the integrity of spermatogenesis.

The result of the histopathological ranking in groups A, C and D showed normal spermatogenesis in some seminiferous tubules while group B depicts slight reduction in the number of germ cells present.

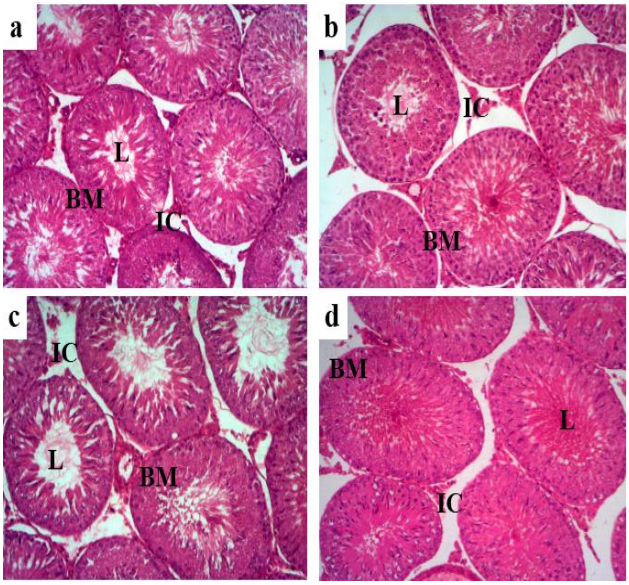

Figure 1: (A-D) Photomicrographs of the testis of adult male Wistar rats treated with distilled water, control (A). Cadmium Sulfate (B), *Polyalthia longifolia* leaf extract (C), and Cadmium sulfate with *Polyalthia longifolia* leaf extract concurrently (D). BM: Basement membrane; IC: Intesrstitial cells; L: Lumen. (H and E x 200).

Table 2. Categorization of Testicular Biopsy According to Johnsen's Scoring System

Groups	A	B	C	D
10	2	-	2	1
9	2	-	2	1
8	2	-	-	2
7	-	1	1	1
6	-	1	1	1
5	-	2	-	-
4	-	1	-	-
3	-	1	-	-
2	-	-	-	-
1	-	-	-	-

Feulgen's reaction observation

For demonstrating DNA content, sections were stained with Feulgen's reaction. The control (A) group showed normal content of DNA of spermatogenic cells and spermatozoa. Group B shows reduction of DNA content in spermatogenic cells and spermatozoa appeared with condensed DNA (pyknotic nuclei) content with the spermatogonia shrunken (sign of apoptosis). Group C and D shows restoration of DNA content.

Immunohistochemical observation

The control (a) group showed a large population of immunopositive cells. In group (b) there was no expression of the marker at all. There was little expression of the marker in group (c) and Group (d).

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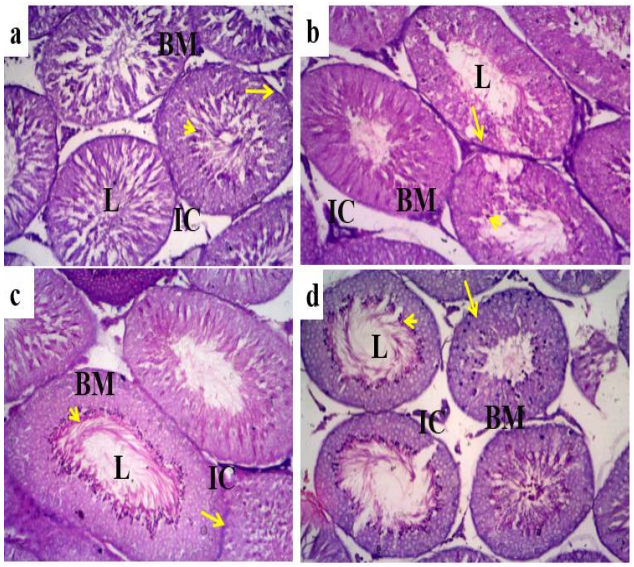

Figure 2: (A-D) Photomicrographs of the testis of adult male Wistar rats treated with distilled water, control (A). Cadmium Sulfate (B), *Polyalthia longifolia* leaf extract (C), and Cadmium sulfate with *Polyalthia longifolia* leaf extract concurrently (D). BM: Basement membrane; IC: Interstitial cells; L: Lumen. (H and E x 200). (Feulgen reaction x 200). Long arrow: Spermatogenic cells; Shoer arrow: Spermatozoa.

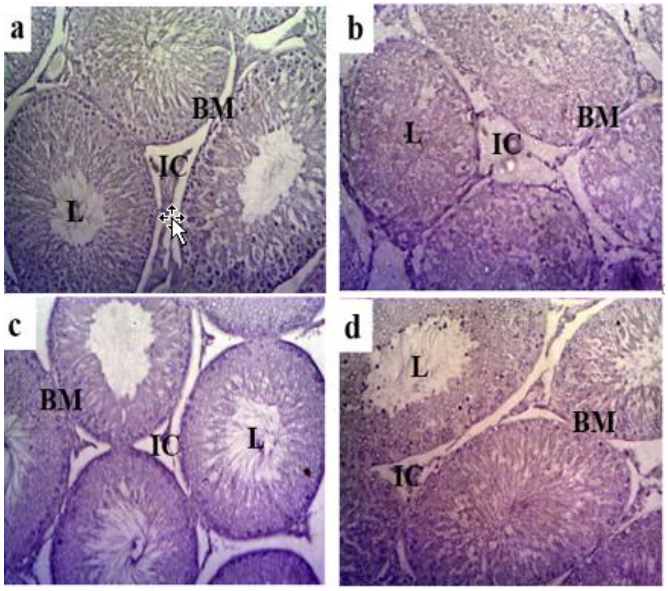

Figure 3: (A-D) Photomicrographs of the testis of adult male Wistar rats treated with distilled water, control (a). Cadmium Sulfate (b), *Polyalthia longifolia* leaf extract (c), and Cadmium sulfate with *Polyalthia longifolia* leaf extracts concurrently (d) groups expressing Ki67 marker. (x 200).

Sperm analysis

Administration of cadmium sulfate significantly reduce sperm concentration count, sperm motility, sperm morphology and life death ratio as compared with the control group. *Polyalthalongifolia* administration on the other hand restored the depleted counts in affected animals. Below (Table 3) are representations of the mean sperm parameters of controls and treated groups for their respective period of treatment.

Serum hormonal level

The effect of treatments on serum levels of FSH and LH were determined and presented in Table 4. There was significant decrease in the serum levels of FSH and LH in group B (Cadmium sulfate treated) when compared to group A (Control)

Discussion

The histological sections from this study demonstrates that the exposure of rats to cadmium bears directly on the microanatomy of the testicular tissues. The testes possesses distinctive histological appearance that can become invariably altered secondary to hormonal dysregulation and inflammatory reactions which could be caused by cadmium administration which agrees with previous study on the role of cadmium on the histology of the testes (21, 22). Such alterations as those observed in this study and the improvement with administration of *Polyalthia longifolia leaf extracts* provide insight into how *Polyalthia longifolia* ameliorates the impact of cadmium on the histology of the testes. There were vast differences in the testicular histology of rats in the experimental groups. The group treated with cadmium only (Group B) in Figure 1 displayed improper arrangement of the basement membrane of the seminiferous tubules. There was also disruption of testicular histoarchitecture and arrest of spermatogenesis was also observed. Most of the seminiferous tubules appeared twisted with signs of partial collapse. Interstitial cells were also impaired in the group treated with cadmium only with loss of Leydig cells. The impairment of interstitial cells may have caused the inhibition of spermatogenesis.

The aforementioned findings were different from the observations made in the groups treated with *Polyalthia longifolia* (Group C) only and *Polyalthia longifolia* with cadmium sulfate concurrently (Group D) in Figure 1. The histoarchitecture of the animals in this group was well defined, the basement membrane was properly arranged indicating intact spermatogenic process. Interstitial cells could also be observed in abundance. These shows an improvement in the histoarchitecture of the seminiferous tubules as compared with that of the group treated with cadmium only. These improvements would have been made possible by some phytochemicals present in the *Polyalthia longifolia* (23). The reparatory mechanism of the body makes use of phytochemicals such as flavonoids, polyphenols and anthraquinones to repair damaged parts of itself which are abundant in *Polyalthia longifolia* leaf and that is why positive improvement was possible.

In routine andrological examination of reproductively dysfunctional men, testicular biopsy is usually not among the first line of approach especially with the development of hormonal assays. In the laboratory settings with animal models, however, there exist the rare opportunity to understand the causes of reproductive dysfunction from examination of testicular histoarchitecture by means of histology (24). In this study, the qualitative descriptions applied to the micrographs were further supplemented with quantitative histopathological evaluations with the aid of Johnsen's score.

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Table 3. Effect of Cadmium and *Polyalthia longifolia* leave extract on the semen level of Wistar rats in all experimental group.

Sperm parameters	Sperm count x 10^6/ml	Sperm motility (%)	Sperm morphology (%)	Life/death ratio (%)	Progressivity
Group A	71.50±3.54	81.58±0.13	86.80±1.90	91.65±2.05	B
Group B	27.95±17.8*	71.25±3.46*	64.21±6.13*	69.36±5.15*	C
Group C	117.67±9.30	80.77±1.21	84.08±1.02	86.04±0.64	B
Group D	103.65±15.89*#	77.95±5.00#	81.95±2.18#	83.14±3.18*#	B

Data were represented as mean and standard error of mean (mean ± SEM). *($P<0.05$), significantly different when compared to control group, #($P<0.05$), significantly different when compared to cadmium sulfate treated group. Group A – Control group received 2ml/kg body weight; Group B – Cadmium Sulfate treated group received 0.3ml/kg body weight; Group C – *Polyalthia longifolia* leaf extracts treated group received 500mg/kg body weight; Group D - Cadmium Sulfate and *Polyalthia longifolia* leaf extracts treated group received 0.3mg/kg of $CdSO_4$ and 500mg/kg of *Polyalthia longifolia* body weight

Table 4. Effect of Cadmium and *Polyalthia longifolia* leave extract on the hormonal assay of Wistar rats in all experimental groups.

Hormonal assay	Group A	Group B	Group C	Group D
LH (µmol/L)	0.076±0.001	0.053±0.001*	0.048±0.002	0.055±0.007*
FSH(µmol/L)	0.518±0.003	0.545±0.004*	0.646±0.001	0.674±0.001*#

Data were represented as mean and standard error of mean (mean ± SEM). *($P<0.05$), significantly different when compared to control group, #($P<0.05$), significantly different when compared to cadmium sulfate treated group. Group A – Control group received 2ml/kg body weight; Group B – Cadmium Sulfate treated group received 0.3ml/kg body weight; Group C – *Polyalthia longifolia* leaf extracts treated group received 500mg/kg body weight; Group D - Cadmium Sulfate and *Polyalthia longifolia* leaf extracts treated group received 0.3mg/kg of $CdSO_4$ and 500mg/kg of *Polyalthia longifolia* body weight.

Johnsen's Score provides objective basis for comparison between treated groups with control (25). Quantitative analysis of seminiferous tubules based on Johnsen's score (Table 2) was, therefore, applied to grade the impact of stress on spermatogenesis. The lower scores, 5-1, were recorded in the group treated with cadmium sulfate only. Lower Johnsen's scores depicts disruption in spermatogenesis, reduced zones of spermiation, low testosterone levels and distorted seminiferous tubules. These findings are in line with the results in this study. In the group treated with *Polyalthia longifolia* and cadmium sulfate, higher Johnsen's scores, 6-10, were observed. Also, the group given *Polyalthia longifolia* only had high Johnsen's scores.

For demonstrating deoxyribonucleic acid (DNA) contents, section of testis were taken through Feulgen's reaction. The photomicrograghs revealed the content of DNA during the process of spermatogenesis among control, Cadmium sulfate, *Polyalthia longifolia,* and Cadmium sulfate with *Polyalthia longifolia* groups. The result of this study showed that there is condensed DNA content revealing shrunken spermatogonia which is a sign of apoptosis while spermatogenic cells and spermatozoa exhibit weak DNA content in cadmium sulfate treated group (group B) in Figure 2. Previous study reveals that cadmium stimulate free radical production, resulting in oxidative deterioration of lipids, proteins and DNA initiating various pathological conditions in humans and animals (26, 27). The observed changes in this study with the treatment of rats with cadmium have been previously revealed that administration of cadmium induce fragmentation of genomic DNA (28). Thymidine kinase (TK) which is an enzyme responsible for the phosphorylation deoxythymidine and its subsequent incorporation into DNA, has been involved in the inhibition of DNA synthesis in cadmium treated rats (28). The co-treatment of rat with cadmium sulfate and *Polyalthia longifolia* (group D) in Figure 2reveals a restoration of the weak DNA content when compared with $CdSO_4$ treated group. This may be as a result of high content of phytochemical components such as alkaloid and flavonoids present in the plant extract as discovered by Wu et al. (29, 30).

Ki67 is a proliferative marker used to detect proliferative cells (31). The cells that are usually picked by this marker are Type-B spermatogonia. The results obtained from the immunohistochemical studies showed abundant proliferative cells in the group treated with cadmium sulfate and *Polyalthia longifolia* concurrently (group D) in Figure 3, while the group treated with cadmium sulfate only (group B) in Figure 3, had no expression of the marker which is an indication of the arrest of spermatogenesis at the level of differentiation from Type-A to Type-B spermatogonia. The group given *Polyalthia longifolia* only (group C) in figure 3, also had some expression of the marker. The immunohistochemical observations further points to the fact that cadmium is indeed injurious to male reproductive health(20), because of the total absence of proliferative cells which is in contrast to the group given *Polyalthia longifolia* only which had expression of the proliferative marker. This could have been as a result of the antioxidant properties of *Polyalthia longifolia* (32).

Under a predefined experimental condition for this study, semen analysis was undertaken to gain insight into the success of the process of spermatogenesis. The indices evaluated provided information about the success of this study. Statistical analysis reveals that there is significant differences in semen parameters among, control, Cadmium sulfate, *Polyalthia longifolia,* and Cadmium sulfate with *Polyalthia longifolia* groups. The results of this study revealed that the administration of cadmium sulfate ($CdSO_4$) showed a drastic reduction in Sertoli cells, which could be the cause for the significant reduction in sperm count, sperm motility, sperm morphology and life and death ratio (Table 3) of the rats as compared with the control and Cadmium sulfate with *Polyalthia longifolia*. The decrease in the mean sperm count may have been as a result of interference with spermatogenesis and

testicular tissue degeneration leading to a reduction in the amount of germinal epithelium and the number of matured sperm cells. The changes observed in this study with the administration of $CdSO_4$ agrees with previous reports, which demonstrated that cadmium impairs testicular function (21, 33). The significant reduction in sperm count, motility morphology and life and death ratio observed in this study following $CdSO_4$ administration may also be associated to impairment of spermatogenesis consequent to reduced secretion of testosterone (from testes) caused by administration of $CdSO_4$ (34). This also conforms to the cigarette smoking-induced impairment of steroidogenesis and spermatogenesis documented in previous study (35). The concurrent administration of $CdSO_4$with *Polyalthia longifolia* significantly increased semen parameters when compared with the $CdSO_4$ treated group, this may be due to the reparative effect of the phytochemicals present in it. These findings were corroborated by Khaki*et al.*(36), when he observed an increase in semen parameters in rats treated with flavonoids.

From the data obtained from this study, administration of *Polyalthia longifolia* fails to improve the level of FSH and LH (Table). In the analysis of FSH there was a significant increase (0.674±0.001) in the group treated with cadmium sulfate and *Polyalthia longifolia* concurrently (group D) as compared with the cadmium sulfate treated group (group B) with 0.545±0.004. In the analysis of LH, a significant reduction (0.053±0.001) was observed in the group treated with cadmium sulfate (group B) as compared to the control group (group A) with 0.076±0.001, also a significant reduction was also observed in the cadmium sulfate and *Polyalthia longifolia* treated group. Though, as surprising as this may seem, it is worthy of note that the normal physiological endocrine response to reduction in blood testosterone level is a reactive elevation in the blood levels of gonadotropins and vice versa (37). The reason for the discrepancy in the increase and decrease of FSH and LH across the experimental groups may be due to idiopathic factors.

Conclusion

As documented in previous studies and corroborated in this study that cadmium sulfate is injurious to male reproductive health, this current study pushed the boundaries of previous knowledge on the antibacterial activity, cytotoxicity, antifungal activity, acute toxicity, anti-inflammatory and hepatoprotective activity of *Polyalthia longifolia* leaf extract by investigating its antitoxic on testes adversely affected by cadmium toxicity. Several parameters were introduced in this study such as immunohistochemistry, Johnsen's score, stereology, semen analysis and DNA localization which revealed the toxicity of cadmium sulfate and ameliorative properties of *Polyalthia longifolia* leaf extract and may be used as subfertility drug.

Conflict of interest

The authors declare that there is no conflict of interests.

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