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Melatonin and garlic cytoprotective-ameliorative effects on dibutyl phthalate intoxication on sperm DNA and testicular biomakers of rabbits

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Abstract

The study investigated the cytoprotective and ameliorative effects of melatonin and *Allium sativum* (garlic) on dibutyl phthalate (DBP)-induced oxidative stress, its impact on sperm DNA integrity and testicular oxidative stress biomarkers. Forty two rabbit bucks were randomly divided into 7 groups of 6 bucks each labeled as A, B, C, D, E, F and G: The treatment were as follows: A (served as negative control, received olive oil for 16 weeks); B (served as positive control, exposed to DBP for 16 weeks, no treatment); C (given melatonin for 8 weeks, thereafter DBP for 8 weeks); D (administered garlic for 8 weeks, thereafter DBP for 8 weeks); E (exposed to DBP for 8 weeks, thereafter melatonin for 8 week); F (exposed to DBP for 8 weeks, thereafter garlic for 8 weeks); and G (exposed to DBP for 8 weeks, thereafter melatonin + garlic for 8 weeks). Ejaculated semen was collected on the last day (112th) using artificial vagina for rabbit and pooled for each group was used for sperm DNA fragmentation index (SDFI) determination, rabbits were sacrificed and the testes harvested for determination of superoxide dismutase activity, reduced glutathione and malondialdehyde concentration. Results showed a significant increase ($P = 0.0018$) in the mean SDFI in group B (78.20 ± 4.72), compared to other groups. A significant increase ($P \leq 0.0001$) in superoxide dismutase activity, increase reduced glutathione concentration and decrease malondialdehyde concentrations in the treatment groups compared to the DBP exposed group without treatment (group B) were observed. Melatonin and garlic demonstrated cytoprotective and ameliorative effects against DBP-induced oxidative stress in rabbit bucks.

Keywords: Dibutyl phthalate, Garlic, Melatonin, Sperm DNA, Testicular biomarkers

Introduction

Oxidative stress (OS) has been implicated in numerous disease processes, including: sepsis, mastitis, enteritis, pneumonia, respiratory and joint diseases (Lykkesfeldt & Svendsen, 2007). In a healthy body, reactive oxygen species (ROS) and antioxidant concentration/activities remain in a balanced state,

but when the balance is disrupted towards an overabundance of ROS, OS occurs. In most cases, OS results from increased generation of ROS rather than a depletion of antioxidant (Agarwal & Gupta, 2006). OS affects many physiological processes in the male (sperm motility, fertilizing ability and deoxyribonucleic acid (DNA) integrity) and female (from oocyte maturation to fertilization, embryo development, implantation and foetal growth). (Agarwal & Gupta, 2006).

Antioxidants play an important role in preventing the damaging effect of free radicals in humans and animals (Noori, 2012). A lot of natural products have protective effects against different medication or chemically-provoked toxicities (Mansour *et al*., 2012; Hosseinimehr, 2014; Baiomy & Mansour, 2015). *Allium sativum* (garlic) is rich in antioxidants, which help scavenge free radical particles that damage cell membranes and DNA and therefore may be beneficial to the ageing process (Capasso, 2013). The antioxidant activity of fresh garlic is due to the unstable and irritating organosulphur compounds (Benkeblia, 2004; Capasso, 2013; Ifesan *et al*., 2014). The ability of garlic to lower blood pressure and offer cardio-protection seems to come from its ability to counteract oxidative stress (Dhawan & Jain, 2005).

Another important agent with free radical scavenging and broad-spectrum antioxidant activity is melatonin (Tan *et al*., 1993; 2002). It is an effective antioxidant for the protection of testicular function (Aitken & Roman, 2008). Melatonin has been reported to reduce oxidative stress in the testes induced by ethanol (Oner-Iyidogan *et al*., 2001), indomethacin (Othman *et al*., 2001), X-irradiation (Hussein *et al*., 2006), and streptozotocin-induced diabetes (Armagan *et al*., 2006).

Several studies have linked declining reproduction, especially male fertility to toxicants found in the environment, particularly endocrine-disrupting chemicals (EDCs) such as phthalates (Wong & Cheng, 2011; Nordkap *et al*., 2012). One of the phthalates, Di-n-butyl phthalate (DBP) has attracted special attention due to its abundance in the environment resulting from high production volume running into millions of tons annually (Swan & Elkin, 1999). It is used extensively as plasticizers in many consumer plastic products, including: Children toys, food wrapping materials, cosmetics, even some biomedical devices like dialysis tubing and intravenous bags. They are also used in enteric coating of some pharmaceutical preparations (Hauser *et al*., 2004; Oehlmann *et al*., 2009; Umar *et al*., 2014). As a result, human and animal exposure becomes inevitable with its attendant negative consequences on reproduction (Kolaric *et al*., 2008; Guerra *et al*., 2010; Zhou *et al*., 2011; Wang *et al*.,2012a;2012b; Asghari *et al*., 2015; Hamdy *et al*., 2015; Rehani *et al*., 2015). In addition, DBP was reported to increase the generation of ROS within the testes, concomitantly decreasing antioxidant concentration, resulting in impaired spermatogenesis (Lee *et al*., 2007; Zhou *et al*., 2011).

The objectives of the study were to determine the protective and ameliorative effects of melatonin and *A. sativum* on dibutyl phthalate-induced testicular oxidative stress and its impact on sperm DNA fragmentation in rabbit bucks.

Materials and Methods

Study location

The study was carried out in the Department of Theriogenology and Production, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria.

Study animals

Forty-two (42) apparently healthy, New Zealand White rabbit bucks (*Oryctolagus cuniculus*), with mean age of 10.0 ± 2.0 months and mean body weight of 1.80 ± 0.1kg were used for the study. The bucks were housed in a standard rabbit cage with a dimension of 1.8m × 0.6m, one buck per cage.

Grouping

The rabbit bucks were randomly divided into seven (7) groups of six (6) bucks each, designated as groups A, B, C, D, E, F and G, which received different treatments as described below:

Group A: served as negative control, each buck received Olive oil only at 1.5 ml once a day, five consecutive days in a week for 16 weeks.

Group B: served as positive control, each buck received 1.5 ml Olive oil + DBP (750 mg/kg) once a day, five consecutive days in a week with no treatment for 16 weeks.

Group C: each buck received pretreatment with 0.5 mg/ml melatonin daily for 8 weeks, followed by 1.5 ml Olive oil + DBP (750 mg/kg) once a day, five consecutive days in a week administered for another 8 weeks.

Group D: each buck received pretreatment with 5.0 % *A. sativum*, daily for 8 weeks, followed by 1.5 ml Olive oil + DBP (750 mg/kg) once a day, five consecutive days a week administered for another 8 weeks.

Group E: each buck received 1.5 ml Olive oil + DBP (750 mg/kg) once a day, five consecutive days a week for 8 weeks, followed by treatment with 0.5 mg/ml

melatonin, once a day, seven days a week for another 8 weeks.

Group F: each buck received 1.5 ml Olive oil + DBP 750 mg/kg, five times a week for 8 weeks, followed by treatment with 5.0 % *A. sativum* daily for another 8 weeks.

Group G: each buck received 1.5 ml Olive oil + DBP 750 mg/kg five consecutive days a week for 8 weeks, followed by treatment with 0.5 mg/ml melatonin + 5.0 % *A. sativum* daily for another 8 weeks.

The rabbit bucks were acclimatized for 30 days before commencement of the study. All rabbits were fed diets corresponding to their groups as shown in Table 1, as described by Shinkut *et al*. (2016a). The diets were of isonitrogenous and isocaloric values. Dried bulbs of *Allium sativum* were then weighed and added to the feed raw materials and ground together to form the experimental diets (5% or 5 kg of garlic was weighed and added to 95% or 95 kg of other feed ingredients to make up 100 kg of the experiment diet for garlic treatment groups). Approval for the study was sought and obtained from the Ahmadu Bello University Committee for Animal Use and Care with the approval number: ABUCAUC/2018/059.

Chemical acquisition and preparation for administration

Di (n-butyl) phthalate DBP (CAS Number 84-74-2) was purchased from Sigma Aldrich USA. The dosage of 750 mg/kg to be given to the experimental bucks, were calculated and reconstituted in olive oil (Goya Extra Virgin Olive Oil, Sevilla, Spain) to form a solution of 50 % DBP as described by Nair (2015) with modification. Melatonin (MEL, 5 mg/Tablet, Nature made, USA) was obtained and dissolved in 10 ml of distilled water to make 0.5 mg/ml suspension daily before administration to the experimental bucks (Umosen *et al*., 2012). All preparations were administered to the animals using gastric tube between the hours of 8.00am-10.00am each day of administration.

Sperm DNA integrity assay

On the last day of the study (112th day), ejaculates were collected with the aid of artificial vagina (AV), pooled together according to the groups and subjected to Acridine Orange (AO) staining for sperm

Table 1: Composition of Experimental Diets for the individual groups

Treatment groups Composition (%)	A	B	C	D	E	F	G
Maize	30.16	30.16	30.16	28.57	30.16	28.57	28.57
Groundnut cake	28.12	28.12	28.12	26.64	28.12	26.64	26.64
Rice offals	35.32	35.32	35.32	33.46	35.32	33.46	33.46
Crude *Allium sativum*	0.00	0.00	0.00	5.0/0	0.00	0/5.0	0/5.0
Vitamin premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Palm oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Bone meal	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Methionine	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Total	100	100	100	100	100	100	100
Dry matter	89.50	89.50	89.50	87.89	89.50	87.89	87.89
Crude protein	16.81	16.81	16.81	18.75	16.81	18.75	18.75
Ether extract	1.27	1.27	1.27	1.10	1.27	1.10	1.10
Crude fibre	8.65	8.65	8.65	8.54	8.65	8.54	8.54
Nitrogen free extract	53.96	53.96	53.96	52.46	53.96	52.46	52.46
Ash	7.20	7.20	7.20	8.65	7.20	8.65	8.65
ME (kcal/kg)	2,640.42	2,640.42	2,640.42	2,645.18	2,640.42	2,645.18	2,645.18

Metabolisable energy was calculated according to the formula of Pauzenga (1985):
ME = 37 × %CP + 81 × %EE + 35.5 × %NFE. ME = Metabolizable energy; CP = Crude protein; EE = Ether extract; NFE = Nitrogen free extract; Crude *A. sativum* 5.0/0 = 5% *A. sativum* in diet for 8 weeks followed by 0% *A. sativum* in diet from 9-16 weeks. Crude *A. sativum* 0/5.0% = 0 % *A. sativum* in diet for 8 weeks followed by 5.0 % *A. sativum* in diet from 9-16 weeks

DNA integrity determination.

Smears of the semen (ejaculate) were made on clean, grease free glass slides and fixed in Carnoy's solution (3:1, methanol:glacial acetic acid) for 2-3 hours, then washed and air-dried. The slides were stained for 5 min with freshly prepared acridine orange (AO) stain as follows: 10 mL of 1% AO in distilled water was added to a mixture of 40 mL of 0.1 M citric acid and 2.5 ml of 0.3 M Na2HPO4.7H2O. The slides were washed with distilled water then covered with glass cover and examined under a fluorescent microscope at ×10 and ×40 magnifications. Averages of 200 sperm cells were evaluated on each slide. Sperm cells which showed green fluorescence were considered to have normal DNA content, whereas sperm cells that displayed a spectrum of yellow-orange to red fluorescence were considered to have damaged DNA. The ratio of yellow to red/green + yellow to red fluorescence were considered as DNA fragmentation index (DFI) expressed in percentage (Liu & Baker, 1994; Essam-elden *et al*., 2015).

Testicular oxidative stress biomarkers

At the end of the study (112th day), five bucks were sacrificed from each group, the left testes were carefully collected before analysis. One gram of testis was homogenized in 9 volumes of buffered saline and centrifuged at 3000rpm for 15 min. The supernatant was separated and used for assessments of the oxidative stress biomarkers [superoxide dismutase (SOD), reduced glutathione reductase (GSH) and malondialdehyde (MDA)] (Oda & Waheeb, 2017).

Lipid peroxidation

MDA serves as index of intensity of lipid peroxidation, a product which reacts with thiobarbituric acid, producing a complex coloured compound which absorbs light at 535nm and can be measured.

Approximately 150 µl of tissue homogenate was treated with 2 ml of 37% Thiobarbituric acid (TBA) solution- 15% Trichloroacetic acid (TCA) solution- 0.25N HCl reagent (1:1:1 ratio) and placed in water bath at 90˚C for 60 minutes. The mixture was then cooled and centrifuged at 3000rpm for 5 minutes and the absorbance of the pink supernatant (TBA-MDA complex was then measured at 535nm using spectrophotometer (The color cube bench top spectrophotometer produced by Colorlite innovative Farbmesstechnik Germany). MDA concentration was then calculated using the molar extinction coefficient of 1.56 ×10-5 M. Protein was determined as described by Lowry *et al*. (1951).

MDA Concentration (nmol/mg protein) = Absorbance of sample/1.56×10-5 × protein concentration (mg).

Testicular antioxidant assay

Superoxide dismutase: Superoxide dismutase (SOD) was determined by the method described by Fridovich (1989), based on the ability of SOD to inhibit auto oxidation of adrenaline at pH 10.2. An aliquot of the sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer (The color cube bench top spectrophotometer produced by Colorlite innovative Farbmesstechnik Germany). The reaction was initiated by the addition of 0.3 ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm due to the adrenochrome formed was monitored every 30 sec for 150 sec. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of epinephrine to adrenochrome during 150 sec.

Calculation:

Increase in Absorbance (per min.) = (A3−A0)/2.5

A0= absorbance after 30 seconds

A3 = absorbance after 150 seconds

% Inhibition = Increase in absorbance of substrate Increase in absorbance of blank 100.

One unit of SOD activity was defined as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 min, expressed in U/mg protein.

Reduced glutathione: (GSH) concentration measurement was done as described by Rajagopalan *et al*. (2004). This is based on the reaction between Ellman's reagent (5, 5-dithiobis nitro benzoic acid) (DNTB) and compounds containing sulfihydryl group yielding a mixed disulphide (GS-TNB) and 2-Nitro-5-thiobenzoic acid (TNB).

About 0.2 ml aliquot of the sample was added to 1.8 ml of distilled water and 3 ml of the precipitating reagent was mixed with the sample. The mixture was then allowed to stand for 5 mins and then filtered. At the end of the fifth minutes, 1ml of the filtrate was added to 4 ml of 0.1M phosphate buffer and finally, 0.5 ml of the Ellmans' reagent was added. A blank was prepared with 4ml of the 0.1M phosphate buffer, 1 ml of diluted precipitating solution and 0.5 ml of the Ellman's reagent. The absorbance was measured at 412 nm using spectrophotometer (The color cube bench top spectrophotometer produced by Colorlite innovative Farbmesstechnik Germany). Using a molar extinction coefficient of 14,150-1cm-1, GSH

concentration was calculated and expressed in µg/mg protein).

Statistical analysis

Data collected were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test using GraphPad Prism version 5.0 for windows 2003, from GraphPad Prism software, San Diego, California (www.graphpad.com). Values were expressed as mean ± standard deviation (SD) and were considered significant at P ≤ 0.05.

Results

Sperm DNA fragmentation index (%)

A high percentage of sperm cells with normal DNA were observed in group A (Plate I). However, group B had the highest percentage of abnormal DNA observed as pinkish to yellowish in colour (Plate II). The group B animals had the highest SDFI (78.20 ± 10.55) and was significant when compared to other groups (Table 2; p < 0.05).

Testicular superoxide dismutase (SOD) activity

Superoxide dismutase activity (U/mg protein) was significantly higher (P ≤ 0.05) in groups C (41.43 ± 6.66), D (36.68 ± 4.90), G (43.25 ± 3.54), compared with groups A, B, E, F, moderately, high in groups E (32.15 ± 2.60), F (32.45 ± 5.20) and significantly low in group B (21.38 ± 5.58), (Table 3).

Testicular reduced glutathione concentration (GSH)

Reduced glutathione concentration (µg/mg protein) was significantly high (P ≤ 0.05) in groups C (43.80 ± 3.36), D (39.60 ± 3.35), E (38.30 ± 4.71), F (36.23 ± 2.88), G (41.90 ± 3.28), moderate in group A (31.28 ± 3.10) and significantly lower in group B (20.10 ± 1.60), (Table 3).

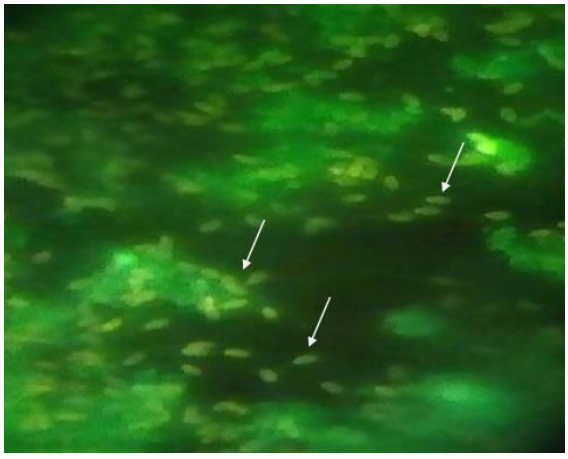

Plate I: Fluorescent micrograph of rabbit buck spermatozoa administered Olive oil for 16 weeks. Sperm cells with normal DNA appeared greenish (white arrows) after staining with acridine orange stain (×40).

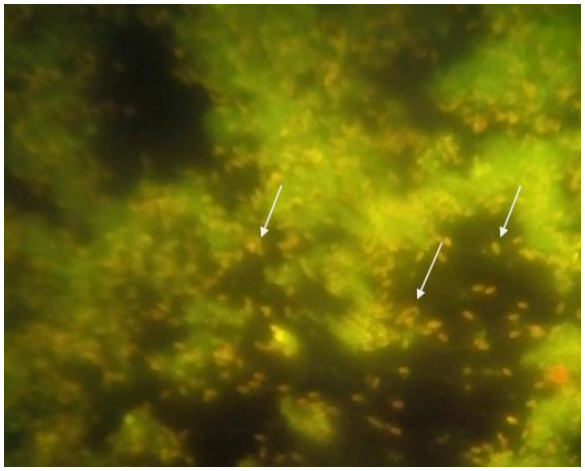

Plate II: Fluorescent micrograph of rabbit buck spermatozoa administered Olive oil and DBP for 16 weeks. Sperm cells with abnormal DNA appeared pinkish-yellowish (white arrows) after staining with acridine orange stain (×40)

Table 2: Mean ± SD of sperm DNA fragmentation index in dibutyl phthalate treated rabbit bucks administered with different regiments of melatonin and *Allium sativum* for 16 weeks

A	B	C	D	E	F	G
15.40±3.58[a]	78.20±10.55[b]	22.00±4.18[a]	23.60±2.51[a]	28.20±2.39[a]	32.00±2.74[a]	24.80±1.30[a]

[ab] rows with different superscripts are significantly different at p≤0.05

Table 3: Mean ±SD of testicular oxidative stress biomarkers in dibutyl phthalate treated rabbit bucks administered with different regimens of melatonin and *Allium sativum* for 16 weeks

	A	B	C	D	E	F	G
SOD	29.73±0.56[a]	21.38±5.58[b]	41.43±6.66[c]	36.68±4.90[c]	32.15±2.60[a]	32.45±5.20[a]	43.25±3.54[c]
GSH	31.28±3.10[a]	20.10±1.60[b]	43.80±3.36[c]	39.60±3.35[c]	38.30±4.71[c]	36.23±2.88[ac]	41.90±3.28[c]
MDA	33.70±1.19[a]	46.18±4.15[b]	26.08±2.77[a]	29.30±2.77[a]	35.88±3.71[a]	36.55±2.82	30.33±3.52[a]

[abc] rows with different superscripts are significantly different at p≤0.05

Testicular malondialdehyde concentration (MDA)
Malondialdehyde concentration (µmol/mg protein) was significantly higher (P≤ 0.05) in group B, 46.18 ± 4.15), moderate in groups A (33.7 ± 1.19), E (35.88 ± 3.71), F (36.55 ± 2.82) and G (30.33 ± 3.52) and significantly lower in groups C (26.08 ± 2.77), D (29.30 ± 2.77), (Table 3).

Discussion

In the present study, the observed high DNA fragmentation index in the group exposed to DBP without treatment (group B) may have been due to oxidative stress induced by DBP. Sperm DNA first becomes susceptible to damage, if chromatin packing is not completed during spermatogenesis, when protamine replacement is occurring in elongating spermatids (Marin *et al*., 2012). High sperm DNA fragmentation index is associated with reduced fertility (Malm *et al*., 2017). Oxidative stress has been reported as a strong factor for DNA breaks (Sakkas *et al*., 1998; Aitken *et al*., 2004; Malm *et al*., 2017). Toxic hydroxyl radical modifies purine and pyrimidine bases and the sugar back bone, causing DNA strand breaks and DNA damage (Agarwal, 2004; Nenkova & Alexandrova, 2013). Melatonin treated groups exhibited significantly decreased level of SDFI, which may be due to the direct antioxidant effect of melatonin on Leydig's cells, as the presence of melatonin receptors have been reported on rat Leydig cells (Balik *et al*., 2004). Mohammadghasemi & Jahromi (2018), reported that melatonin increased the integrity of sperm DNA in nicotine treated mice. Also, the observed decreased SDFI in garlic-treated groups may be due to the reported antioxidant effect of garlic.

The observed significant decrease in SOD activity, and GSH concentration and the significant increase in MDA concentration (Table 3) in DBP exposed rabbit bucks (group B) in the present study is indicative of oxidative stress (OS) and testicular lipid peroxidation respectively. Several studies reported that administration of DBP alters reproductive functions by increasing ROS hunting for DNAs and lipids, leading to decreased SOD, GSH and increased MDA in the testis (Akingbemi *et al*., 2004; Asghari *et al*., 2015). It is known that testes and spermatozoa are extremely sensitive to ROS-induced damage (Kumar *et al*., 2009). The elevated ROS has strong affinity to cellular component made up of polyunsaturated fatty acids, resulting in their damage by lipid peroxidation (Aly *et al*., 2009). MDA is one of the most important products of lipid peroxidation which interfere with protein biosynthesis by forming adducts with DNA, RNA and proteins (Doreswamy *et al*., 2004). Exposure to DBP in this study was observed to cause several fold increases in MDA formation, suggesting enhanced lipid peroxidation which may probably be due to the several intermediates being formed such as superoxide, singlet oxygen in peroxidation reaction (Nair, 2015).

The observed increase in SOD activity, GSH concentration and decreased levels of MDA in melatonin treated rabbit bucks (C, E and G) could be that melatonin has an antioxidant effect of long duration of time or that the concentration attained in the testes before exposure to DBP counteracted the effect of DBP on the testes. Similarly, flavonoid, one of the active constituents of garlic is reported to confer protection against the harmful effects of ROS (Shinkut, 2015). In vitro studies also showed that flavonoid have potent antioxidant and free radical scavenging activity (Prochazkova *et al*., 2011). This may have been responsible for the increase SOD activity, GSH concentrations and decreased MDA concentrations observed in groups D, F and G in the present study, which corroborate the findings of Borek (2001). Ide *et al*. (1996) reported that garlic and its major organosulphur constituents have a scavenging effect on hydrogen peroxide and inhibited the chain of oxidation induced by a hydrophilic radical initiation.

In conclusion, DBP caused increase in sperm DNA fragmentation index, induced oxidative stress by decreasing the concentration of SOD activity, GSH concentration while increasing MDA concentration in the DBP exposed group that was not treated with melatonin and garlic. Furthermore, the present study clearly demonstrated the potential use of melatonin and garlic as cytoprotective and ameliorative agents against oxidative stress induced pathologies in the reproductive system. It further affirmed DBP as a potent environmental health hazard with devastating consequence on reproduction.

It is recommended that male animals used for breeding should be given melatonin supplement and 5.0% garlic in-feed to protect against oxidative stress inducers in the environment. Better policies by government at all levels should be put in place, to promote the production of DBP-free products by Pharmaceutical and Plastic companies and also sensitize the populace on proper use and disposal of plastics materials to safe-guard the environment.

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