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Melatonin and *Phoenix dactylifera* attenuates Bisphenol A induced Testicular and sperm toxicity in Male Wistar Rats.

Godam ET, Young-Harry BO, Sapira-Ordu L, Woha BJ, Bolaji MS, Opusunju BH, Pepple IA and Wofuru CD

1. Histology and Molecular Biology Unit, Department of Human Anatomy, Rivers State University Port Harcourt.
2. Department of Obstetrics and Gynaecology, Faculty of Clinical Sciences Rivers State University
   Port Harcourt.
3. Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Rivers State University Port Harcourt Rivers State Nigeria.
4. Department of Medicine, School of Medicine and Health Sciences Eden University Zambia

Corresponding Author: Godam ET
E-mail: elvis.godam@ust.edu.ng; +2348063339602

ABSTRACT

Infertility is a disease of the reproductive system defined by the lack of ability of a non-contraceptive couple to conceive after 12 months or more of regular unprotected sexual intercourse. Bisphenol A(BFA) which is one of the most widely used industrial chemical in the production of plastic materials has been associated with male infertility by causing increase in reactive oxygen species level and oxidative stress, melatonin and *phoenix dactylifera* (date palm) has shown to have many useful medicinal properties, including antioxidant effects. This study aimed at evaluating the effects of melatonin and *Phoenix dactylifera* on the cellular changes associated with bisphenol A induced testicular and androgen toxicity in male Wistar rats. Twenty- five (25) adult male Wistar rat with a weight range of 120- 150g were randomly divided into five groups of five rats each in this research study. Group 1 received distilled water orally, Group 2 received BFA only; 4 mg/kg orally, Group 3 received BFA; 4 mg/kg plus melatonin; 10mg/kg, orally, Group 4 received BFA; 4mg/kg plus date palm; 500mg/kg, orally and Group 5 received BFA; 4mg/kg plus melatonin; 10mg/kg and date palm; 500mg/kg, orally. Administration was done for 14 days. The animals were humanely sacrificed using chloroform, 24 hours after last administration. The testes and epididymis were dissected following abdominal incision. The epididymis was used for semen analysis while the testes was processed for histological analysis. The data was presented as mean values ± SEM and analyzed by ANOVA using the SPSS package version 25 software. Differences were considered significant at $p<0.05$. Bisphenol A administration caused significant increase in the body weight. There was also a reduction in sperm parameters with degenerative changes in the seminiferous tubules of the testes in the BFA treated group compared to the control. However, administration of melatonin and *phoenix dactylifera* showed significant improvement in sperm parameters and restored the richness of the histology of the testes tissues.

Key words: Bisphenol A, infertility, *P. dactilifera*, melatonin, testes, epididymis, sperm.

INTRODUCTION

Infertility is a global health issue affecting millions of people of reproductive age worldwide (1). Available data suggests that between 48 million couples and 186 million individuals have infertility globally (2). Over the years there has been a significant increase in the rate of infertility and is becoming more common, especially since many couples are waiting to have children later in life (3). Some doctors and researchers would say that infertility is becoming an epidemic (4) and infertility treatments are becoming more popular as couples look for ways to start a family (5).Infertility is a disease of the reproductive system defined by the lack of ability of a non-contraceptive couple to conceive after 12 months or more of regular unprotected sexual intercourse (1). Primary infertility is the inability to have any pregnancy, while secondary infertility is the inability to have a pregnancy after previously successful conception (1). Infertility may occur due to male factors, female factors, a combination of male and female factors or may be unexplained (6) For both male and female, however, environmental and lifestyle factors such as smoking, excessive alcohol intake, obesity, exposure to environmental pollutants and industrial chemicals such as Bisphenol A (BPA), an endocrine-disrupting chemical have been associated with low fertility rates (7).Bisphenol A (BFA) is one of the most widely used industrial chemicals in the production of plastic materials (8), prolonged exposure to BFA is said to have harmful effects on male reproductive system due to increased reactive oxygen species level and oxidative stress (OS) (9). Several research groups and pharmacological approaches in the past years, have focused their investigations to prevent and/or revert male infertility and BPA-induced testicular toxicity based on the use of compounds and several plant extracts containing valuable antioxidant properties such as Melatonin, and *Phoenix dactylifera* (date palm) (10).Melatonin is an endogenous hormone derived from tryptophan, which was first exposed in the vertebrate pineal gland (11). It regulates many important biological functions, such as sleep, circadian rhythm, reproduction, immunity, and oncostatic processes (12; 13; 14). Melatonin exerts an antioxidant effect on organs and anti-apoptotic effect on cells (15) and can easily move across the cell membrane blood–brain barrier, protectecting various biomolecules by detoxifying Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) (16). It enhances the antioxidant defense system by increasing the expression of antioxidant enzymes (17). The role of melatonin in reproduction in many species is related to seasonal reproductive cycles. In man, it has been demonstrated that changes in secretion of melatonin by the pineal gland can modulate the activity of the reproductive neuroendocrine axis (18). *Phoenix dactylifera* (Date palm) fruit, is one of the plants suggested in traditional medicine for the improvement of fertility potential. Date palm fruits were traditionally claimed to be aphrodisiacs and fertility enhancers and have been used in the Middle East as a natural medicine for the treatment of male infertility and promoting fertility in women (19). The Iranian traditional medicine revealed that date palm has refreshing and nutritional value and is beneficial for the treatment of infertility in both males and females. However, it is widely used for the treatment of male infertility (20). Date palm fruit extract (DPFE) has been shown to contain high concentration of antioxidant compounds (21) and may be a suitable and cost-effective alternative to orthodox drugs for the protection of fertility potential (22).

MATERIALS AND METHODS

The following materials were used in the study, Plastic Cages, blood sample containers, Temperature controlled refrigerator, Microwave oven, humidity chamber, microscope.

Bioactive compounds, drugs and assay kits used in this study were: Melatonin M5250-1G (Sigma Aldrich, USA), Bisphenol A (Sigma Aldrich, USA), Haematoxylin and Eosin Stain (H&E). Leica Auto processor, Leica Auto stainer.

Bioactive Compounds – Bisphenol: Bisphenol was sourced from Sigma Aldrich USA. Before use, bisphenol was dissolved in distilled water at a dose of 4mg/kg bodyweight and administered to the rats at a single dose orally.

Melatonin: Melatonin was sourced from Sigma Aldrich USA with batch no. M5250-IG. Before use, 10 mg/kg body weight of melatonin was dissolved in 2ml of water and administered orally to the wistar rats.

Animal Source and Handling: Twenty-five male wistar rats weighing between 120-150g, were obtained from the Animal Housing Facility, Faculty of Basic Medical Science, College of Medical Sciences Rivers State University Port Harcourt, Nigeria. They were acclimatized for two weeks before starting the experiment. During this period, the rats were housed in metal cages at room temperature, maintained under 12 hours' light/dark cycle, they had free access to standard rat food/diet and water till the end of the experiment. All rats were handled in accordance with the standard guide for the care and use of laboratory animals.

Acquisition and extraction of *Phoenix dactilyfera* fruits: Fresh fruits of Phoenix dactilyfera (date palm) was purchased from Mile Three (3) market, Port-Harcourt. The fruits were washed, air dried, minced and powdered using laboratory mortar. 1000g of the powdered leaves was extracted in 1.5 liters of 80% ethanol using a soxhlet extractor. These were further filtered using a Whatman filter paper (24mm). The filtrate was dried in a laboratory water bath set at 68°C and total yield of (55.8g) was obtained per 1000g of the powdered fruits.

Phoenix dactylifera (date palm) fruit was purchased from mile three (3) market, Port-Harcourt, Nigeria. Plant material was authenticated in the Herbarium unit of the Department of Biological Sciences, Faculty of Sciences, Ahmadu Bello University, Zaria Kaduna State, Nigeria with the Voucher Specimen Number of 7130. Preparation of *Phoenix dactylifera* Fruit extract was conducted in the Department of Chemistry Faculty of Sciences, Rivers State University Nigeria

Experimental Design: Twenty- five (25) adult male wistar rat with a weight range of 120- 150g were randomly divided into five groups of five rats each, as follows:

Group 1: Control group (distilled water, orally for 14 days).

Group 2: Bisphenol A group (BFA; 4 mg/kg orally for 14 days).

Group 3: Treatment group I (BFA; 4 mg/kg + Melatonin; 10mg/kg, orally for 14 days.

Group 4: Treatment group II (BFA; 4mg/kg + Date palm; 500mg/kg, orally for 14 days).

Group 5: Treatment group III (BFA; 4mg/kg + Melatonin; 10mg/kg + Date palm; 500mg/kg, orally for 14 days).

Administration was by gavage using metal oral cannulas. The body weights of the rats were measured just before administration, after 7 days of administration and after 14 days of administration. The rats were sacrificed after 14 days of administration.

Sample Collection - Animal Sacrifice: At the end of the experiment, the animals were humanely sacrificed using chloroform, 24 hours after last administration. Blood samples were taken by cardiac puncture from all groups for hormonal assay (androgens). The testes were harvested after abdominal incision, to evaluate the sperm parameters and histological examination

respectively. The testes were then fixed in 10% buffered formalin.

Semen Analysis: The caudal epididymis was dissected out; several incisions (1mm) were made in the caudal epididymis, which was suspended in 1ml of normal saline solution according to Olaniyan *et al.* (2018) for sperm motility and morphology. The sperm concentrations were determined by fixing the sperm in 10% formo-saline in a 1:9 ratios. The counting was done using the newly improved nebular hemocytometer (Olaniyan *et al*., 2018).

Histological Analyses: The testes were fixed properly in 10% buffered formalin for 48 hours and grossed. They were further dehydrated through changes of alcohol, cleared in changes of xylene and infiltrated in molten paraffin wax at 2 degrees centigrade higher than the melting point of wax using automated tissue processor.

The tissues were further embedded on embedding machine, trimmed and sectioned using the rotary microtome at 5 microns and floated out using water bath at temperature lower than the melting point of wax. Floating out was done and air-dried before heat fixing it using hot plate.

Sections were taken to water and stained using Hematoxylin and Eosin (H&E) staining method. Photomicrograph sections were taken Leica DM750, Camera ICC50 E

Histological investigations were carried out on the tissues fixed in formal saline. The tissue blocks were sectioned for routine Hematoxylin and Eosin (H&E) staining and periodic acid schiff staining. The fixed organs were cut in about 0.5cm cross-sections and transferred to 70% alcohol for dehydration. The tissues were rinsed in 90% and absolute alcohol and xylene for different durations before they were transferred into two changes of molten paraffin wax for 1 hour each, in an oven at 65oC for infiltration. They were subsequently embedded and sliced in serial sections using a rotary microtome at five microns (5μ). The tissues were transferred onto albumenized slides and allowed to dry on a hot plate for 2 minutes. The slides were dewaxed with xylene and passed through absolute alcohol (2 changes); 70% alcohol, 50% alcohol, and then water for 5 minutes. The slides were then stained with hematoxylin and eosin and periodic acid schiff stain.

Statistical Analysis: The data was presented as mean vaues ± SEM. ANOVA was carried out on the Statstical Package for the Social Sciences (SPSS package version 21) and we checked for the occurrence of significant differences between the results. Differences were considered significant at $p<0.05$.

RESULTS

Effect of Melatonin and *Phoenix dactylifera* on Body Weight of Bisphenol A Induced Toxicity in Wistar Rats: Bisphenol A treatment caused a significant increase ($P<0.05$) in animal body weight compared with the control. The body weight of the rats showed a dose dependent reduction in the treatment group animals ($P<0.05$) compared with the control group animals who maintained a continuous increase in weight throughout the period of experiment as shown in table 1. Group 1 (control group), showed a continuous increase in weight when comparing the initial to the final body weight and the finial body weight was significantly different ($P>0.05$) with relative increase. Group 2 (bisphenol only), caused a significant increase ($P<0.05$) in body weight when compared to the control group. Group 3 (bisphenol + melatonin), showed a significant decrease ($P<0.05$) in weight during treatment compared with the bisphenol group animals. Group 4 (bisphenol + date Palm), caused a reduction in weight significantly different from the animals in group (bisphenol group). Group

5 (bisphenol + melatonin + date palm) showed a significant decrease (P<0.05) in weight during treatment compared with the bisphenol and control group animals.

Effect of Melatonin and *Phoenix dactylifera* on the Testicular Weight of Bisphenol A Induced Toxicity in Wistar Rats: Table 2 shows a significant increase in the testicular weight of rats in group administered with Bisphenol only (group 2), when compared with the control (P>0.05). The group administered with Bisphenol and Melatonin (group 3) showed a significant decrease at (P>0.05) from the control (group 1). The group administered with Bisphenol and Date Palm (group 4) caused a decrease significantly different from control group (P<0.05). Group 5 (Bispheno+ Melatonin + Date Palm) showed a decrease, significantly different (P<0.05) from the control (group 1).

Effect of Melatonin and *Phoenix dactylifera* on the Relative Weight of Bisphenol A Induced Toxicity in Wistar Rats: The results obtained in table 3 shows a significant increase in the relative weight of rats in group administered with Bisphenol only (group 2), when compared with the control (P>0.05). The group administered with Bisphenol and Melatonin (group 3) showed a significant decrease at (P>0.05) from the control (group 1). The group administered with Bisphenol and Date Palm (group 4) caused a decrease significantly different from control group (P<0.05). Group 5 (Bispheno+ Melatonin + Date Palm) showed a decrease, significantly different (P<0.05) from the control (group 1).

Table 1: Mean Body Weight of Wistar Rats Treated with Bisphenol A, Melatonin and *Phoenix dactylifera*: GROUPS

	1 DAY (g)	7 DAY (g)	14 DAY(g)
1	104.2 ± 5.08	110 ± 4.82	116.4 ± 6.85
2	128 ± 7.13^{c}	131 ± 3.85^{d}	155 ± 3.11^{b}
3	120.4 ± 2.50^{bc}	117.8 ± 1.98^{c}	153.8 ± 2.60^{b}
4	111.2 ± 1.32^{b}	107.8 ± 1.20^{bc}	132 ± 6.00^{a}
5	94.8 ± 5.00^{a}	92.4 ± 5.03^{a}	119.4 ± 8.97^{a}

Values expressed as mean ± SEM; n = 5; for body weights of wistar rats in grams. ^{a}, ^{b}, ^{c}, ^{d} denote significant variation ($p<0.05$).

Group 1 = Control, Group 2 = Bisphenol, Group 3 = Bisphenol + Melatonin, Group 4 = Bisphenol + Date Palm, Group 5 = Bisphenol + Melatonin + Date Palm.

Table 2: Mean Testicular Weight of Wistar Rats Treated with Bisphenol A, Melatonin and *Phoenix dactylifera*.

GROUPS	WEIGHT(g)
1	1.58 ± 0.18
2	1.78 ± 0.13^{d}
3	1.69 ± 0.15^{cd}
4	1.42 ± 0.21^{ab}
5	1.32 ± 0.22^{a}

Values expressed as mean ± SEM; n = 5; for testicular weights of wistar rats. [a], [b], [c], [d] denote significant variation ($p<0.05$).

Table 3: Mean Relative Weight of Wistar Rats Treated with Bisphenol A, Melatonin and *Phoenix dactylifera*.

GROUPS	RELATIVE WEIGHT(g)
1	0.116 ± 0.002
2	0.125 ± 0.002^{c}
3	0.107 ± 0.001^{bc}
4	0.097 ± 0.002^{ab}
5	0.053 ± 0.001^{a}

Values expressed as mean ± SEM; n = 5 in grams, for testicular weights of Wistar rats. [a], [b], [c], denote significant variation ($p<0.05$).

Effect of Melatonin and *Phoenix dactylifera* on Sperm Parameters (Motility, Morphology, Vitality and Count) of Bisphenol A Induced Toxicity in Wistar Rats: The results obtained in Table 6 indicate a significant decrease ($P<0.05$) in sperm motility, morphology, vitality and count in group 2 (Bisphenol) compared to the control group. There was no significant difference in sperm count and motility between the control, group 3 (Bisphenol + Melatonin), group 4 (Bisphenol + Date Palm) and group 5 (Bisphenol + Melatonin + Date Palm). Administration of Melatonin and Date Palm maintained the Spermatogenic characteristics as reflected in the sperm concentration and vitality compared with Bisphenol treated groups. A sperm is alleged to be morphologically abnormal if the head is detached.

Table 6: Mean Sperm Parameters (Motility, Morphology, Vitality and Count) of Wistar Rats Treatment with Bisphenol A, Melatonin and *Phoenix dactylifera*.

GROUP	MOTILITY (%)	MORPHOLOGY (%)	VITALITY	COUNT (x10ˆ6/ml)
1	36.50 ± 12.76	30.75 ± 6.99	29.75 ± 11.92	89.00 ± 5.07
2	25.05± 8.75*	13.50 ± 10.57*	12.25 ± 11.92*	51.00 ± 45.46*
3	38.00 ± 10.00	23.33 ± 13.33	21.67 ± 1.67	82 .00+ 6.20
4	42.50 ± 0.00	38.25 ± 7.76	25.61 ± 0.00	79.20 ± 0.50
5	40.00 ± 2.00	42.00 ± 2.16	28.05 ± 1.50	70.25 ± 14.69

Values expressed as mean ± SEM; n = 5; for semen analysis of Wistar rats. * denote significant variation (*p<0.05)* from control.

Histological Analysis of the Testes: The results obtained from the histological investigations of the testes showed photomicrograph sections of the testes of wistar rat from control group (group 1) given distill water, which revealed normal seminiferous tubules with sertoli cells, spermatogonia, primary spermatocytes and spermatids. The interstitium showed Leydig cells. Germ cell maturation is variable around the tubule as shown in figure 1. Figure 2 shows a photomicrograph section of seminiferous tubule of testes of wistar rats treated with bisphenol A alone (group 2), section showed sloughing of germ cell elements into tubule lumen showing hypolastic and degenerative changes in germ cells with intersitium, leydig cells lesions. Figure 3 shows a photomicrograph section of seminiferous tubule of testes of wistar rats treated with Bisphenol A plus melatonin (group 3). The section displayed recovered germ cells with less sloughing of sertoli cells and protected interstitium with Leydig cells. Figure 4 shows a photomicrograph section of testes of wistar rat treated with bisphenol A plus date palm (group 4). Section revealed recovered seminiferous tubules with sertoli cells, spermatogonia, primary spermatocytes and spermatids. The interstitium displayed Leydig cells and connective tissues. Figure 5 shows a photomicrograph section of testes of wistar rat from group given melatonin plus date palm and bisphenol A (group 5). Section showed seminiferous regenerated tubules with sertoli cells, spermatogonia, primary spermatocytes and spermatids. The interstitium connective tissues are regenerated with Leydig cells.

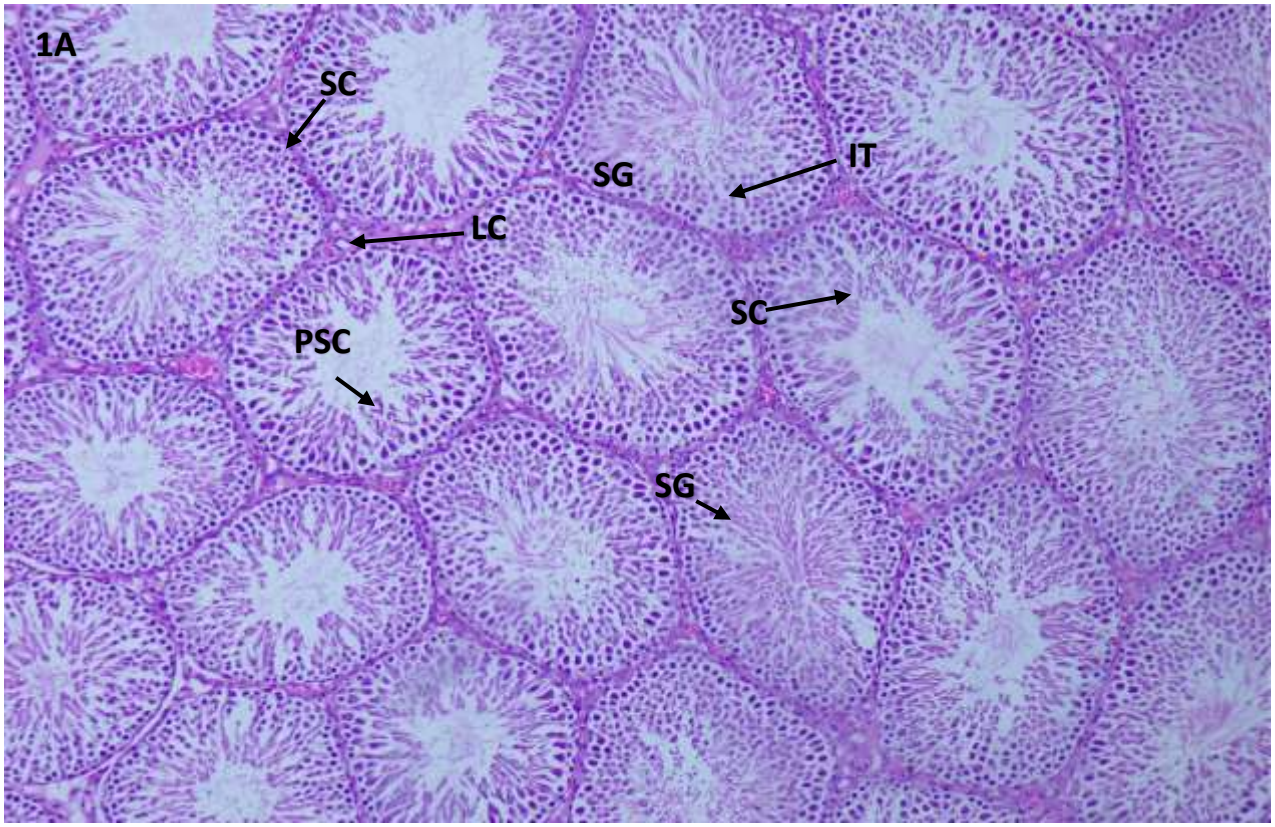

Figure 1: Photomicrograph section of testes of Wistar rat from control group given distill water. Section showed seminiferous tubules with sertoli cells (SC), spermatogonia, (SG) primary spermatocytes (SPC) and spermatids. The interstitium (IT) showed Leydig cells (LC). Germ cell maturation is variable around the tubule. H&E X100.

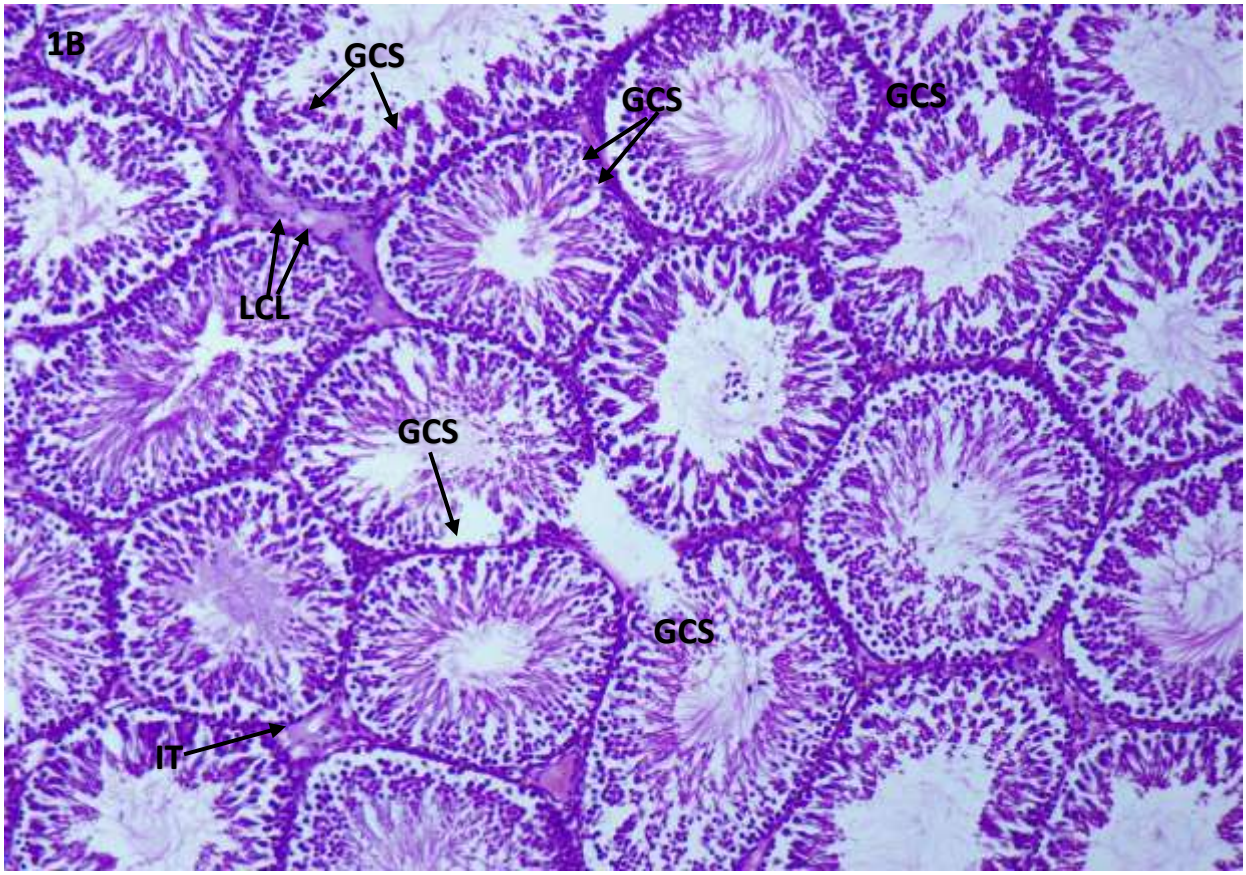

Figure 2: Photomicrograph section of seminiferous tubule of Testes of Wistar rats treated with Bisphenol A alone. Section showed artifactual sloughing of germ cell (GCS) elements into tubule lumen showing hypolastic and degenerative changes in germ cells and lesions (LS). IT (Intersitium), Leydig cells lesions (LCL). H&E X100.

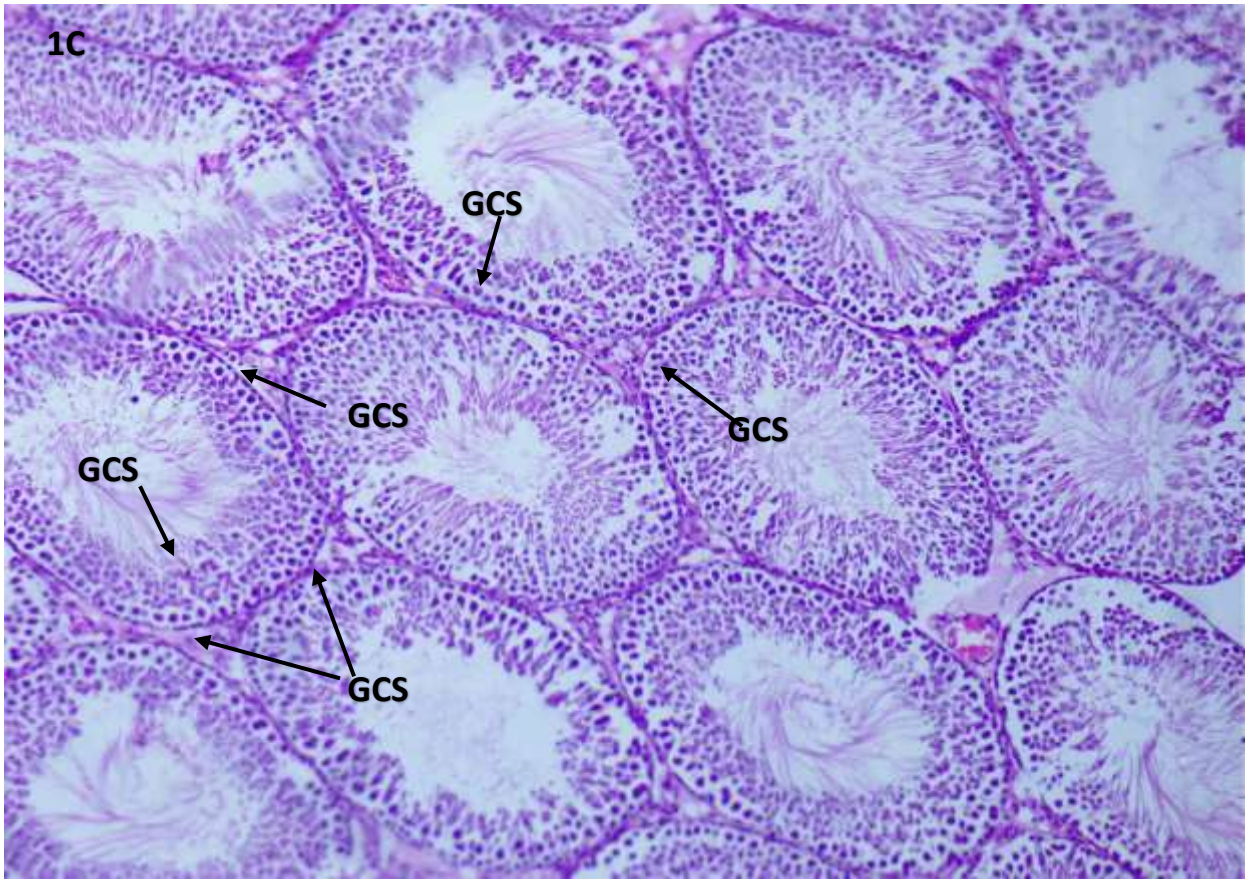

Figure 3: Photomicrograph section of seminiferous tubule of Testes of Wistar rats treated with Bisphenol A plus melatonin. Section displayed recovered germ cells with less sloughing of sertoli cells and protected interstitium with Leydig cells. H&E X100.

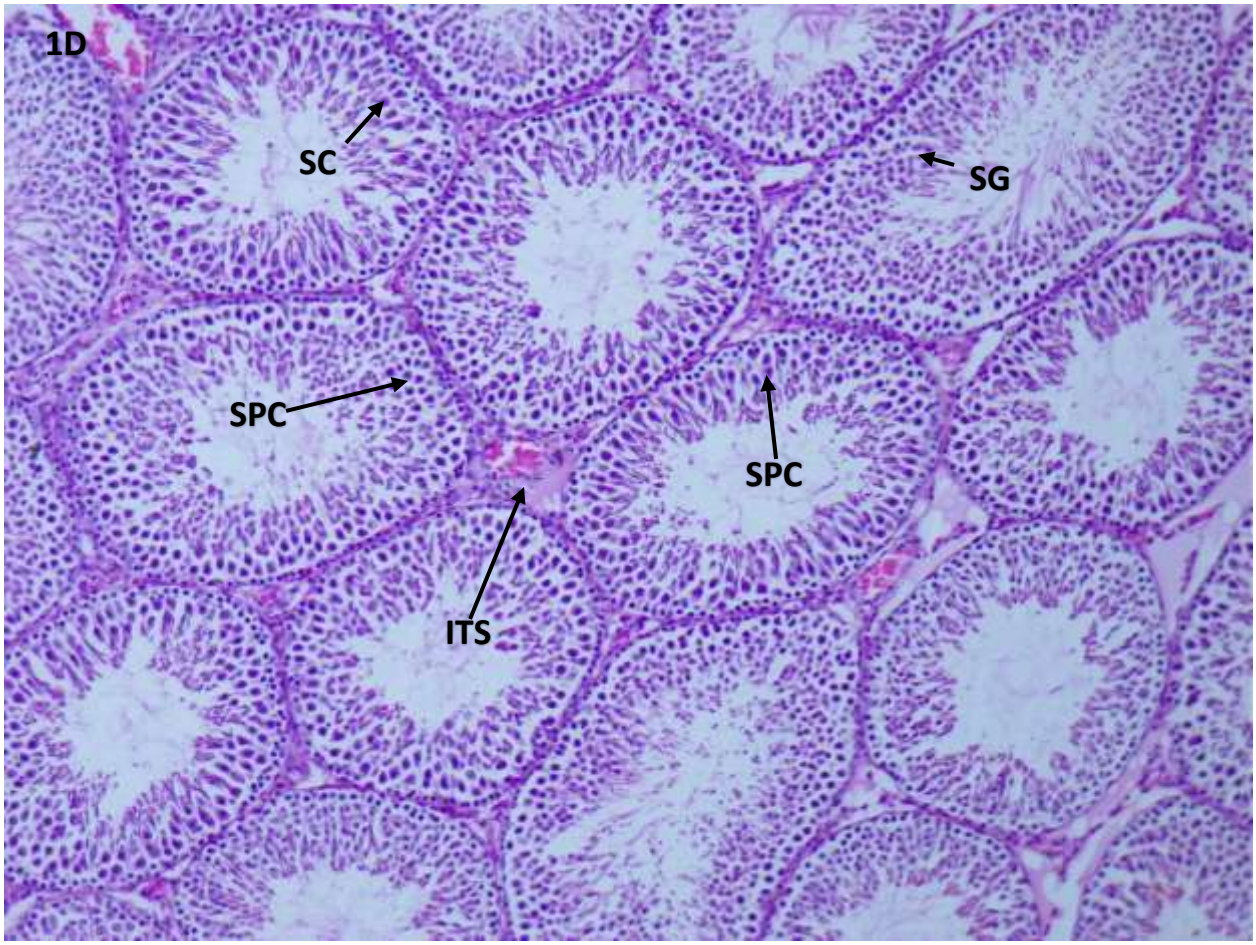

Figure 4: Photomicrograph section of testes of Wistar rat treated with Bisphenol A plus date Palm. Section showed recovered seminiferous tubules with sertoli cells (SC), spermatogonia, primary spermatocytes (SPC) and spermatids. The interstitium displayed Leydig cells (LC) and connective tissues. H&E X 100.

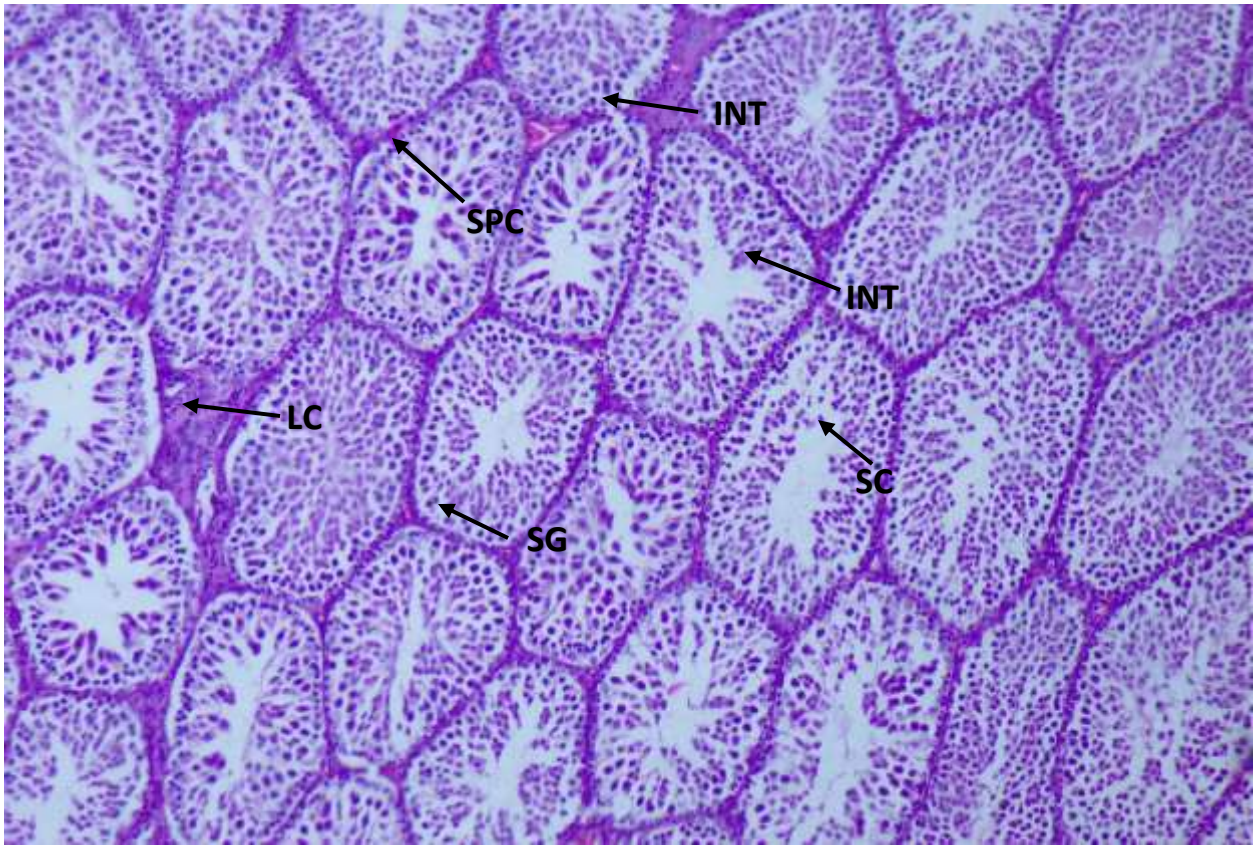

Figure 5: Photomicrograph section of testes of Wistar rat from given melatonin plus Date Palm and Bisphenol A. Section showed seminiferous regenerated tubules with sertoli cells (SC), spermatogonia (SG), primary spermatocytes (SPC) and spermatids. The interstitium (INT) connective tissues are regenerated (IT) with Leydig cells (LC). H&Ex100.

DISCUSSION

The present study showed that Bisphenol A (BFA) administration in adult male wistar rats is capable of inducing reproductive alterations that could lead to infertility, while the co-treatment with melatonin and *phoenix dactylifera* (date palm) ameliorates these pathological changes.

The findings showed an increase in body weight in the bisphenol A treated group which became progressive with the duration of administration compared to the control. This agrees with the findings of [23] and [25] who cited the positive association of bisphenol A (BFA) with obesity, as BFA is capable of stimulating adipocyte cell differentiation and hypertrophy leading to excess fat accumulation. Obesity has been shown to disrupt male fertility by causing changes in hormonal levels, poor sperm quality and sperm molecular composition as well as, impaired spermatogenesis and sexual dysfunction [26, 27]. Administration of melatonin in the treatments groups showed significant reduction in body weight. This is in agreement with the studies by [28]. Research work by [29] revealed that increased metabolism and anti-inflammatory effects of melatonin could be the cause of the weight reduction. Administration of date palm also caused reduction in body weight. This correlates with studies carried out by [30, 31, 32] who stated that dates contain high fibers and unsaturated fatty acids which helps to boost metabolism and reduced inflammation as seen in obesity.

The study revealed that the steroids, saponins and flavonoid contents of the extract might be the cause of this normalization as steroids are reported to decrease testosterone level. Flavonoids are also reported to decrease plasma testosterone levels in wistar rats [33, 34]. This is also an indication that the extract could affect spermatogenesis as testosterone is necessary for the normal development of spermatogenic cells [35].

Bisphenol A caused a reduction in sperm parameters such as morphology, motility, vitality, and cell count of spermatocytes significantly different from the control group. These observations supports the report made by [36] who revealed that exposure to BFA impairs proliferation of spermatogonia and spermatocytes, resulting in poor sperm quality. Previous studies also showed that BFA induced the reduction and degeneration of spermatocytes and other spermatogenic cells as well as disturbing steroidogenesis and spermatogenesis in testes of Wistar rats, which can lead to poor fertility outcomes [37,38].Melatonin treatment showed improvement in sperm morphology, motility, vitality, and cell count of spermatocytes when compared to the bisphenol A treated group by allowing the occurrence of normal spermatogenesis. This study is in agreement with the findings of [39, 40] and [41]

Melatonin therapy may be the most effective way to prevent the potential fertility related disorders caused by occupational and environmental exposure to BFA [40,42,43]. Administration of Date Palm maintained the spermatogenic characteristics in the testes of Wistar rats, this agrees with the findings of [21] who suggested the protective effects to be related to antioxidant compounds present in date palm. Reports indicate that date palm contain estradiol and flavonoid components, which have positive effects on the sperm quality and the scavenging properties of date palm is said to be the main important effects on the sperm parameters [44,45]. The result obtained from this present study confirmed that date palm has beneficial effects on sperm parameters in wistar rats. Therefore, it may be useful to solve infertility problems.

Results from the histological analysis revealed degenerative changes in the germ cells and leydig cells in the seminiferous tubules of the testes treated with bisphenol A when compared to the normal control

group. This agrees with the findings of [46, 47] who stated that BFA induced germinal cell debris and congestion in the testes. This effect was also demonstrated in previous studies conducted by [48, 38]. They all revealed that BFA disrupts spermatogenesis by inhibiting androgen production and reducing sertoli cell number and function and that bisphenol A exposure decreased the seminiferous tubule diameters and increased tubule atrophy and damage in the testes of Wistar rats according to reports published by [41]. The deleterious effects caused by BFA was reversed in the groups administered with Melatonin which showed recovering of the seminiferous tubules with sertoli cells, spermatogonia, primary spermatocytes and spermatids. This finding is consistent with the study from [49,50] who stated that melatonin improves histopathological alterations and allows the occurrence of normal spermatogenesis thereby, highlighting its importance in male reproductive system. Administration of date palm showed regenerated seminiferous tubules in the testes of Wistar rats. This correlates with the study carried out by [21] who suggested this protective effect to be associated with the antioxidant compounds present in date palm.

CONCLUSION

This study has shown that Melatonin and *Phoenix dactylifera* (Date Palm) has attenuating effects on the cellular and hormonal changes against Bisphenol A induced testicular and androgen toxicity in male wistar rats. These effects may be attributed to the antioxidants properties of melatonin and antioxidant compounds present in *Phoenix dactylifera* (date palm). The compounds ameliorate free radicals generated by Bisphenol A, hence increasing SOD and GPX levels in vivo. These cascades of reactions reduced the MDA levels, reversed the pathological changes in the testes and normalized hormonal levels of the male wistar rats.

RECOMMENDATION

Further studies should be carried out to evaluate the molecular mechanism of action and the likely pathways responsible for these antioxidant effect.
Individuals are advised to increase intake of dietary supplements rich in antioxidants compounds as a therapeutic approach for the prevention of these reproductive disorders. Finally, we recommend the limit of using products containing Bisphenol A.

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