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# MORPHOMETRIC CHANGES IN MALE REPRODUCTIVE ORGANS OF WISTAR RATS FOLLOWING COMBINED MELATONIN AND BISPHENOL-A TREATMENT

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**Ethical considerations:** When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

## ABSTRACT

**Bisphenol A (BPA) is a widespread endocrine disruptor known to cause reproductive toxicity. Melatonin has shown promise in ameliorating BPA-induced reproductive damage, but its effects on BPA-induced morphometric changes in reproductive tissues require further investigation. This study investigated the effects of combined melatonin and BPA administration on morphometry of male reproductive organs in Wistar rats. Twenty-four adult male Wistar rats were divided into four groups: control, BPA-treated (10 mg/kg/day), melatonin-treated (10 mg/kg/day), and BPA + melatonin-treated. After 45 days, testes, epididymides, and spermatozoa were analyzed for morphometric parameters. BPA significantly reduced epididymal and testicular tubular density compared to control ($p < 0.05$). Melatonin treatment alone increased epididymal germinal epithelial height and decreased luminal diameter ($p < 0.05$). The combined BPA + melatonin treatment showed the lowest epididymal luminal diameter ($p < 0.05$) and highest testicular tubular density. Spermatozoa head diameter was significantly reduced in the melatonin group compared to the BPA group ($p < 0.05$). The combined treatment group showed a significant decrease in entire sperm length compared to all other groups ($p < 0.05$). Melatonin administration mitigated BPA-induced morphometric anomalies in male rats, primarily by increasing testicular and epididymal tubular density and modulating epididymal luminal diameter. This study demonstrated morphometric effects of BPA and melatonin on male reproductive systems, but the molecular mechanisms remain unexplained. Future research should explore biochemical pathways and long-term reproductive impacts.**

**Keywords: Bisphenol A; melatonin; morphometry; reproductive toxicity**

## INTRODUCTION

Bisphenol-A (BPA) has received great attention due to its widespread presence in several consumer products and its adverse effects on reproduction [19]. Reproductive and endocrine disruptions are the major kinds of toxicity induced by BPA [1, 15]. Testicular toxicity induced by BPA has been reported and may account for the increasing frequency of infertility [4]. The reproductive toxicity of BPA is caused through multiple signaling pathways and may lead to the alteration in the process of spermatogenesis in rats [16].

Moreover, BPA reversibly perturbs the integrity of the blood-testis barrier in Sertoli cells *in vitro* [25]. It also induces uterotrophic effects in rats following high oral/and or subcutaneous dosing [6]. In rodents, developmental exposure to BPA leads to increased prostate weight [9], decreased epididymal weight and decreased sperm production [18]. Toxicity of BPA has been reported in mammals through increasing the hydroxyl-radical formation in the rat striatum, it also depletes the endogenous antioxidants in the epididymal sperms and causes defects in the hepatic detoxification mechanism of rats [17]. BPA exhibits endocrine like properties that raise concern about its suitability in consumer products and food containers. It is present in several products, including the interior coatings of food cans, milk containers and baby formula bottles, as well as in dental sealants [24]. It has also been reported to be present in water, feed, dust, and other related media [20, 21] by which animals are also at risk of exposure. When taken orally, it is readily absorbed from the digestive system and can be detected in various body fluids, organs and tissues.

Melatonin (N-acetyl-5-methoxytryptamine, MLT) has been reported to be useful in ameliorating BPA-induced reproductive toxicity in various studies [5]. In addition, Melatonin has been shown to possess antioxidant and prophylactic properties against oxidative stress in several experiments [2]. In addition to its use as an anti-stress, anti-aging, and immune-modulatory agent, MLT has also been used for sexual dysfunctions, gallbladder stones, obesity, and even tumors [13, 22].

Melatonin has demonstrated specific protective effects on testicular tissue and seminiferous epithelium in various studies. It has been shown to maintain the integrity of the seminiferous tubules by protecting Sertoli cells and developing germ cells from oxidative damage [2, 5]. Studies have demonstrated that melatonin helps preserve the height and organization of the seminiferous epithelium, supports spermatogenic cell development, and maintains the blood-testis barrier function [12]. The hormone acts directly on the testes through melatonin receptors present on Leydig cells and seminiferous tubules, helping to regulate local testosterone production and spermatogenesis [10]. Additionally, melatonin's powerful antioxidant properties protect the highly sensitive seminiferous epithelium from reactive oxygen species that can disrupt spermatogenesis [2, 13]. These specific effects on testicular tissue make melatonin a promising protective agent against reproductive toxicants like BPA that are known to disturb seminiferous epithelium structure and function [5, 12].

Although many studies have already shown how melatonin may exert its already well-documented beneficial effects in the treatment of a number of conditions [10, 12], a comprehensive analysis of melatonin's effects on BPA-induced morphometric alterations across the male reproductive system remains to be fully elucidated. Hence, the present work aimed at investigating the effects of combined melatonin and Bisphenol-A (BPA) administration on detailed morphometry of male reproductive organs in Wistar rats, with particular focus on testicular, epididymal, and spermatozoal parameters.

## MATERIALS AND METHODS

### Experimental animals

Twenty-four (24) adult male Wistar rats (n= 6) weighing between 160 ± 180g were used in this study. The animals were obtained from the Experimental Animal House of the Department of Physiology, University of Ibadan, Nigeria. The rats were housed in plastic cages (60*60*50 cm) where they were acclimatized for 14 days. All the rats were kept under controlled conditions of temperature (25 +/- 2 ˚C), relative humidity (50 +/- 5 %) and normal photoperiod (12 hour light and 12 hour dark). The rats were fed on a standard rat diet (commercial pellet diet) and water was provided *ad libitum*.

### Experimental Protocol

The twenty-four (24) male rats were randomly assigned into four (4) groups of six animals each. Group A: rats were orally administered 0.2 mL of olive oil, serving as control. Group B (BPA-intoxicated rats): rats received

a dose of 10 mg.kg$^{-1}$ body weight per day of BPA reconstituted in 0.2 mL olive oil, administered orally. Group C: rats received 10 mg.kg$^{-1}$ body weight of intraperitoneal melatonin (Sigma Aldrich, 98 % pure, dissolved in 0.5 % ethanol in normal saline). Group D (BPA-Melatonin treated rats): rats received orally administered BPA (10 mg.kg$^{-1}$ body weight per day) concomitantly with intraperitoneal melatonin (10 mg.kg$^{-1}$ body weight) [12]. At the end of the 45-day treatment, the rats were fasted overnight and weighed before sacrifice.

**Sample collection**

The rats were euthanized using an overdose of anesthetic ether [3]. The tunica vaginalis and tunica albuginea of the testes were removed exposing the testicles and epididymis. The testicles were then properly cleaned with tissue paper to remove traces of blood as much as possible in order to prevent interference with the procedure of spermatozoa harvest. The sperm cells were harvested from the cauda epididymis by cutting it open with a clean razor blade and gently placed on a warm glass slide.

**Morphometric Analysis (Testes, Epididymis and Spermatozoa)**

The testes and epididymis were harvested and weighed. The relative weights of these organs were obtained by standardizing them against the body weight of the rats. These organs were subsequently fixed in buffered neutral formalin solution and processed for histological evaluation. The tissues were embedded in melted paraffin wax, the wax block was then cut on a microtome to yield a thin slice of paraffin containing the tissue. The specimen slice was then applied to a microscope slide, air dried, and heated to cause the specimen to adhere to the glass slide. Residual paraffin was then dissolved which was usually followed by rinsing with acid-alcohol. This was rinsed with water to remove the acid-alcohol. Testes and epididymis (germinal height, luminal diameter and tubular density), the sperm (complete sperm length, head length and head diameter), were measured using analysis world Graph Pad Prism 5.01 software.

**Morphometric measurement of the testes (Germinal height, Tubular density and Luminal diameter)**

Micrographs of testes was taken at x 40 magnification to specify the parts of the organ, it was retaken at x 100 magnification in which the measurement was made. A straight-line measurement was made by placing the cursor on the basement membrane to the luminal border. About five to six measurements were made on different cell on the same slide with same magnification and recorded. Micrographs of the testes was taken at x 40 magnification after which the number of cells in a tubule was counted and recorded. About five to six counts of the tubules on a slide of the same magnification was made and recorded. Micrographs of the testes was taken at x 40 magnification to specify the parts of the organ to be captured. The capture was then taken at x 100 magnification after which the cells were measured using a straight-line measurement from one end of the basement membrane to the adjacent end.

**Morphometric measurement of the epididymis (Germinal height, Tubular density and Luminal diameter)**

Micrographs of epididymis were taken at x 40 magnification to specify the parts of the organ, it was retaken at x 100 magnification in which the measurement was made. A straight-line measurement was made by placing the cursor on the basement membrane to the luminal border. About five to six measurements were made on different cells on the same slide with same magnification and recorded. The micrographs of the epididymis were taken at x 40 magnification after which the number of cells in a tubule were counted and recorded. The micrographs of the epididymis were taken at x 40 magnification to specify the parts of the organ to be captured. The capture was then taken at x 100 magnification after which the cells were measured using a straight-line measurement from one end of the basement membrane to the adjacent end.

**Morphometric measurement of the sperm cell (Sperm length, Head length, Head diameter and Mid-piece length)**

The micrograph of the sperm was taken at x 100 magnification after which an irregular line measurement was used to get the length of the sperm, measured from the acrosome to the extreme end of the end piece). The micrograph of the head was taken at x 100 magnification, after which an irregular line measurement was used to get the length of the head, measured from the acrosomal head to the margin between the head and the mid-piece.

The micrograph of the sperm was taken at x 100 magnification after which a straight-line measurement was used to get the diameter of the sperm head, measured from one end to the adjacent head of the head.

The micrograph of the sperm was taken at x 100 magnification after which an irregular line measurement was used to get the length of the mid-piece, which is the junction between the head and the tail.

**Data analysis**

The Statistical Package for Social Sciences (SPSS®, version 26) was used for the analysis. The Shapiro-Wilk test was done and it showed that the parameters were Gaussian. Given the normal distribution of the data, parametric tests were employed for further analysis. The 4 groups were subjected to a One-Way Analysis of Variance (ANOVA) to compare their means. Specific group differences were identified by conducting post-hoc pairwise comparisons using Tukey's Honest Significant Difference (HSD) test. Each group's data is represented by mean ± standard deviation (SD), offering information on both the central tendency and dispersion. Mean values of $p \leq 0.05$ were considered significant.

## RESULTS

**Effect of melatonin against Bisphenol-A induced morphometric anomalies of epididymis of albino rats**

The results indicate that the epididymal germinal epithelial height did not differ significantly between the control group (A: 103.3 ± 13.0 µm) and the Bisphenol-A-treated group (B: 118.9 ± 11.8 µm) ($p > 0.05$). However, the melatonin-treated group (C: 189.3 ± 95.1 µm) exhibited a significantly greater germinal height than groups A and B ($p < 0.05$). The combination treatment group (D: 135.3 ± 17.4 µm) had an intermediate value, which was not statistically different from any other group ($p > 0.05$). In terms of epididymal luminal diameter, no significant differences were observed between the control (A: 1088.0 ± 45.3 µm) and Bisphenol-A (B: 1002.3 ± 73.5 µm) groups ($p > 0.05$). However, the melatonin group (C: 855.2 ± 207.3 µm) exhibited a significant reduction in diameter compared to groups A and B ($p < 0.05$), while the combination group (D: 585.7 ± 32.0 µm) had the lowest luminal diameter, which was significantly different from all other groups ($p \leq 0.05$). Regarding epididymal tubular density, the Bisphenol-A group (B: 10.3 ± 0.6) had a significantly lower density compared to all other groups ($p < 0.05$). The control (A: 16.0 ± 1.73), melatonin (C: 16.0 ± 1.0), and combination treatment (D: 19.0 ± 2.0) groups did not differ significantly from each other ($p > 0.05$) (Table 1).

**Effect of melatonin against Bisphenol-A induced morphometric anomalies of testes of albino rats**

The results for the height of the germinal epithelium showed no significant differences across the groups ($p > 0.05$). The combined treatment group (D: 311.2 ± 39.4 µm) had the lowest value, while the melatonin-treated group (C: 326.6 ± 31.5 µm) had the highest. For testicular luminal diameter, the control (A: 1131.8 ± 17.4 µm), Bisphenol-A (B: 1012.5 ± 66.6 µm), and combined treatment (D: 1077.4 ± 40.5 µm) groups did not differ significantly from one another ($p > 0.05$). However, the melatonin group (C: 890.0 ± 265.7 µm) showed a significant decrease compared to the control ($p \leq 0.05$), though it did not differ significantly from the Bisphenol-A or combined treatment groups ($p > 0.05$). Regarding testicular tubular density, the control group (A: 16.3 ± 1.5) was not significantly different from the melatonin (C: 14.0 ± 1.0) or combined treatment (D: 18.3 ± 2.1) groups ($p > 0.05$). However, the Bisphenol-A group (B: 11.0 ± 1.7) showed a significant decrease compared to the control and combined treatment groups ($p < 0.05$) but was not significantly different from the melatonin group ($p > 0.05$). The combined treatment group (D) had the highest value, which was significantly different from the Bisphenol-A group ($p < 0.05$) (Table 2).

**Effect of melatonin against Bisphenol-A induced morphometric anomalies of spermatozoa of albino rats**

The spermatozoa head diameter in the melatonin group (C: 34.9 ± 0.8 µm) was significantly reduced compared to

**Table 1. Effect of melatonin against Bisphenol-A induced morphometric anomalies of epididymis of albino rats**

| Parameters | Group A (mean ± SD) | Group B (mean ± SD) | Group C (mean ± SD) | Group D (mean ± SD) |
|---|---|---|---|---|
| Epididymal germinal epithelial height (µm) | $103.3 \pm 13.0^{a}$ | $118.9 \pm 11.8^{a}$ | $189.3 \pm 95.1^{b}$ | $135.3 \pm 17.4^{ab}$ |
| Epididymal luminal diameter (µm) | $1088.0 \pm 45.3^{a}$ | $1002.3 \pm 73.5^{a}$ | $855.2 \pm 207.3^{b}$ | $585.7 \pm 32.0^{c}$ |
| Epididymal tubular density | $16.0 \pm 1.73^{a}$ | $10.3 \pm 0.6^{b}$ | $16.0 \pm 1.0^{a}$ | $19.0 \pm 2.0^{a}$ |

*Values with different superscript ($^{a,b,c}$) letters are significantly different ($p \leq 0.05$).*

**Table 2. Effect of melatonin against Bisphenol-A induced morphometric anomalies of testes of albino rats**

| Parameters | Group A (mean ± SD) | Group B (mean ± SD) | Group C (mean ± SD) | Group D (mean ± SD) |
|---|---|---|---|---|
| The height of the germinal epithelium (μm) | 317.8 ± 54.4 | 315.2 ± 42.5 | 326.6 ± 31.5 | 311.2 ± 39.4 |
| Testicular luminal diameter (μm) | 1131.8 ± 17.4$^{a}$ | 1012.5 ± 66.6$^{ab}$ | 890.0 ± 265.7$^{b}$ | 1077.4 ± 40.5$^{a}$ |
| Testicular tubular density | 16.3 ± 1.5$^{ac}$ | 11.0 ± 1.7$^{b}$ | 14.0 ± 1.0$^{ab}$ | 18.3 ± 2.1$^{c}$ |

*Values with different superscript ($^{a,b,c}$) letters are significantly different ($p \leq 0.05$).*

the Bisphenol-A group (B: 45.3 ± 6.9 μm) ($p < 0.05$). The control (A: 37.5 ± 1.6 μm) and combined treatment (D: 37.9 ± 1.0 μm) groups did not differ significantly from any other group ($p > 0.05$). For spermatozoa head length, no significant differences were observed across the groups ($p > 0.05$), with values ranging from 225.5 ± 5.7 μm in the melatonin group (C) to 249.7 ± 5.8 μm in the Bisphenol-A group (B). Similarly, spermatozoa mid-piece length did not show significant differences among groups ($p > 0.05$), with values ranging from 293.8 ± 32.1 μm in the control group (A) to 320.0 ± 24.5 μm in the Bisphenol-A group (B). In terms of entire sperm length, the combined treatment group (D: 839.2 ± 77.2 μm) displayed a significant decrease compared to all other groups ($p < 0.05$). The control (A: 920.3 ± 47.3 μm), Bisphenol-A (B: 874.2 ± 52.4 μm), and melatonin (C: 898.6 ± 38.0 μm) groups were not significantly different from each other ($p > 0.05$) (Table 3).

## DISCUSSION

Results from the present study indicated significant levels of variations, especially in the epididymal parameters for the different treatment groups. The melatonin-treated group had an epididymal germinal epithelial height that is significantly increased as compared with what was seen in both control and BPA-treated groups. Such could potentially indicate that melatonin has a stimulatory effect on the epididymal epithelium. These agree with other studies that were reported by Q i et al. [14] where melatonin promoted the proliferation and migration of the epithelial cells. The most striking result was that the epididymal luminal diameter was significantly reduced only in the BPA + melatonin group when compared to all other groups; this may be considered as a suspected synergistic action of BPA and melatonin on the epididymis, possibly affecting sperm maturation and storage. Similar structural changes in the epididymis following BPA exposure have been reported by G u r m e e t et al. [7], who observed decreased epithelial height and increased lumen diameter in rats exposed to BPA. BPA treatment significantly reduced epididymal tubular density, consistent with previous studies. For instance, O l u k o l e et al. [11] demonstrated that BPA exposure caused degenerative changes in the epididymis, including a reduced tubular density. Thus, maintenance of tubular density by melatonin treatment alone or combined with BPA at the level comparable to the control group suggests a protective role of melatonin against BPA-induced damage.

Regarding testicular parameters, no differences were statistically significant in the height of the germinal epithelium among all groups. Thus, it may indicate that neither BPA nor melatonin has succeeded in showing significant changes in the thickness of the seminiferous epithelium during this study. However, the result obtained disagreed with some earlier studies. For instance, J i n et al. [8] indicated that mice had a decrease in the height of the seminiferous epithelium following BPA exposure. There was, however, a significant decrease in the testicular luminal diameter in the melatonin-treated group when compared with the control, which was not evident in the BPA and BPA + melatonin groups. What this actually means bio-

**Table 3. Effect of melatonin against Bisphenol-A induced morphometric anomalies of spermatozoa of albino rats**

| Parameters | Group A (mean ± SD) | Group B (mean ± SD) | Group C (mean ± SD) | Group D (mean ± SD) |
|---|---|---|---|---|
| Spermatozoa head diameter (μm) | 37.5 ± 1.6$^{abc}$ | 45.3 ± 6.9$^{ab}$ | 34.9 ± 0.8$^{c}$ | 37.9 ± 1.0$^{abc}$ |
| Spermatozoa head length (μm) | 225.9 ± 10.5 | 249.7 ± 5.8 | 225.5 ± 5.7 | 237.0 ± 30.3 |
| Spermatozoa mid piece length (μm) | 293.8 ± 32.1 | 320.0 ± 24.5 | 305.5 ± 3.2 | 296.7 ± 12.6 |
| Entire sperm length (μm) | 920.3 ± 47.3$^{a}$ | 874.2 ± 52.4$^{a}$ | 898.6 ± 38.0$^{a}$ | 839.2 ± 77.2$^{b}$ |

*Values with different superscript ($^{a,b,c}$) letters are significantly different ($p < 0.05$)*

logically remains speculative and as such requires further study. Melatonin may affect the fluid dynamics in the seminiferous tubules; however, this is purely hypothetical and needs to be verified in future. BPA treatment significantly reduced testicular tubular density compared to the control and BPA + melatonin groups, aligning with previous studies showing that BPA exposure can lead to testicular damage. For example, T a i n a k a et al. [23] observed that prenatal exposure to BPA resulted in reduced seminiferous tubule volume in mice. The BPA + melatonin group had the highest tubular density, suggesting a potential protective effect of melatonin against BPA-induced testicular damage.

Regarding spermatozoa parameters, the melatonin-treated group showed a significantly reduced spermatozoa head diameter compared to the BPA-treated group, indicating that melatonin might influence sperm head morphology. No significant differences were observed in spermatozoa head length or mid-piece length across the groups. The BPA + melatonin group showed a significant decrease in entire sperm length compared to all other groups, suggesting a potential interaction between BPA and melatonin that affects sperm morphology, which could impact sperm motility and fertility.

## CONCLUSION

In conclusion, melatonin mitigates some of the morphometric changes induced by BPA in male rats with respect to the maintenance of the density of both the epididymal and testicular tubules. However, the study again revealed unexpected effects when BPA and melatonin were administered together – for example, the significant reduction in the diameter of epididymal lumina and the whole length of sperm with regard to the BPA + melatonin group. This study has indeed shown the morphometric effects of BPA and melatonin on the male reproductive system, but it has not explained 'how' it is done at a molecular level. Thus, future studies may investigate the biochemical and molecular pathways through which these interactions occur and further determine the long-term implications of such morphological changes with regard to reproductive function and fertility.

## ETHICAL APPROVAL

All the animals (rats) used in this study were handled in accordance with good animal practice requirements of the Animal Ethics Procedures and Guidelines with approval obtained from the Animal Care and Use Research Ethics Committee (UI-ACUREC /17/0069) of the University of Ibadan.

## CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

## DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

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## AUTHORS CONTRIBUTIONS

All authors contributed to the study revision. Material preparation, data collection and analysis were performed by Olumide Samuel Ajani, Dideolu Osunkoya, and Olumide Odunayo Akinniyi. All authors read and approved the final manuscript.

## GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

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