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Mitochondrial oxidative damage by co-exposure to bisphenol A and acetaminophen in rat testes and its amelioration by melatonin

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Objective: Human exposure to multiple xenobiotics, over various developmental windows, results in adverse health effects arising from these concomitant exposures. Humans are widely exposed to bisphenol A, and acetaminophen is the most commonly used over-the-counter drug worldwide. Bisphenol A is a well-recognized male reproductive toxicant, and increasing evidence suggests that acetaminophen is also detrimental to the male reproductive system. The recent recognition of male reproductive system dysfunction in conditions of suboptimal reproductive outcomes makes it crucial to investigate the contributions of toxicant exposures to infertility and sub-fertility. We aimed to identify toxicity in the male reproductive system at the mitochondrial level in response to co-exposure to bisphenol A and acetaminophen, and we investigated whether melatonin ameliorated this toxicity.
Methods: Male Wistar rats were divided into six groups (n=10 each): a control group and groups that received melatonin, bisphenol A, acetaminophen, bisphenol A and acetaminophen, and bisphenol A and acetaminophen with melatonin treatment.
Results: Significantly higher lipid peroxidation was observed in the testicular mitochondria and sperm in the treatment groups than in the control group. Levels of glutathione and the activities of catalase, glutathione peroxidase, glutathione reductase, and manganese superoxide dismutase decreased significantly in response to the toxicant treatments. Likewise, the toxicant treatments significantly decreased the sperm count and motility, while significantly increasing sperm mortality. Melatonin mitigated the adverse effects of bisphenol A and acetaminophen.
Conclusion: Co-exposure to bisphenol A and acetaminophen elevated oxidative stress in the testicular mitochondria, and this effect was alleviated by melatonin.

Keywords: Acetaminophen; Bisphenol A; Mitochondria; Oxidative stress; Spermatozoa; Testis

Introduction

The widespread exposure of humans to xenoestrogens and endocrine-disrupting chemicals (EDCs) has led to a number of deleterious health effects, among which declining fertility is of grave concern. Decreased male reproductive capacity is on the increase, accounting for half of the cases of infertility, with a frequency of one in every 20 men in the general population [1]. Exposure to EDCs has been established as one of the reasons for decreased male fertility. EDCs are a variety of exogenous and anthropogenic chemicals, which are known to disrupt endocrine function [2]. These chemicals are responsible for various metabolic, reproductive, immune, and behavioral disorders, the prevalence of which has increased over the last few decades. The exposure to EDCs is increasing with the rise in con-

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sumerism and the modern lifestyle [3].

Humans may be exposed to multiple EDCs during their lifetime or during a particular window of development. One of the EDCs to which humans are most frequently exposed is bisphenol A (BPA). BPA is a plasticizer and is a well-known EDC that exerts its endocrine-disrupting effects chiefly by binding both the nuclear estrogen receptors (ERs): ERα and ERβ. The testes are a sensitive site for BPA, as BPA inhibits testicular steroidogenesis acting presumably via the ER [4]. Apart from altering steroidogenesis, BPA is well-known to induce reactive oxygen species (ROS) and cause oxidative damage in testicular tissues. BPA is known to increase hydroxyl-radical formation in the striatum and deplete the endogenous antioxidants in epididymal sperm [5,6]. It has also been reported to have an impact on Leydig cell steroidogenesis by affecting 17α-hydroxylase/17,20-lyase and aromatase expression and interfering with luteinizing hormone receptor-ligand binding [7]. BPA also causes elevated ROS in rat epididymal sperm, with a negative influence on sperm parameters [8].

Another xenobiotic that is widely used and increasingly suspected as an EDC is acetaminophen (APAP) [9]. It is most widely used as a mild analgesic and antipyretic and is commonly used by women during pregnancy. Regular usage of the drug predisposes to drug toxicity, especially endocrine disruption. A number of studies have indicated that APAP is an EDC. In a murine model, treatment with APAP for 30 consecutive days caused a decrease in libido, sexual vigor, and the fertility index in male rats [10]. Van den Driesche et al. [11] reported that prolonged use of APAP for 1 week may suppress fetal testosterone production in castrated host mice bearing human fetal testis xenografts. Gestational exposure to APAP also leads to a decrease in fetal testosterone production in the rat and human fetal testis, which can result in an increased risk of cryptorchidism following maternal use of APAP during the advanced first or early second trimester of pregnancy [11]. Lister and McLean [12] reported that treatment with APAP was associated with an inhibitory effect on DNA synthesis in testicular tissue in rats. All these studies indicate the deleterious effects of APAP on testicular tissue. Since humans are exposed to BPA for most of their lives and APAP is also widely consumed by all age groups and both genders, an undesirable effect may be caused by co-exposure to both substances. In the current study, we hypothesized that co-administration of BPA and APAP could increase oxidative stress in the testicular mitochondria and that melatonin (MEL) may mitigate this increase in oxidative stress. Sperm parameters were assessed as endpoints of toxicity and amelioration.

Methods

1. Animals and experimental protocol

Sixty male Wistar rats (100 ± 15 g) were obtained from the Animal House Facility of Jazan University. Animals were provided *ad libitum* with drinking water and a standard pelleted diet and were reared and maintained according to standard laboratory conditions (12-hour light and 12-hour dark cycle; temperature 25°C ± 2°C). Animals were house-acclimatized for a period of 1 week and divided into six groups of 10 animals each. BPA was given orally through drinking water, APAP was given intraperitoneally, and MEL was administered orally. The groups were as follows: (1) control; (2) MEL; (3) BPA (10 ppm); (4) APAP (500 mg/kg); (5) BPA (10 ppm) and APAP (500 mg/kg); and (6) BPA (10 ppm), APAP (500 mg/kg), and MEL (20 mg/kg/day). Dosing was carried out for 4 weeks.

2. Necropsy and tissue preparation

Animals were sacrificed under anesthesia. Testes were isolated and cleared of the adhering tissues with an ice-cold isolation medium (0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4). Testes were blotted dry and weighed. To obtain sperm from the epididymis, the caudae were excised, rinsed with ice-cold normal saline, and teased, followed by gentle mincing in phosphate-buffered saline (PBS). The fragments were allowed to sediment, and the sperm were filtered and collected for further evaluation.

3. Isolation of mitochondria

Mitochondria were isolated according to the method of Sayeed et al. [13]. The process was carried out at 4°C on ice. The testes were minced in a fresh isolation medium and were manually homogenized in 10% w/v in an isolation medium. Centrifugation of the homogenate was carried out at 500 × *g* for 10 minutes. The supernatant was retained, and the pellet was washed with fresh isolation medium and recovered by the initial supernatant fraction. The pooled fractions were centrifuged at 500 × *g* for 10 minutes, and the final supernatant obtained was centrifuged at 5,000 × *g* for 15 minutes to obtain the mitochondrial pellet. This pellet was washed with an isolation medium, followed by a respiration reaction buffer. The final purified mitochondrial pellet was obtained by centrifugation at 12,000 × *g* for 10 minutes and was re-suspended in a respiration reaction buffer (1 mL/g tissue) to produce a suspension containing 25 to 40 mg of mitochondrial pellet/mL.

4. Measurement of oxidative stress

1) Measurement of lipid peroxidation

The method as described by Buege and Aust [14] was used to measure lipid peroxidation (LPO) in the testicular mitochondria. An equal volume of the 4 mg/mL mitochondrial preparation and Buege and Aust [14] reagent was mixed and heated for 15 minutes in boiling water. Once cooled, it was centrifuged at 1,000 × *g* for 10 minutes at room temperature and the precipitate was removed. Absor-

bance was measured at 532 nm. Thiobarbituric acid reactive substances (TBARS) were quantified using an extinction coefficient of 1.56×10^5/M•cm and expressed as nmol of TBARS/mg mitochondrial protein.

2) Measurement of reduced glutathione

Reduced glutathione (GSH) was measured by the method described by Tabassum et al. [15]. In this method, 1 mL of sulfosalicylic acid (4%) was used to precipitate the mitochondrial suspension. The suspensions were kept at 4°C for 1 hour and then centrifuged for 15 minutes at 4°C at 1,200 $\times g$. The assay mixture consisted of 0.4 mL mitochondrial suspension, 2.2 mL sodium phosphate buffer (pH 7.4), and 0.4 mL 5,5′-dithiobis-(2-nitrobenzoic acid), with 3 mL as the total volume. The absorbance of the reaction product was read on a spectrophotometer at 412 nm and the GSH content was expressed as micromoles of GSH per gram of tissue.

3) Measurement of antioxidant enzymes in testicular mitochondria

Glutathione peroxidase (GPx) activity was assessed by the method of Flohe and Gunzler [16]. Mitochondria (freshly isolated) were suspended in 1 mL of buffer (5 mM EDTA, 50 mM Tris HCl, 1 mM GSH, 0.22 mM nicotinamide adenine dinucleotide phosphate [NADPH], and glutathione reductase [GR]; pH 7.6). The reaction was initiated by adding tertiary butyl hydroperoxide to a final concentration of 0.22 mM. The activity of the enzyme was calculated as nanomoles of NADPH oxidized per minute per milligram of protein using a molar extinction coefficient of 6.22×10^3/M•cm.

The GR activity was assessed by the method of McFarland et al. [17]. The mitochondrial pellet, homogenizing buffer (250 mM sucrose, 80 mM Tris HCl, 5 mM $MgCl_2$, 250 mM KCl, and 1 mM EDTA; pH 7.4), assay mixture (0.2 M sodium phosphate buffer, 1 mM oxidized glutathione, and 0.1 mM NADPH) in a total volume of 2 mL constituted the reaction mixture. The activity of the enzyme was measured with the disappearance of NADPH at 340 nm and was calculated as nanomoles NADPH consumed per minute per milligram of protein using a molar extinction coefficient of 6.22×10^3/M•cm.

Catalase (CAT) activity was assessed by the method of Claiborne [18] (slightly modified). The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.4), 0.05 M H_2O_2, and 0.05 mL of mitochondria obtained from a 10% homogenate of testicular tissue. The change in the absorbance of the reaction mixture was recorded kinetically at 240 nm. The activity of the enzyme was calculated as micromoles of H_2O_2 consumed per minute per milligram of protein using a molar extinction coefficient of 39.6/M•cm.

Manganese superoxide dismutase (MnSOD) activity was assessed by the method of Govil et al. [19]. In this method, 0.2 mL of mitochondrial suspension was treated with 0.8 mL of 50 mM glycine buffer (pH 10.4) and 0.020 mL of epinephrine. The activity of MnSOD was measured kinetically at 480 nm, and the oxidized product of epinephrine was indirectly measured. The activity of MnSOD was expressed as nanomoles of (–) epinephrine prevented from oxidation per minute per milligram of protein using a molar extinction coefficient of 4,020/M•cm.

5. Sperm parameters

1) Sperm count

The sperm count was measured as described by Bairy et al. [20]. The epididymal sperm suspension was prepared in 1 mL of PBS at pH 7.2. The sperm count was determined in a hemocytometer. An aliquot from the suspension (1 mL) was diluted 1:40 with PBS. A sample of the diluted suspension was charged into a counting chamber (Neubauer's chamber). The total sperm count in eight squares, excluding the central erythrocyte area of 1 mm^2 each, was determined and multiplied by 5×10^4 to obtain the total count.

2) Sperm mortality

Sperm mortality was assessed by the method of Correia et al. [21]. A drop of the epididymal sperm suspension was put on a glass slide and mixed with 3% eosin, followed by mixing it with 10% nigrosin. A small quantity of this was taken and smeared on another slide and let to air-dry. In total, 200 sperm cells from each rat from each group were counted, and the number of live and dead sperm cells was recorded. Using this method, live sperm cells have a whitish color, while dead sperm cells appear pinkish.

3) Sperm motility

The progressive motility of the cauda epididymal sperm was evaluated following the procedure described by Saalu et al. [22]. The fluid obtained from the cauda epididymis was diluted to 2 mL with Tris buffer solution. A slide was placed on a phase contrast microscope, and an aliquot of this solution was placed on the slide. At a magnification of $\times 400$, the percent motility was visually evaluated. Motility estimations were performed in each sample from three different fields. The final motility score was calculated using the mean of the three estimations. The samples for motility testing were kept at 35°C. Sperm motility was recorded as a percentage (%).

6. Protein estimation

Protein content was measured using the method of Lowry et al. [23].

7. Data presentation and statistical analysis

The values are expressed as mean ± standard error. One-way analysis of variance was applied to determine the significance of differ-

ences in the results of various groups compared to the respective controls. A p-value < 0.05 was considered significant. Subsequently, the Tukey test was applied to analyze the significance of changes between various groups.

8. Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Jazan University (number REC42/1/028).

Results

1. Body and organ weight

The various treatments caused no significant changes in the animals' body and organ weights. Furthermore, no mortality was observed during the treatments (Table 1).

2. Lipid peroxidation

Treatment with MEL decreased LPO in the testicular mitochondria when compared to the control. However, treatment with BPA ($p < 0.01$) and APAP ($p < 0.05$) caused a significant increase in the LPO in testicular mitochondria when compared to the control. This increase was significant in the co-treatment group ($p < 0.01$), which had higher basal values. Treatment with MEL was observed to lower LPO significantly when compared to the BPA, APAP, and co-treatment groups ($p < 0.01$) (Figure 1).

3. Glutathione

Treatment with MEL only caused a significant increase in GSH levels when compared to the control. A significant decrease was observed in the GSH levels in the APAP, BPA, and APAP+BPA ($p < 0.01$) treatment groups when compared to the control, with the APAP+BPA group ($p < 0.01$) having a lower basal value. Significant differences were also observed between the BPA and APAP+BPA ($p < 0.01$) and between the APAP and APAP+BPA ($p < 0.01$) groups. Treatment with MEL caused a significant increase in the GSH levels when compared to the APAP ($p < 0.01$), BPA ($p < 0.01$), and APAP+BPA ($p < 0.01$) groups (Figure 2).

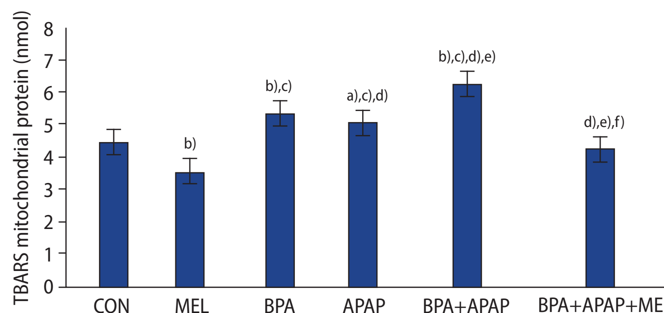

Figure 1. Effects of various treatments on lipid peroxidation in the testicular mitochondria of bisphenol A (BPA)-treated rats. Values are mean±standard error (n=10). Values are expressed as nmol of thiobarbituric acid reactive substances (TBARS)/mg mitochondrial protein. Significant differences are indicated by [a)]p<0.05 or [b)]p<0.01 when compared to control (CON); [c)]p<0.01 when compared to melatonin (MEL); [d)]p<0.05 when compared to BPA; [e)]p<0.01 when compared to acetaminophen (APAP); [f)]p<0.01 when compared to BPA+APAP.

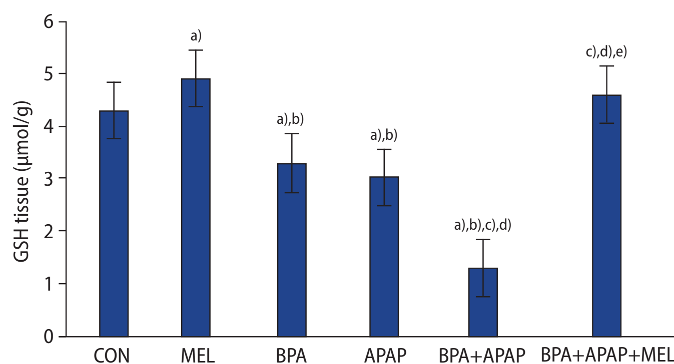

Figure 2. Effect of various treatments on reduced glutathione (GSH) levels in the testes of bisphenol A (BPA)-treated rats. Values are mean±standard error (n=10). Values were expressed as micromoles of GSH per gram of tissue. Significant differences are indicated by [a)]p<0.05 when compared to control; [b)]p<0.01 when compared to melatonin (MEL); [c)]p<0.05 when compared to BPA; [d)]p<0.01 when compared to acetaminophen (APAP); [e)]p<0.01 when compared to BPA+APAP.

Table 1. Changes in body weight, testicular weight, and epididymal weight in rats treated with melatonin and BPA for 4 weeks

	Control	MEL	BPA	APAP	APAP+BPA	APAP+BPA +melatonin
Change in body weight (g)	9.0±3	4.9 ±0.4	2.0±5	5.0±0	6±3.3	4.3±5
Testis (g)	1.8±0.4	1.3±0.2	1.7±0.2	2.8±0.5	2.6±1.5	1.84±0.3
Epididymis (mg)	134.7±10	123±3	133±12	131.7±13	134.4±17	138.4±11.7

Values are presented as mean±standard deviation of 10 animals each. No significant changes in body weight and testicular weight were observed between the control and treatment groups.
BPA, bisphenol A; MEL, melatonin; APAP, acetaminophen.

4. Enzymatic antioxidants of testes mitochondria.

The activity of the antioxidant enzymes significantly decreased in the treatment groups, and the basal values of the co-treatment group were lower than those of other groups. MEL also increased the activity of the enzymes.

5. Catalase

APAP, BPA, and APAP+BPA treatments led to a significant decrease in the CAT activity of testes mitochondria when compared to the control ($p < 0.05$ to $p < 0.01$). However, no decrease was found in CAT activity in the APA+BPA+MEL treatment group when compared to the control. Significantly lower CAT activity was also observed in the APAP+BPA treatment group than in the BPA ($p < 0.01$) and APAP ($p < 0.01$) treatment groups. MEL treatment significantly increased CAT activity as compared to the APAP+BPA group ($p < 0.05$). However, treatment with MEL only increased CAT activity significantly when compared to the control ($p < 0.05$) (Table 2).

6. Glutathione peroxidase

A significant decrease was observed in GPx activity in all the treatment groups when compared to the control ($p < 0.01$), except for the APAP+BPA+MEL treatment group, which did not show a significant difference from the control group. A significant difference was also observed between each of the BPA and APAP treatments and the combined APAP+BPA treatment ($p < 0.01$). Co-treatment of MEL with BPA+APAP significantly attenuated the decrease in GPx activity when compared to APAP+BPA treatment ($p < 0.01$). Treatment with MEL alone significantly increased GPx activity in the testicular mitochondria ($p < 0.05$) (Table 2).

7. Glutathione reductase

A significant decrease in GR activity was observed in response to BPA ($p < 0.01$), APAP ($p < 0.05$), and APAP+BPA ($p < 0.01$) treatment when compared to controls. No significant difference was observed between the MEL+APAP+BPA treatment group and the control. The APAP+BPA group also showed significant differences from the APAP ($p < 0.05$) and BPA ($p < 0.05$) groups. The MEL+APAP+BPA group had a significantly lower decrease in GR activity than the APAP+BPA treatment group ($p < 0.01$). Treatment with MEL alone significantly increased the activity of GR when compared to the control ($p < 0.05$) (Table 2).

8. Manganese superoxide dismutase

The activity of MnSOD significantly decreased with APAP, BPA, and APAP+BPA treatments when compared to the control ($p < 0.01$). The MEL+APAP+BPA group, however, had no significant reduction as compared to the control. Significant decreases in MnSOD in the APAP ($p < 0.01$) and BPA ($p < 0.01$) treatment groups were also observed when compared to the APAP+BPA group. MEL treatment significantly attenuated the decrease in MnSOD activity when compared to the APAP+BPA group ($p < 0.01$). Treatment with MEL alone caused a significant increase in MnSOD activity in the testicular mitochondria ($p < 0.05$) (Table 2).

9. Sperm parameters

Sperm count, mortality, and motility were adversely affected by BPA, APAP, and their co-treatment. MEL ameliorated the adverse effects of the treatments. Sperm count significantly decreased in response to BPA ($p < 0.01$), APAP ($p < 0.01$), and BPA+APAP ($p < 0.01$) treatments when compared to the control. However, MEL treatment attenuated this decrease, as no significant decrease was observed in the BPA+APAP+MEL group compared with the control. A significant increase in sperm mortality was also seen in the BPA+APAP treatment group ($p < 0.01$) when compared to the controls. However, MEL treatment reduced this increase in mortality, and no significant difference was observed between the control and the BPA+APAP+MEL group. A significant decrease was seen in the motility of the sperm in the BPA ($p < 0.01$), APAP ($p < 0.01$), and BPA+APA ($p < 0.01$) treatment

Table 2. Effect of various treatments on the activity of antioxidant enzymes in testicular mitochondria

	Control	MEL	BPA	APAP	BPA+APAP	APAP+BPA +MEL
CAT	4.16±0.09	5.08±0.2[a)]	2.94±0.2[b),c)]	3.22±0.07[a),c),e)]	1.22±0.1[b),c),e),f)]	4.30±0.1[f),h)]
GPx	425.78±1.8	484.12±5.6[a)]	324.38±10.1[b),c)]	331.68±13.7[b),c)]	149.50±25.6[b),c),e),f),h)]	436.74±5.9[e),f),h)]
GR	115.38±1.1	125.44±1.5[a)]	102.38±1.9[b),c)]	104.08±1.8[a),c)]	83.52±3.8[b),c),e),g)]	114.84±2.1[d),e),f),h)]
MnSOD	181.24±12.7	209.70±1.1[a)]	125.40±2.1[b),c)]	137.48±5.3[b),c)]	98.48±0.9[b),c),f)]	176.24±6.3[d),e),f),h)]

Values are presented as mean±standard error (n=10). GPx activity is expressed as nanomoles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized/min/mg protein, GR activity is expressed as nanomoles of NADPH consumed/min/mg protein, CAT activity is expressed as micromoles of H_2O_2 consumed/min/mg protein, MnSOD is expressed as nanomoles of (–) epinephrine protected from oxidation/min/mg protein.
MEL, melatonin; BPA, bisphenol A; APAP, acetaminophen; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; MnSOD, manganese superoxide.
Significant differences are indicated by [a)]p<0.05 or [b)]p<0.01 when compared to control; [c)]p<0.01 or [d)]p<0.05 when compared to MEL; [e)]p<0.05 when compared to BPA; [f)]p<0.01 when compared to APAP; [g)]p<0.05 or [h)]p<0.01 when compared to BPA+APAP.

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groups when compared to the control group. This decrease was mitigated by MEL treatment, as no significant decrease was seen between the control and the BPA+APA+MEL treatment groups. These findings strongly suggest that the various treatments exhibited deleterious effects, which were ameliorated by MEL (Table 3).

Discussion

It is well established that BPA induces the generation of ROS in testicular tissue and impairs male reproductive outcomes [24,25]. BPA is known to alter redox status, increases the production of hydroxyl radicals, and depletes the antioxidant system in the testicular mitochondria [25,26]. However, this is for the first study to report induction of ROS and depletion of antioxidants in the testicular mitochondria by APAP. The oxidative damage induced by exposure to toxicants in the testes may be the underlying cause of infertility and sub-fertility [27]. Ample evidence suggests that mitochondrial integrity and mitochondrial function are critical for maintaining normal testicular function and that exposure to xenobiotics can lead to adverse effects on the testicular mitochondria [28]. In the present study, we observed that chronic co-exposure to APAP and BPA caused a significant increase in oxidative damage and a decrease in the antioxidant capacity of the testicular mitochondria. We also observed a significant increase in the LPO in response to APAP, BPA, and APAP+BPA treatments in the testes of rats. LPO is frequently used as an index of tissue oxidative stress, which results from free radical damage to membrane components of the cell and has been hypothesized as a major mechanism of cell damage [29]. The animals that were treated with MEL along with APAP and BPA; however, had less LPO induced by BPA. The level of GSH, which is an important cellular antioxidant, significantly decreased in the animals treated with APAP, BPA, and APAP+BPA. The availability of GSH is necessary to clear the cell environment of the oxidative species produced in the cell during metabolism [30]. Treatment with MEL attenuated the reduction of GSH levels, indicating that MEL decreases the vulnerability of tissues to oxidative damage by increasing the level of cellular antioxidants. APAP, BPA, and APAP+BPA treatments also reduced the activities of antioxidant enzymes such as CAT, GPx, GR, and MnSOD, indicating a toxic effect. Treatment with MEL, however, mitigated the decrease in antioxidant enzymes. An earlier study by Anjum et al. [26], 2011 reported BPA-induced oxidative stress in the testicular mitochondria, which was ameliorated by MEL. However, the amelioration of oxidative stress-induced by APAP+BPA in testicular mitochondria by MEL has not been assessed earlier to the best of our knowledge. MEL is an antioxidant with considerable potential, as it has direct free radical scavenging activity and enhances the synthesis of antioxidant enzymes such as superoxide dismutase and CAT, which leads to the rapid disposal of H_2O_2 from cells [31]. MEL is five times superior to glutathione in scavenging free hydroxyl radicals, twice as potent as vitamin E in removing peroxyl radicals, and 14 times more effective in scavenging hydroxyl radicals than mannitol [32,33]. MEL has an advantage over other antioxidants as it crosses morphophysiological barriers, such as the blood-brain barrier, cell membranes, and sub-cellular compartments [34,35]. Considering the magnitude of exposure to pharmaceutical agents like APAP and xenobiotics such as BPA in daily life and their deleterious health effects, especially on the reproductive system, it has become necessary to identify the ameliorative effect of one of the most potent antioxidants on the oxidative stress-induced by such co-exposures. BPA has been strongly associated with a rise in infertility, and the male reproductive system merits serious investigation, as oxidative stress is the underlying cause in half of infertile men, despite the fact that this has generally received less attention as a possible cause of infertility [36]. APAP is also strongly suspected to be an EDC and has deleterious effects on male testicular function, leading to decreased testosterone levels with chronic exposure to APAP [11]. Furthermore, MEL levels in seminal plasma are depressed in infertile patients exhibiting poor motility, leukocytospermia, varicocele, and non-obstructive azoospermia, all of which are conditions associated with oxidative stress in the male reproductive tract [37]. The decreased basal values of the enzymes in the co-treatment group indicate an increase in toxicity resulting from the combined treatment of BPA and APAP.

The results of this study have important practical implications; for instance, regular consumption of MEL may lead to a better antioxi-

Table 3. Effect of various treatments on sperm parameters in rats

	Control	MEL	BPA	APAP	APAP+BPA	APAP+BPA+MEL
Total sperm count ($\times 10^6$/mL)	33.96±1.0	35.08±1.0[a)]	27.16±1.31[b),c)]	24.24±1.12[b),c)]	20.18±0.85[b),c),d)]	30.5±2.1[f),h)]
Sperm mortality (%)	22.58±1.3	27.98±0.7[b)]	24.02±1.1	25.04±0.6	30.30±0.7[b),e),f)]	24.7±1.1[h)]
Motility (%)	77.60±0.5	80.50±1.4	68.24±0.4[b),c)]	64.96±1.0[b),c)]	60.16±1.3[b),c),e)]	72.2±2.0[f),g),h)]

Values are presented as mean±standard error (n=10).
MEL, melatonin; BPA, bisphenol A; APAP, acetaminophen.
Significant differences are indicated by [a)] $p<0.05$ or [b)] $p<0.01$ when compared to control; [c)] $p<0.01$ when compared to MEL; [d)] $p<0.05$ or [e)] $p<0.05$ when compared to BPA; [f)] $p<0.05$ or [g)] $p<0.01$ when compared to APAP; [h)] $p<0.01$ when compared to BPA+APAP.

dant status, preventing oxidative stress-induced tissue damage in general and male infertility in particular. Furthermore, MEL could preclude the general predisposition to oxidative stress inflicted by various xenobiotics. It would be worth conducting a prospective clinical trial to test the efficacy of MEL as a supplement to improve reproductive outcomes in various presentations of sub-fertility and infertility.

In conclusion, co-exposure to various classes of xenobiotics in humans is increasing with large-scale consumerism and industrialization. Reproductive physiology is strongly affected by these exposures. We observed that co-exposure to BPA and APAP elevated oxidative stress in the testicular mitochondria in rats, while MEL ameliorated these changes. Thus, it is highly recommended to minimize exposure to xenobiotics and increase the intake of antioxidants, such as MEL, for the public in general and vulnerable groups in particular to have optimal reproductive outcomes.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Author contributions

Conceptualization: HR. Data curation: HR, MSA. Formal analysis: MSA, SA. Funding acquisition: HR. Methodology: MS, MFA, TA, RA, RJHB. Project administration: HR, AK. Visualization: MSA, AK. Writing-original draft: MQ, YN, AK. Writing-review & editing: HR.

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