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Thymoquinone mitigates phthalate-induced reproductive toxicities in male Swiss albino mice

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ABSTRACT

Phthalates (PHA) are common environmental pollutants used extensively in the plastics sector. Exposure to PHA negatively impacts both human and animal health. Thymoquinone (TQ), an active ingredient of black cumin seed, exhibits potential pharmacological effects against many illnesses. The study aimed to investigate how TQ affected the reproductive parameters and histo-morphology of the testes in male Swiss albino mice that has been given PHA. Twenty-four male mice, aged 28 to 30 days, were utilized and allocated into three groups, each including eight animals. Group A (the control) received standard mice pellets combined with olive oil; Group B was supplied PHA; and Group C was given both TQ and PHA mixed with mice pellets. All mice were reared at 26–30°C for 60 days. Blood, serum, and organs were obtained and processed using established protocols. PHA induced an elevation in body weight in male mice. The administration of TQ normalized body weight in PHA-treated mice. Administration of PHA to male mice resulted in a significant decrease in blood levels of thyroxine (T4) and testosterone ($p < 0.01$), whereas the administration of TQ led to an increase in these two hormones. PHA resulted in a substantial ($p<0.01$) decrease in sperm count and motility, accompanied by an increase in abnormalities, whereas TQ mitigated these sperm characteristics. Degenerative and necrotic alterations were observed in the seminiferous tubules of the testis in PHA-treated male mice which was altered by TQ. In conclusion, the integration of TQ may alleviate the adverse effects generated by PHA.

INTRODUCTION

The influence of endocrine-disrupting chemicals (EDCs), such as phthalates (PHA), bisphenol A (BPA), and other plasticizers, on the reproductive health of both humans and animals is evident [1]. PHAs are widely present in our environment and extensively utilized in various consumer products such as plastics, cosmetics, personal care items (e.g., shampoo, lotions, makeup, perfume), toys, PVC pipes, medical devices e.g., IV tubing, IV fluid, total parenteral nutritional bags, catheters, ready foods, formulation of insecticide, and high-fat dairy and meats [2]. Animals are consistently subjected to PHAs via direct contact [3]. The United States Environmental Protection Agency has classified six well-known phthalate compounds, namely di (2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP), dibenzyl phthalate (DBzP), and di-isopropanol phthalate (DiPP), as environmental pollutants [4]. Metabolites derived from DEHP, DEP, DMP, and DBP have been consistently identified in urine obtained from pregnant women [5, 6]. In addition, maternal plasma and urine samples have been found to contain urinary levels of DEHP and its metabolite, mono-ethylhexyl phthalate (MEHP), which have been linked to a reduction in gestational age [7]. PHA metabolites are frequently detected in amniotic fluid and cord blood samples [8]. There exists a correlation between levels of MEHP during pregnancy and a decrease

in testosterone, estradiol, progesterone, inhibin B, and insulin-like factor 3. Additionally, studies have found higher levels of DEHP metabolites in the autism's urine samples compared to those without autism [9]. The impact of gestational exposure to DEHP on animals' anxiety and depression-like behavior has been observed in previous studies [10]. DEHP exerts its effects on multiple tiers of the hypothalamic-pituitary-adrenal (HPA) axis. Prolonged exposure to PHA and BPA has been associated with a decline in fertility and the occurrence of embryo damage in animals, with the severity of these effects being directly proportional to the dosage administered [11].

The pharmacological efficacy of thymoquinone (TQ), which is obtained from *Nigella sativa* seed (usually known as black seed), has been demonstrated in the treatment of diverse medical conditions encompassing anti-parasitic, antimicrobial**,** antioxidant, and anti-inflammatory activities [12–15]. By enhancing the oxidant scavenger system via NF-κB signaling, caspase pathways, and TGF-β signaling, TQ has been shown to consistently have antioxidant effects. Black seed oil has demonstrated remarkable kidney-protective effects in clinical trials, normalizing blood and urine parameters and improving disease outcomes in patients with advanced chronic renal disease [16]. TQ has the potential to enhance fertility by augmenting the quantity of healthy sperm and mitigating the occurrence of sperm abnormalities [17]. TQ, in a dose-dependent manner, has positive effects on bleomycin (BL)-induced toxicity of the testis in mouse models [18]. Prepubertal administration of DBP led to testicular damage, while vitamin E and selenium restored normal spermatogenesis [19]. It was observed that the administration of a suspension containing *Nigella sativa* seeds resulted in a notable augmentation of ejaculation volume, sperm activities, and motility in rams [20]. Similarly, Sujan *et al.* [21] found that male rats exhibited improved testes function and a reduction in sperm abnormalities upon the consumption of *Nigella sativa*.

Therefore, the present study examined the impact of TQ on hormonal levels, reproductive functions, and testicular histopathology in mice treated with PHA.

MATERIALS AND METHODS

Chemicals

PHA metabolites and TQ were acquired from Sigma Aldrich Company, Hamburg, Germany, and subsequently dissolved in olive oil (vehicle) from F. Faiges SL, Tortosa, Tarragona, Spain, to create a stock solution before administration.

Ethical approval statement

The study was carried out in the Department of Physiology of Bangladesh Agricultural University, Mymensingh, Bangladesh, with the approval of the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Mymensingh 2202, Bangladesh [No: AWEEC/BAU/2022(22)].

Animals

A total of twenty-four male Swiss Albino mice (*Mus musculus*), of 28-30 days of age were used. The mice were procured from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B) in Dhaka, Bangladesh. Prior to being utilized in the experiment, mice were subjected to a 7-day period of acclimation in order to adapt to their new environment. During this time, mice were housed in mouse cages

located in a well-ventilated room that received natural daylight. The environmental conditions within the room were maintained at a temperature of 28 ± 2°C and relative humidity ranging from 70% to 80%. The body weight of mice was measured and documented prior to their categorization. The mice were subsequently divided into three distinct groups, denoted as A, B, and C, with each group comprising a total of eight mice. Group A was considered as non-treated control and was given a normal mice pellet combined with olive oil. Group B was administered a PHA mixture composed of DEP 33%, DBP 32%, DIBP 31%, and DPP 4% formulated in olive oil and dose rate was 0.2 ml/kg/day (212 mg/kg/day), while group C received the PHA mixture and TQ at doses 0.2ml/kg/day (212 mg/kg/day) and 50 mg/kg/day, respectively. Mice from each group were kept together in individual cages. The experiment was conducted over a period of 60 days. The mice were provided with commercially available standard mice pellets obtained from ICDDR'B in Dhaka. The diet was provided on a daily basis, and unrestricted access to both diet and clean drinking water was ensured. The feed was stored in hermetically sealed polyethylene bags to maintain its freshness. The provision of feed and drinking water was consistently maintained throughout the entire 24 h period.

Body weight measurement

A digital balance was employed to measure the body weight of each mouse. Body weight was measured on the first day of treatment and repeated every 15 days until the experiment was completed.

Blood collection and serum preparation

Blood collection and serum separation were done as per standard protocol [21]. Briefly, the mice fasted overnight before being individually placed in a vacuum-sealed container containing cotton soaked with diethyl ether. Following confirmation of unconsciousness, blood was drawn directly from the heart with a sterile syringe. To separate serum, approximately 1.5 milliliters of blood were drawn and transferred to an Eppendorf tube devoid of anticoagulant. The blood tubes were kept in an inclined upright position at room temperature for 6 h. The samples were incubated overnight at 4ºC in a refrigerator. Serum samples were centrifuged for separation and collected with 200 µl pipettes. Serum samples were maintained in sealed tubes at -20ºC for further hormonal analysis.

Hormonal assay

Serum levels of T4 and testosterone were assessed at the Institute of Nuclear Medicine & Allied Sciences (INMAS; Mymensingh Medical College, Mymensingh, Bangladesh using a radioimmunoassay kit (Berthold, Bad Wildbad, Germany & Co. KG) following the standard protocol [21], with an assay sensitivity of 0.10 nmol (0.0288 ng/ml). The inter-assay coefficient of variation for testosterone was 12.2%.

Analysis of sperm physiological parameters

Upon ending the experiment, all mice were euthanized under anesthesia, and their testes were excised to obtain the epididymis for sperm parameter analysis. The motility of sperm was assessed by applying a tiny volume of the diluted suspension onto a pre-

warmed microscope slide and thereafter covering it with a cover slip. The percent of motility was recorded [22]. Mice cauda epididymis was collected and minced with sharp scissors in a petri dish. The ruptured epididymis was inserted in a test tube with 4 mL of physiological saline at 37°C. The sperms were then allowed to disseminate for 5-10 minutes before counting them with a pipette in a Neubauer's counting chamber. Sperms were observed at a high magnification using a microscope. Sperm morphology was assessed using the Williams staining method.

Histopathological studies and microscopy

Testicles were collected from each group of mice, and complete blood removal was carried out through perfusion with phosphate-buffered saline. The testicles obtained were subsequently immersed in 10% neutral buffered formalin for 15 days. Subsequent to the preservation period, the adequately fixed tissues underwent processing, sectioning, and staining (hematoxylin and eosin) in conjunction with the Department of Pathology at Bangladesh Agricultural University, Mymensingh, according to the standard protocol described [23]. The stained slides were analyzed using an Olympus Photomicroscope (Model CX43) at the Department of Physiology, Bangladesh Agricultural University, Mymensingh, and images of these slides were captured.

Statistical analysis

The data was recorded and saved in Microsoft Excel-2010 (Microsoft, USA) before being imported into GraphPad Prism 8 software (GraphPad Software Inc., USA) for statistics. The analysis was conducted using a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison test. P-value ≤ 0.05 is considered as statistical significance.

RESULTS

Effect of TQ on body weight in PHA-treated mice

The average body weight gain in male mice upon being treated with PHA and PHA with TQ is shown in Figure 1. PHA treatment resulted in a significant increase in body weight in male mice in a time-dependent manner compared to the control. The higher body weight gain was observed in the PHA group in 60 days (54.0 ± 4.58 g), as compared to the PHA+TQ and control groups (43.33 ± 1.52 g and 42.33 ± 2.51 g). Interestingly, the addition of TQ to PHA treatment resulted in a decrease in body weight, indicating a potential interaction between these two factors. The data were statistically significant at $p < 0.01$ and $p < 0.05$ levels.

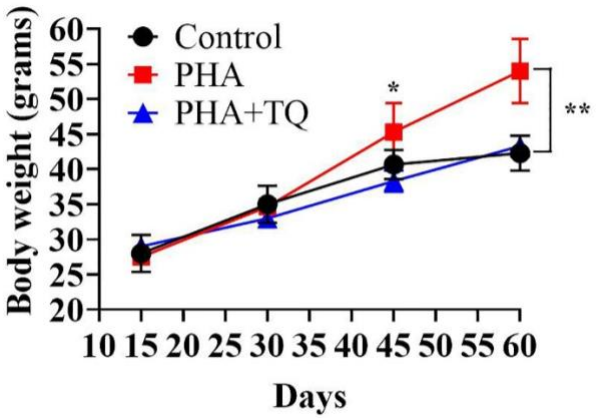

Figure 1. Effect of TQ on body weights (grams) (Mean± SEM) in PHA-treated male mice in a time-dependent manner. Swiss albino mice were treated with TQ and PHA daily for 60 days (details in materials and methods). Body weights were measured at every 15-day interval. * p<0.05 (control versus PHA / PHA+TQ at day 45) ** P<0.01 (control versus PHA/PHA+TQ at day 60 of age).

Effects of TQ on reproductive parameters in PHA-treated mice

The effects of TQ on sperm parameters in male mice treated with PHA are depicted in Figure 2. Mice that were administered PHA for a duration of 60 days exhibited a statistically significant reduction (p<0.01) in sperm count (21.86 ± 0.32 million/ml), a decrease in sperm motility (48.4 ± 4.39 %), and an increase in sperm abnormalities (38.66 ± 3.05 %) when compared to the control group of mice (27.2 ± 0.8 million/ml; 72.4 ± 5.03 % and 23.0 ± 2.64%, respectively) (Figure 2A-C). Normal sperm morphology was observed in both the non-treated control group and the TQ group (Figure 3A-C). However, in the group of mice treated with PHA, various abnormalities were observed in the sperm, including those without a hook and with a folded or coiled tail, small or immature sperm, decapitated sperm, sperm with two heads and a single tail, abnormal head with a coiled tail, folded sperm, and sperm without a head (Figure 3D-J). The parameters exhibited a significant improvement (p<0.05) toward normal levels when treated concurrently with TQ and PHA (Figure 2A-C).

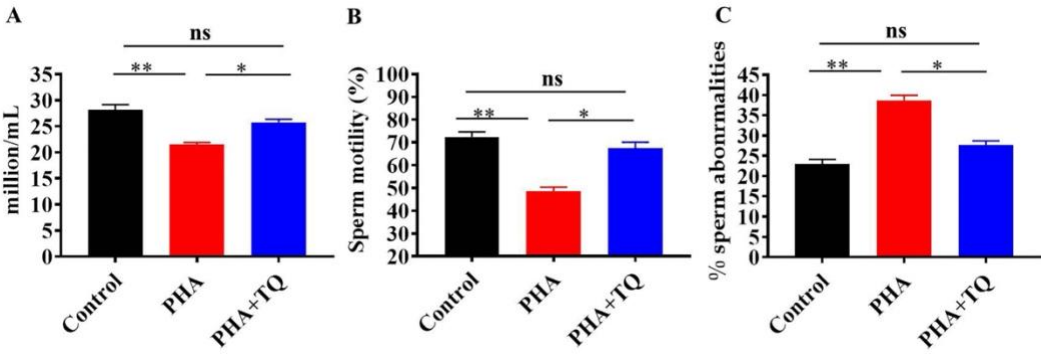

Figure 2. Effects of TQ on (A) sperm count, (B) sperm motility, and (C) sperm abnormalities in PHA-treated male mice. Swiss albino male mice were treated with TQ and PHA daily for 60 days (details in materials and methods). Testes were isolated at day 60 and analyzed for sperm parameters *p<0.05 PHA+TQ versus PHA); #p<0.01 (control versus PHA); ns = not significant (control versus PHA+TQ).

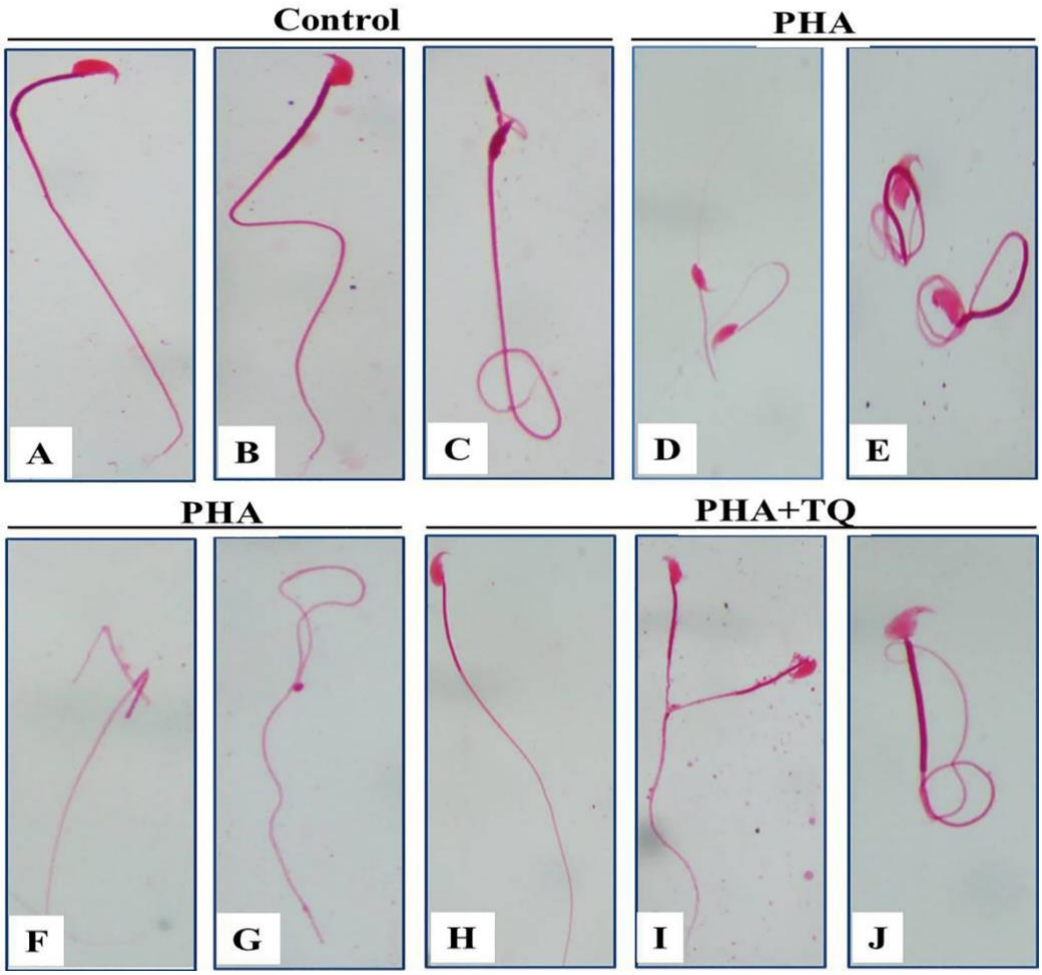

Figure 3. Representative images of normal and abnormal sperm in male albino mice treated with PHA and TQ. Swiss albino male mice were treated with TQ and PHA daily for 60 days (details in materials and methods). Testes were isolated at day 60; sperms were separated and stained with William staining. A, B, and H represent normal sperms, and D-F, I, and J represent abnormal sperms. C, sperm without a hook and a folded or coiled tail; D, small or immature sperm; E, folded sperm; F, sperm without a head; G, decapacitated sperm; I, two heads with a single tail; and J, an abnormal head with a coiled tail.

Effects of TQ on reproductive hormone levels in PHA-treated mice

Figure 4 shows how TQ changed the amount of testosterone and T4 in male mice that had been given PHA. The mean value of testosterone in control mice was 2.97 ± 1.31 ng/ml. The values were 0.76 ± 0.26 ng/ml and 1.83 ± 0.35 ng/ml in the PHA and PHA+TQ groups, respectively (Figure 4A). Results demonstrated that mice treated with PHA had a significantly ($p<0.01$) lower amount of testosterone, which was prevented by the treatment with TQ. Similarly, serum T4 levels also significantly decreased in PHA-treated mice (61.37 ± 5.89 nmol/L) compared to the values in control mice (81.28 ± 5.97 nmol/L) and PHA-and TQ-treated mice (69.56 ± 6.62 nmol/L) (Figure 4B).

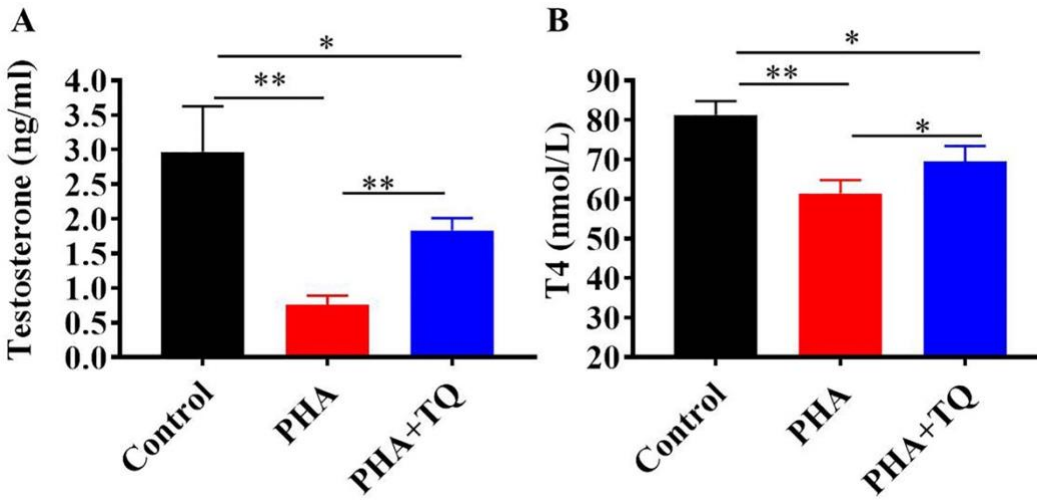

Figure 4. Effects of TQ on (A)Testosterone and (B) T4 level in PHA-treated male mice. Swiss albino mice were treated with TQ and PHA daily for 60 days (details in materials and methods). Blood samples were collected on day 60; sera were separated and analyzed for testosterone and T4 by radioimmunoassay. *p<0.05 (control versus PHA+TQ for both and PHA versus PHA+TQ only for T4); ** p<0.01 (control versus PHA for both and PHA versus PHA+TQ only for testosterone).

Effect of TQ on histo-structure of testis in PHA-treated mice

Histological studies of the testis were performed to determine the effects of PHA and TQ at the tissue level (Figure 5). The control group exhibited seminiferous tubules within the testis that displayed a typical morphology and organization of Sertoli cells (Figures 5A1 and A2). The presence of observable spermatozoa within the seminiferous tubules exhibited intact tissue structures with no discernible alterations observed. However, in the group treated with PHA, degenerative and necrotic modifications were evident in the seminiferous tubules, leading to a reduction in both tubular and luminal diameters. Sertoli cells exhibit an irregular morphology characterized by the presence of vacuoles (Figure 5B1, B2). The groups treated with PHA and TQ exhibited a restoration in germ-line cells with spermatozoa, and slight discernible alterations were observed in the seminiferous tubules' lumen (Figures 5C1 and C2).

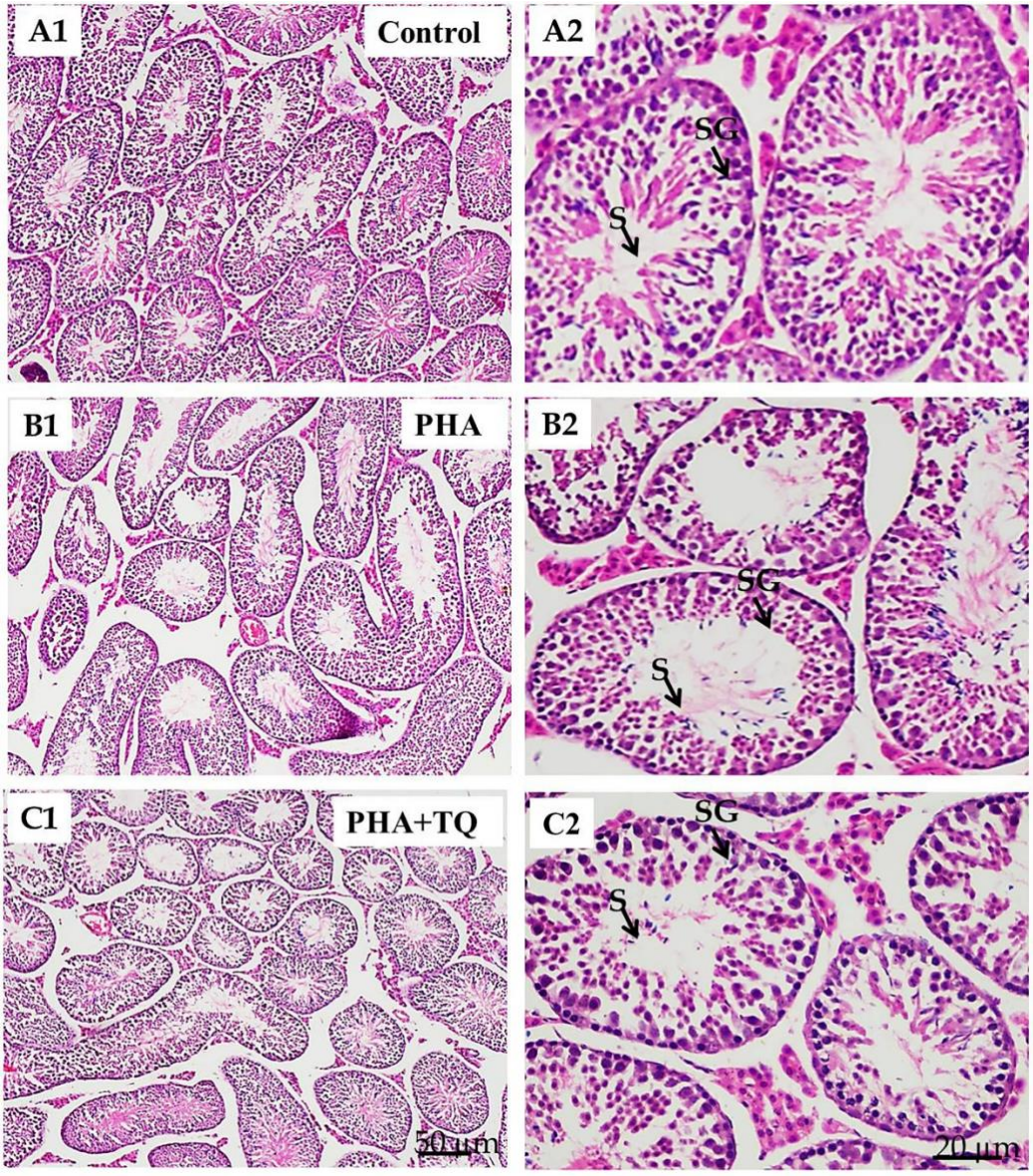

Figure 5. Effect of TQ on testis in PHA-treated male albino mice. Photomicrograph of histopathological sections of testicular tissues of different groups of mice after 60 days of study. Swiss albino male mice were treated with TQ and PHA daily for 60 days (details in materials and methods). Testis was isolated at day 60; formalin-fixed, sectioned, and stained with Hematoxylin and Eosin staining non-treated control (A1, A2); PHA (B1, B2); PHA+TQ (C1, C2) at 100X and 400X magnification. S, spermatozoa; SG, spermatogonium.

DISCUSSION

The observed increased body weight in PHA-treated mice and the counteracting action of TQ on PHA-induced body weight in the present study are consistent with those of the researchers who claimed that PHA has a significant effect on body weight by increasing adipose tissue deposition and lipid accumulation in mice [4, 24]. In DEHP exposure groups, there was a significant reduction in adiponectin mRNA expression in adipose tissue [25]. Because of the presence of the phytochemical TQ, black seed oil has anti-obesity and lipid peroxidation activity [26]. Black seed oil might assist in losing weight by suppressing appetite [27]. According to Bano et al. [28], appetite suppression could be linked to neural circuits that regulate the catecholaminergic, serotonergic, and peptidergic systems or to circulating leptin hormone signaling the brain's satiety center to produce hypophagic effects. It may also reduce body weight by

decreasing the amount of glucose absorbed by the intestines, lowering blood glucose levels, serum cholesterol, and triglyceride levels, and inhibiting gluconeogenesis in the liver.

Exposure to PHA resulted in hormonal and reproductive abnormalities in male mice, whereas TQ compensated or prevented such abnormalities. The present findings are in line with the findings of Abd-Ellah et al. [29], which found that the treatment with DEHP resulted in a significant decrease in sperm count, daily sperm production, and decrease in serum testosterone levels. Chiu et al. [30] found a significant reduction in estradiol levels as a result of exposure to PHA. Furthermore, it has been observed that DEP induces significant fluctuations in testosterone levels, leading to the manifestation of these abnormalities [31], and alters the hemato-biochemical parameters in goats [32]. The decline in plasma testosterone levels may be attributed to reduced expressions of enzymes and proteins, as well as decreased levels of plasma LH. The administration of TQ resulted in a substantial elevation in both plasma testosterone levels and epididymal sperm count. TQ is implicated in the augmentation of Leydig cell quantity and testosterone concentrations, ultimately leading to enhancements in spermatogenesis [12]. The potential increase in testosterone hormone levels may be attributed to the impact of TQ on key enzymes involved in testicular metabolism and steroid secretion. TQ demonstrated the ability to improve the adverse impacts caused by lead acetate [33] and morphine [34]. TQ has been found to induce the release of follicle-stimulating hormone (FSH) and testosterone, as reported by Al-Sa'aidi [36]. There was a gradual deterioration of the seminiferous tubules of the testis of the PHA group, and TQ prevented the degeneration of seminiferous tubules caused by PHA.

The current study indicates that TQ might possess protective properties against the detrimental effects induced by PHA. This is supported by the observed restoration of sperm motility and concentration, normal histo-morphology of the testis, and the maintenance of normal testosterone levels.

CONCLUSIONS

It can be concluded that PHA has detrimental effects on male reproductive health in mice. TQ may play a vital role in improving reproductive health in males by regulating the hypothalamo-pituitary-gonadal hormone axis. This research work opens a new window for future research areas and may act as a research track for filling information gaps regarding PHA and TQ. Further investigation is needed to determine the specific mechanisms by which PHA and TQ affect male reproductive health. Additionally, studying the long-term effects of TQ supplementation on male fertility could provide valuable insights for potential therapeutic interventions.

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AUTHOR CONTRIBUTIONS

MAM designed the experiment, supervised it, analyzed the data, and revised the final draft of the manuscript. MA, SH carried out the experiment, analyzed data, and wrote the first draft of the manuscript; AM critically revised the manuscript; MAHNAK

performed the histology of the mouse testis and revised the manuscript; FYB performed staining of the mouse sperm and revised the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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