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Oxidative Stress Effect on the Spermatogenesis Genes Expression in the Mouse Model

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Abstract

We aimed to evaluate the effects of oxidative stress induced by tertiary-butyl hydroperoxide on the DNA Fragmentation Index (DFI) and expression of spermatogenesis genes. Totally, 15 mice of BALB/c strain were categorized into 3 equal groups. To induce oxidative stress (OS), the case group was intraperitoneally injected with 1:10 (Lethal Dose) LD50 (100 µl) of tert-butyl hydroperoxide (t-BHP) for 14 days. Sterile water (200µl) was given intraperitoneally as a placebo in the control group. Whether OS is responsible for gene expression alteration, a third group of mice was treated simultaneously with t-BHP and Taurine for 14 days. In each group, the OS level was determined by the isolation of testicular cells and measurement of H_2O_2 and $O_2^{\bullet-}$ levels by flow cytometry. In order to evaluate the incidence of the DFI and apoptosis, tunnel assay was performed on prepared testis tissue samples. By the completion of the treatment phase, the mice were sacrificed and the expression of *Dazl*, *Ddx3y*, *Smcy* and *Usp9y* genes was measured by RT-PCR in each group.

Flow cytometry indicated an increase in reactive oxygen species (ROS) in testicular cells following t-BHP treatment ($p < 0.009$) and a reduction after Taurine co-administration ($p < 0.008$). Also, the TUNEL assay showed an increase in DFI in sperm DNA following t-BHP treatment and a reduction after Taurine co-administration ($p < 0.008$). Our results showed that the ROS decrease ($p<0.001$) the expression of the mentioned genes and Taurine treatment adjacent to t-BHP significantly reduced the ROS level and protected against downregulation of the *Dazl*, *Ddx3y*, *Smcy* and *Usp9y* genes ($p<0.001$, $p = 0.030$, $p = 0.002$, $p = 0.011$ respectively). The oxidative stress may reduce the expression of these Y chromosome genes significantly that are involved in spermatogenesis, and the use of the antioxidant may be a protection against downregulation.

INTRODUCTION

The male factor is responsible for 30% of all infertility problems [1]. Oxygen is necessary for the maintenance of normal cellular function [2]. Previous studies have been demonstrated that oxygen metabolite production such as reactive oxygen species (ROS), free radicals and peroxides increase oxygen ions and decrease antioxidants leading to oxidative stress, sperm DNA damage and reduced sperm motility [3]. ROS are known as a reactive chemical species that contain H_2O_2, $O_2^{\bullet-}$ and OH, which can damage intracellular lipids, proteins, and nucleic acids via oxidation-reduction reaction [5]. During normal spermatogenesis, reactive oxygen species are produced. Although, a small level of ROS is needed for capacitation, acrosome reaction and fertilization [5], a high level of ROS may associate with lipid peroxidation and damages of the plasma membrane integrity, genomic expression profile and DNA damages that may lead to a spermatozoa malfunction and infertility [6]. Different factors such as chemotherapy drugs, environmental pollution [7], fertilizers, waste material burners, fossil fuel power plants, and other industrial activities [8] may induce reactive oxygen species in the testis. In addition, diabetes has been reported as an oxidative stress inducer in testis [9].

It has been shown that Hydro peroxides such as t-BHP) can induce significant DNA damages on sperm ad testicular tissue [10]. Moreover, it has been shown that t-BHP induces OS in the other biological systems of mice [11]. The human Y chromosome harbors genes involved in the development of the testis and spermatogenesis are *Dazl*, *Ddx3y*, *Smcy* and *Usp9y*.

DAZL (Deleted in Azoospermia like), a member of highly conserved DAZ family genes, plays important role in the human fertility due to the control of differentiation, growth, maturation of germ cells and maintain spermatogonia stem cells (SSC) [12] by regulating stage-specific gene expression. Another important gene is DDX3Y, a member of the DEAD-box protein family of ATP-dependent RNA helicases, which plays an essential role in the development, maintenance, and fate of early germ cells [13].

Smcy or *KDM5D,* a Y - link gene, is essential for the spermatogenesis progress that plays an important controlling role in the epigenetic changes during spermatogenesis. Evidence suggests that during the spermatogenesis, SMCY may be involved to some extent in inactivating and concentrating chromosomes before entering meiosis [14]. Another important gene through spermatogenesis procedure is *USP9Y,* a Y-linked gene encoding the ubiquitin-specific peptidase 9. This testis-specific gene is a major member of the *AZFa* gene family [15]. This gene encodes a member of the C19 peptidase family and removes ubiquitin residues from ubiquitin-related precursors. It is a vital modulator of a spermatogenesis procedure that plays role in the survival, stability, and protection of germ cells [16]. To our best knowledge, there are two published studies, which reported expression alterations of Dazl and Ddx3y under influence of antibiotics and arsenic and DFI {Seidel, 2019 #38}. Here, for the first time, we investigated the effect of directly induced oxidative stress on the expression of genes involved in the spermatogenesis and DFI before and after treatment in a mouse model.

MATERIALS AND METHODS

This experimental study was approved by the Royan institutions ethical committee of the Research Council, Tehran, Iran (No.: Ec/92/1084). Guidelines of the ethics committee and the declaration of Helsinki were followed for the Care and Use of animals (DHEW publication, NIH, 80–23).

Animals and care

Fifteen adult male mice (Balb/C strain, 8-12 weeks old, 22-25 g) were provided from the Royan mouse stock. One week prior to the experiment initiation, they were kept under controlled condition, including ambient temperature of 25˚C ± 3˚C, 50% humidity, and an 12:12-hour light/dark cycles. They had free right to use food and water.

Animal treatment

We randomly distributed these 15 mice into 3 equal experimental groups, including: (1) the t-BHP treated group which received t-BHP (Sigma., USA, CAS Number: 75-91-2) at doses equivalent to 1: 10 (LD50) which was determined in our previous study [17] (daily, intraperitoneal (IP) injection of 508 µmol per 100 g/w, for two consecutive weeks. (2)- received a combination of t-BHP (same dose of t-BHP as first group) and Taurine (Sigma Co., USA, CAS Number: 107-35-7) [18], and (3): control group and received only distilled water (200,ip/14 days). Following treatment completion, animals were sacrificed through cervical dislocation and testes were collected.

Evaluation of reactive oxygen species in testes

The induced ROS level of testes tissue samples was measured by flow-cytometry (BD FACS Calibur; Becton-Dickinson, USA) with utilization of the 2',7'-dichloro-dihydro-fluorescein di-acetate (DCFH-DA; Sigma, USA. CAS Number: 4091-99-0) for H_2O_2 and di-hydro-ethidium (DHE; Sigma, USA. CAS Number: 104821-25-2) for $O_2^{\bullet-}$ using a previously described procedure [19,20] with some modifications. Briefly, after enzymatic digestion of testicular tissue by the method of Bellvt and colleagues [19], about 1-3 million testicular cells were homogenized with DCFH-DA (10 mmol) and DHE (1/25 µmol) and then incubated for 15 min at 37 °C in a shaker incubator to allow the probe connected to the membrane-bound vesicles in the darkness. Then the cells were washed several times with PBS (Phosphate Buffer Saline; USA. CAS Number: 21600-010), and the percentage of fluorescent cells was measured by flow cytometry [20]. Green fluorescence (DCF) was evaluated between 500 and 530 nm in the FL-1 channels, and the red fluorescence (HE) was evaluated between 590 and 700 nm in the FL-2 channels.

Sperm Collection and DNA Fragmentation Index (DFI)

Following scarification, the cauda epididymides were dissected and resuspended in a 2 mL of pre-warmed Tissue Culture Medium (TCM -Sigma, USA. CAS Number: M 2520), containing 10% Human serum albumin (HAS; Biotest, Germany. CAS Number: B 05AA01). Mature spermatozoa using swim up, density gradient and simple wash steps were selected and evaluated for the DFI. The DNA fragmentation test was performed by TUNEL assay (Cell Death Detection Kit: Roche, Mannheim, Germany). Briefly, sperm sample (1-3× 10^6 sperm cells/sample) were washed from seminal plasma by low-speed centrifugation (600 g; 5 minutes), fixed with 4% PBS-buffered for 60 min at room temperature and permeabilized with 0.25% Triton X-100 for 10 min at 4°C. Then the specimen incubated (individually) in a TUNEL reaction mixture in the dark at 37 °C for 1 hour followed by evaluation in a fluorescence microscope. Using a fluorescence microscope (Nikon, Japan), the DFI was calculated as the percentage of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive sperm [21]. Images were captured by a video camera (Basler Vision, A312FC at 50 fps; Tecnologie Co., Ahrensburg, Germany) x 10 magnification.

RNA extraction and cDNA synthesis

The biopsy pieces were frozen immediately using liquid nitrogen. Total RNA was extracted from tissue using the Trizol reagent (Invitrogen Life Technologies; USA. CAS Number: 15596026). RNA samples were quantified by Nano Drop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was evaluated by the ratio of 28S/18S ribosomal RNA bands after electrophoresis in a normal 1 % agarose gel. The cDNA was synthesized from 1 µg of the total RNA, treated with deoxyribonuclease I (DnaseI; Fermentase: Gran Island, NY, USA. CAS Number: EN0521) according to the manufacturer protocol (Fermentase: Gran Island, NY, USA, CAS Number: 4368814) and stored at -20 ˚C until use.

Quantitative real-time PCR

A primer builds upon existing primer design software for q real time PCR primers, such as Primer3 (Table A2). The *β-Actin* was gene selected as the housekeeping gene. Quantification

of cDNAs was conducted two times by quantitative real time-polymerase chain reaction (qRT-PCR), using the SYBR Green PCR kit (ABI Applied Biosystems, USA. CAS Number: 4309155) and 7500 Real-time PCR System (ABI Applied Biosystems, USA). The reaction conditions were followed by 40 cycles: denaturation at 95°C for 10 min, annealing at 60°C for 1 min. Data were analyzed using the 2ˆ(−ΔΔCt) method [22] (Table A2).

Statistical Analysis

For comparison of the flow cytometry and DFI results between control and treated groups, the data were analyzed by Mann-Whitney (Non parametric) test using SPSS software (SPSS V.16, Inc, Chicago, IL, USA). For comparison of the expression results between control and treated groups, the data were analyzed by One Way ANOVA (supplemented with a Tukey– HSD multiple comparison test) using SPSS software (SPSS V.16, Inc, Chicago, IL, USA).

DISCUSSION

Oxidative stress is one of the male infertility causes [23]. Previously, we showed fourteen days injection of the t-BHP can induce oxidative stress in the mouse testis by a significant increase in H_2O_2 and $O_2^{\bullet-}$ levels, which can lead to a significant reduction in adult sperm parameters [5]. Rectifying gene-expression changes and consequently sperm parameters change and also, the sperm DNA fragmentation index, in vivo studies are necessary. Normally, the double-stranded DNA breakage repairs in a short time through homologous recombination. Since the Y chromosome haploid genome contains key spermatogenesis-specific genes and is unable to repair the lost genetic information, therefore it is very sensitive to the deletion of genes and compensating for their reduced expression. It has been observed that increasing the ROS levels may lead to the conversion of guanine to 8-OH guanine (8-OHG), 2 ′-deoxyguanosine and DNA fragmentation, and if not repaired, can cause Y chromosome microdeletions which affect the expression of genes in this region and eventually, cause infertility [24,25].

In this study, to establish a ROS model, after two weeks of peritoneal injection of t-BHP in mice, H_2O_2 and $O_2^{\bullet-}$ levels increased significantly in the testes, which proves the induction of oxidative stress. Also, a significant increase in DNA strand breaks was confirmed by comparing case and control groups with the TUNNEL test. A reduction in sperm parameters was also observed. To examine this theory, the alterations of the expression of the specific *Ddx3y, Smcy,* and *Usp9y* genes on the mice Y chromosome may cause infertility, a group of genes as mentioned in the introduction, which has high homology with human Y chromosome genes and also *Dazl* gene on chromosome 3 were tested.

In this study, the downregulation of the *Dazl* gene was observed after the induction of oxidative stress in the t-BHP treated group, which may lead to a decrease in sperm count. Similarly, in previous studies, two antibiotics, amoxicillin and gentamicin, and also mono-butyl phthalate were associated with increased oxidative stress and sperm morphology, while, decreased sperm motility and the *Dazl* mRNA expression level [26,27].

Following induction of oxidative stress, a significant decrease in the *Ddx3y* gene expression was observed, which is probably associated with oligospermia due to a decrease in the mitotic division in germ cells [Table 2]. Deletion of the *DDX3Y* gene is associated with the complete loss of germ cells and the development of Sertoli cell-only syndrome (SCOS), that leads to azoospermia in humans [28]. Based on a previous study, the arsenic induction of oxidative stress reduces the expression of the *Ddx3y* gene and sperm count in addition to an increase in the sperm tail and head abnormalities [29].

After induction of oxidative stress in our study, decreased expression of the *Smcy* gene was observed. Since this gene is involved in the cell cycle controlling and the onset of meiosis [14], a decrease in sperm count [Table 2]. After ROS induction is probably due to this decrease in the *Smcy* gene expression. In addition, loss or reduction of the *SMCY* gene expression due to disruption of the regulation of H3K4me3 transcription marker may lead to cell cycle irregularities and increases replication stress. It is possible that the observed increase in the breakdown of double-strand DNA, which remains unrepaired, is associated with a decrease in the expression of this gene, results in the formation of sperm with unrepaired DNA [30] (Table A1).

As mentioned in other studies, spot mutations and deletion of *USP9Y* can be associated with different phenotypes such as decreased motility (asthenozoospermia), diminished concentration of sperm (oligospermia to azoospermia), and spermatid maturation arrest [31]. In this study, after the induction of oxidative stress, changes in the spermogram were associated with a decrease in *Usp9y* gene expression.

It has been observed that ROS is neutralized by the enzymatic antioxidant system. Therefore, treatment with antioxidant agents is associated with reducing oxidative stress and has protective effects against ROS-induced damage in the different tissues such

Table A1: Mean (± SD) values of sperm count and vitality in control and t-BHP treated groups

Groups	Concentration (millions per ml)	Vitality (%)
Control	3.8 ± 0.7	82% ± 0.09
Treatment	1.6 ± 0.5	51% ± 0.1

Table A2: Specific Primer Sequences for each gene

Sequences of Primer (5' → 3')	Gene
FOR: 5′ATC CGT AAA GAC CTC TAT GC 3′	β-Actin
REV: 5′AAC GCA GCT CAG TAA CAG TC 3′	
FOR: 5′ TCAGTCTTCATCAGCAACCA 3′	Dazl
FOR: 5′ AGACAAATCCATAGCCCTTCG3′	
FOR: 5′ AACTTACTCGTTACACTCGTCC 3′	Ddx3y
REV: 5′ TTTCCAGACCCTGTTTGAGC 3′	
FOR: 5′ACT TGT CAC TCT GAT GAA TCC T 3′	Smcy
REV: 5′TCC AAC AGG TAG CCA ATC G 3′	
FOR: 5′ CAGTGTCCTCAAGTGTTCAG 3′	Usp9y
REV: 5′ TGTCCAAACGGCTTAATAAGAG 3′	

as testis. Taurine via NADPH oxidase may alter ROS production through two different ways: first, it may suppress ROS production by mitochondrial electron transfer chains and second: it protects the antioxidant enzymes against oxidative stress damage [32].

After Taurine injection, no significant difference in the expression of specific *Dazl, Ddx3y, Smcy,* and *Usp9y* genes were observed in the control group. It seems that the oxidative stress production under the t-BHP injection is deactivated by the Taurine and the normal condition of gene expression and sperm production will return. This also confirms, no other pathways may have influenced the results of our experiments, other than oxidative stress induced by t-BHP.

Although, using inbred mouse rather than outbred, eliminates the effect of background genetic in our animal model, but using human sample will provide more accurate information. We suggest using the Testicular sperm extraction (TESE) samples of those obstructive azoospermia patients with high level of oxidative stress for the next step of this research.

RESULTS

DCFH and DHE processing by the testis cells

The intracellular induction of ROS was detected by flow cytometry using fluorescent probes DCFH-DA and DHE to determine H_2O_2 and $O_2^{\bullet-}$ levels. Figure A1 shows the level of H_2O_2 and $O_2^{\bullet-}$ in a mouse selected from each group. A significant increase in H_2O_2 and $O_2^{\bullet-}$ level was observed in the t-BHP-treated group in comparison with the control group ($p < 0.009$). And also, in the t-BHP+Taurine group, the level of H_2O_2 and $O_2^{\bullet-}$ was significantly reduced in comparison with the t-BHP group ($p < 0.008$). The percentage of H_2O_2 and $O_2^{\bullet-}$ in the testis was higher in the t-BHP group in comparison with the t-BHP+Taurine group (77%-99% versus 34%-58%) (Figure A2) ($p < 0.008$).

DFI and TUNNEL Assay

One hundred spermatozoa were counted in duplicate slides. Green- colored sperm cells (TUNEL positive) and red-colored sperm cell (TUNEL negative) were calculated by the counting machine (Figure A3). The percentage of DNA fragmentation in the sperms was higher in the t-BHP group in comparison with the t-BHP+Taurine group (52%-80% versus 16%-23%) (Figure A4) ($p < 0.008$).

Gene Expression analysis

qRT-PCR analysis shows that the expression of *Dazl, Ddx3y, Smcy,* and *Usp9y* were significantly downregulated in the t-BHP treated group in comparison with the control group ($p<0.001$). However, the expression of the same genes was significantly upregulated in the t-BHP+Taurine group in comparison with to the t-BHP group ($p<0.001$, $p = 0.030$, $p = 0.002$, $p = 0.011$ respectively) (Figure A5).

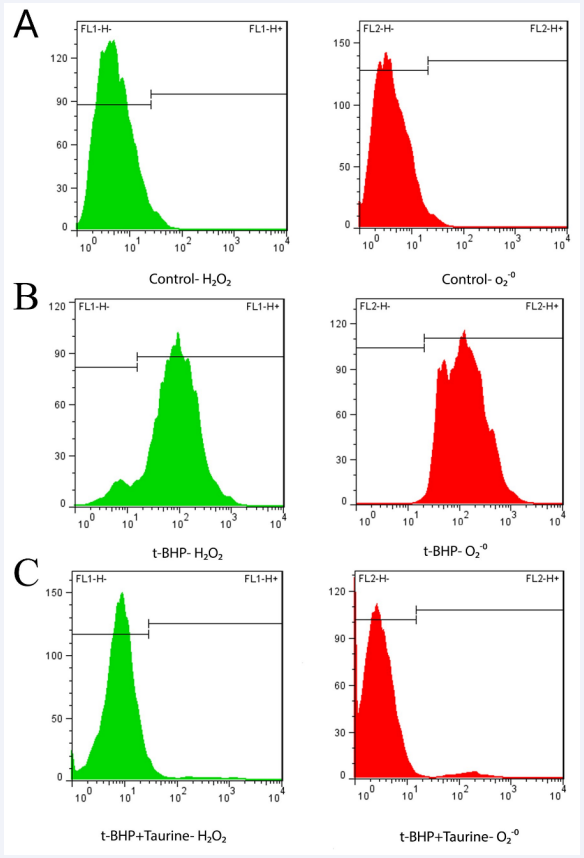

Figure A1 Histogram graphs of flow cytometry of mouse testis stained with DCFH and DHE. The green line shows development of fluorescent dye of DCFH which was collected in fluorescence detectors 1 (FL1) and red line shows the development of fluorescence dye of DHE which was collected in fluorescence detectors 2 (FL2). (Date analyzed by Mann-Whitney (Non parametric) test).
A: shows the level of H2O2 and O_2•- in control group. B: A significant increase in H_2O_2 and O_2•- levels in the t-BHP treated compared to the controls group ($p < 0.009$). C: A significantly reduced H2O2 and O_2•- in the t-BHP+Taurine treated compared to the t-BHP group ($p < 0.008$).

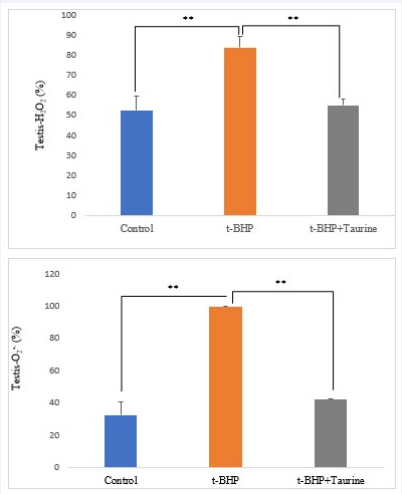

Figure A2 Status of ROS measured as H_2O_2 and O_2•- levels (measured using DCFH and DHE) in testis treated with t-BHP and t-BHP+Taurine groups compared to control group by flow-cytometry. (Data analyzed by Mann- Whitney test, Error bar: STDV).

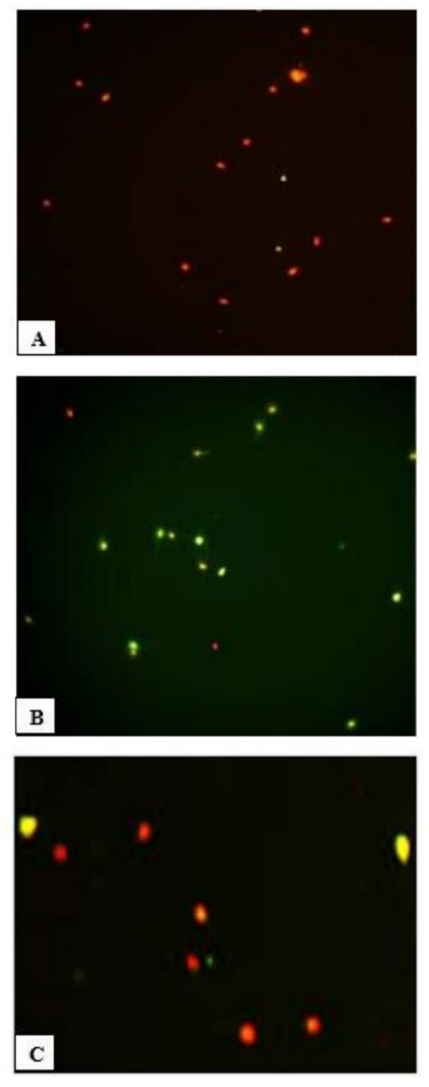

Figure A3 The incidence of sperm DNA damage by TUNNEL staining image: Image A shows the control group, image B shows the treated group with t-BHP and image C shows treated group with t-BHP+Taurine). Florescent microscopy.

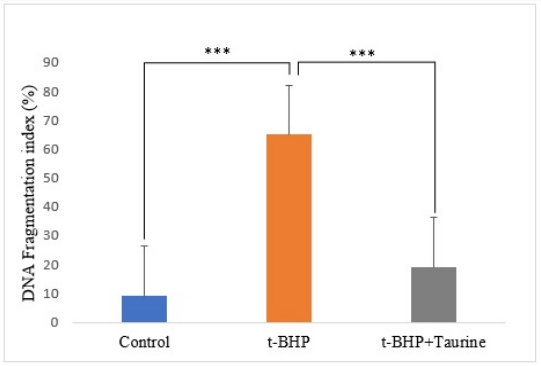

Figure A4 The compression of DNA Fragmentation in the t-BHP and t-BHP+Taurine groups with the control group by TUNNEL assay. (Data analyzed by Mann-Whitney test, Error bar: STDV).
*** Shows a significant difference at level p < 0.008.

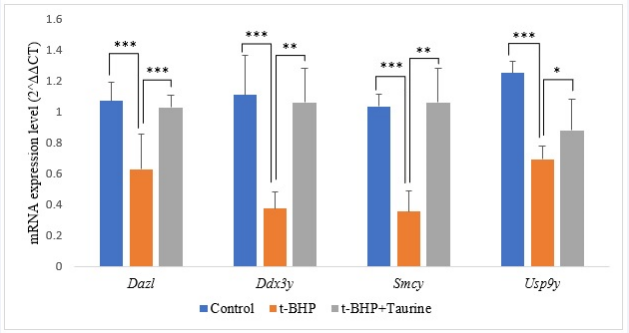

Figure A5 mRNA expression of Dazl, Ddx3y, Smcy, & Usp9y in mice testis.
The expression level of Dazl, Ddx3y, Smcy, & Usp9y were significantly downregulated in the t-BHP treated mice compared to those in the control group. (Data analyzed by One Way ANOVA Error bar: STDV) (p<0.001).
The expression level of Dazl, Ddx3y, Smcy, & Usp9y were significantly upregulated in the t-BHP+Taurine treated compared to the t-BHP treated. (Data analyzed by One Way ANOVA Error bar: STDV) Respectively (p<0.001, p = 0.030, p = 0.002, p = 0.011).
* Shows a significant difference at level p<0.05.
**Shows a significant difference at level p<0.01.
***Shows a significant difference at level p<0.001.

CONCLUSION

Oxidative stress can influence gene expression such as *Dazl, Ddx3y, Smcy,* and *Usp9y* in the testes and change spermatogenesis in the mouse model. Antioxidants such as Taurine prevent adverse effects of ROS and can be recommended to regulate gene expression and maintain normal spermatogenesis.

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