



Exposure to bis (2-ethylhexyl) phthalate causes sperm quality deteriorations, oxidative stress and down-regulates the expression of Catsper2 gene and protein in testis of mice

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General Note

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ABSTRACT

Purpose: Environmental factors such as exposure to heavy metals and air pollutants are factors influencing the male fertility. The present study was conducted to determine the effect of bis (2-ethylhexyl) phthalate administration on sperm parameters, testicular histopathology, expression of Catsper2 gene and protein and oxidative stress in male adult mice. **Methods:** In this study, 32 male NMRI mice were randomly divided into 4 groups of control and phthalate 1-3. The control group received the solvent of bis(2-ethylhexyl) phthalate (corn oil). The phthalate 1-3 groups received 50, 250, and 500 mg/kg of bis(2-ethylhexyl) phthalate for 14 days, respectively. Sperm, CatSper2 gene and protein expression, testicular histopathology and oxidative stress assessments were performed 5 weeks after the intervention. **Results:** Our results showed that, the administration of phthalate reduced sperm parameters as well as interstitial space and congestion with incomplete spermatogenesis in seminiferous tubules. Mice received phthalate showed a significant decrease in CatSper2 gene expression compared to the controls ($p < 0.05$). The changes identified in CatSper2 protein expression levels were consistent with their relative mRNA level. A significant difference was found in the mean levels of malondialdehyde and superoxide dismutase enzyme between the phthalate 2 and 3 groups and the control group. A significant increase was observed in the mean thiol level in phthalate 1 ($p = 0.04$), phthalate 2 ($p < 0.001$) and phthalate 3 ($p < 0.001$) groups compared to the control group. **Conclusion:** The exposure to bis(2-ethylhexyl) phthalate reduces sperm quality especially sperm with tail defects and degenerative changes in testicular tissue and increases oxidative stress in male mice. A dose-dependent reduction was also observed in CatSper gene and protein expression.

Keywords: Phthalate; CatSper; Sperm; Testis; Mice

1. INTRODUCTION

About 10% of couples face infertility, approximately half of which is attributed to the genetic factors, infections, and male factors (Borghet & Wyns 2018; Safarinejad, 2008). Environmental factors such as exposure to lead, formaldehyde, nickel, cadmium, and mercury can have adverse effects on the male fertility (Mohammadi et al., 2016; Mohammadi et al., 2014; Mohammadi et al., 2017). Phthalates are endocrine disruptors, widely used in the manufacturing of cosmetic products (e.g. nail polishes, sunscreens, moisturizers, hair sprays, and shampoos), can coatings, plastic food containers and cellophane, soft drinks, wall papers, glues, teflon coatings, paints, toys and disposable medical supplies (blood transfusion, hemodialysis and angioplasty). People are unwittingly exposed to phthalates via oral and skin contact, and inhalation. Studies have shown that, exposure to phthalate reduces testicular weight and it causes sperm DNA damage. Previous studies have also reported a decrease in the diameter of seminiferous tubules and degenerative changes along with the prevention of testosterone production by leydig cells after administration of phthalate (Jurewicz & Hanke, 2011; Zare et al., 2009; Lee et al., 2009; Pan et al., 2017; Koniecki et al., 2011; Tickner et al., 2001). Calcium ions play a central role in the regulation of sperm cells behaviors (Darszon et al., 2006). A novel family of four unique channel-like proteins (CatSper1 - CatSper4) as well as two auxiliary subunits (CatSper β and CatSper γ) has been identified to be merely expressed in sperm cells (Ren et al., 2001; Quill et al., 2003; Lobley et al., 2003).

Studies on the effects of environmental factors on CatSper gene and protein expression indicated that, exposure to environmental factors such as bisphenol, (Wang et al., 2016) cadmium, (Zare et al., 2009) and lead (Mohammadi et al., 2018) influences the CatSper gene and protein expression and sperm motility. This study was designed to evaluate the effects of phthalate administration on CatSper gene and protein expression, testicular histopathology, sperm parameters, and oxidative stress in male mice.

2. MATERIALS AND METHODS

After approval by the Ethics Committee of the Mashhad University of Medical Sciences (code of ethics: IR.MUMS.fm.REC.1396.467), 32 male NMRI mice (25-30 g) were kept under standard nutritional and environmental conditions. Then, they were randomly divided into 4 groups of control and phthalate 1-3. The control group received the solvent of bis(2-ethylhexyl) phthalate (corn oil). The phthalate 1-3 groups received 50, 250, and 500 mg/kg of bis(2-ethylhexyl) phthalate (Sigma, England), respectively for 14 days (Wang et al., 2016; Lee et al., 2009). Study duration was 17 months. Start date was 2018-01-10 and End (Completion) date was 2019-06-10.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Ethical approval number IR.MUMS.fm.REC.1396.467, Date: Wednesday, November 1, 2017.

Sperm Quality Assessment

The tail of epididymis were placed in the saline and incubated for 20 minutes in a CO₂ incubator. Sperms were analyzed according to the World Health Organization (WHO) protocols (Attari et al., 2018).

Histopathology of Testis

The mice were anesthetized, and the testes were removed and placed in a fixative after opening the abdominal cavity. The dehydration and clearing steps were carried out by passing through alcohol and xylene, respectively. Then, the tissues were molded and cut into 5-micron-thick sections using a microtome (Leica, Germany). The sections were stained with hematoxylin and eosin and a blind pathologist evaluated the sections by a Zeiss microscope, Germany (Mohammadi et al., 2015).

Real-time Polymerase Chain Reaction (PCR)

RNA was extracted from the left testes according to the manufacturer's protocol, and then cDNA was obtained from it (Pars Tous kit, Iran). Real-Time PCR was performed for both CatSper and β -actin genes using SYBR Green (Pars tous, Iran). Primers explained in our previous study (Mohammadi et al., 2017).

Western Blotting

CatSper proteins were examined using western blotting technique (Amerizadeh et al., 2018). Tissue was homogenized and lysed in radioimmunoprecipitation assay (RIP) buffer supplemented with protease inhibitor, and an equal amount of protein was isolated by SDS-PAGE gel. After electrophoresis, it was transferred to a nitrocellulose membrane. After blocking by Bovine Serum Albumin (BSA) blocking solution and washing with buffer solution, the membrane was incubated with primary antibody (mouse anti-rabbit CatSper2 antibody dilution 1:100; Biorbyt, UK) and secondary antibody (1:1000 dilution; HRP-conjugated goat anti-rabbit IgG; Abcam, USA), and protein bands were visualized using chemiluminescence substrates. β -actin gene used as internal control.

Measurement of Malondialdehyde (MDA) and Thiol Levels

MDA was measured based on our previous study. Dithionitrobenzoic acid (DTNB) reagents were used to measure the level of thiol, and adsorption at 412 nm was assessed using a spectrophotometer (Vafaei et al., 2018).

Measurement of Activity of Superoxide Dismutase and Catalase Enzymes

Activities of superoxide dismutase and catalase enzyme were measured by preparing of various solutions and using a spectrophotometer according to our previous study (Vafaei et al., 2018).

Statistical Analysis

Data was analyzed using Analysis of Variance (ANOVA) and post-hoc Bonferroni in SPSS software. The correlations were evaluated using the Pearson correlation coefficients. *P*-value less than 0.05 were considered as statistically significant.

3. RESULTS

Sperm Parameters

The mean number of sperms in the control group was equal to 4.62 ± 0.37 million/mL, while it significantly reduced after receiving 500 mg/kg of phthalate (3.1 ± 0.52). Sperm counts were significantly lower in the phthalate 1 ($p < 0.001$), phthalate 2 ($p < 0.001$), and phthalate 3 ($p < 0.001$) groups than that of the control group (Table 1). The statistical analysis showed a significant difference in sperm motility between the phthalate 1 ($p < 0.001$), phthalate 2 ($p < 0.001$) and phthalate 3 ($p < 0.001$) groups compared to the control group. A significant difference was found in the abnormal morphology rate of sperms in the phthalate 1 ($p = 0.01$), phthalate 2 ($p = 0.04$) and phthalate 3 ($p = 0.02$) groups compared to the control group (Figure 1).

Table 1 Comparison of sperm parameters in different groups

Sperm parameters	Control group	pthalate 1 group	pthalate 2 group	pthalate 3 group
Sperm Count (million/mL)	4.62 ± 0.37	3.11 ± 0.49*	3.63 ± 0.30*	3.12 ± 0.52*
Sperm motility (%)	80.87 ± 9.01	60.37 ± 4.24*	60.25 ± 6.67*	58.12 ± 4.76*
Normal Morphology (%)	88.29 ± 7.92	78.75 ± 5.7*	80.25 ± 3.91*	79.62 ± 3.99*

Data are expressed as mean ± standard deviations. The ANOVA and post-hoc Bonferroni are used for testing statistic. $p < 0.05$ was significantly different with the control group in the same row.

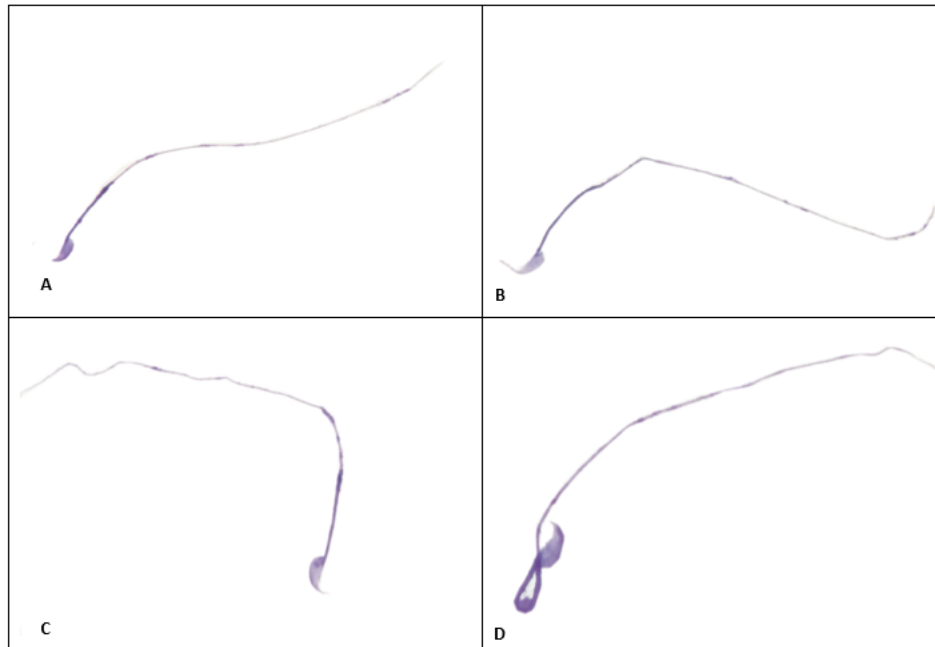


Figure 1 Image of sperm in different groups. ($\times 400$ magnification) A: control group, B: pthalate1 group, C: pthalate 2 group, and D: pthalate 3 group; giemsa staining.

Effects of Phthalate Administration on Testicular Histopathology in Male Mice

Figure 2 shows the tissue sections of seminiferous tubules in different groups. Seminiferous tubules with normal structure and various types of spermatogonia cells, spermatocytes, spermatids, spermatozoa, and sertoli cells were observed in the control group. The interstitial space contained interstitial cells and myoid cells (Fig. 2a). An increased interstitial space with reduced interstitial cells was observed in transverse section of the pthalate 1 group. Seminiferous tubules were not much affected (Fig. 2b). An increased interstitial space with reduced interstitial cells was observed in the image of testes of the pthalate 2 group. Seminiferous tubules were not much affected (Figure 2. C). Some tubules showed incomplete spermatogenesis and lack of spermatozoa in the lumen of the seminiferous tubules and the congestion of blood vessels in interstitial space was also observed in the image of seminiferous tubules of pthalate 3 group. Besides, an increased interstitial space with reduced interstitial cells was noted in this group (Figure 2. (d)-(f)).

Effects of Phthalate Administration on *CatSper 2* Gene Expression in Male Mice

As shown in Figure 3, the relative expression of *CatSper 2* gene was significantly lower in the pthalate 2 ($p < 0.001$) and pthalate 3 ($p < 0.001$) groups compared to the pthalate 1 group. The relative expression of *CatSper 2* gene was lower in the pthalate 2 ($p < 0.001$) and pthalate 3 ($p < 0.001$) groups compared to the control group.

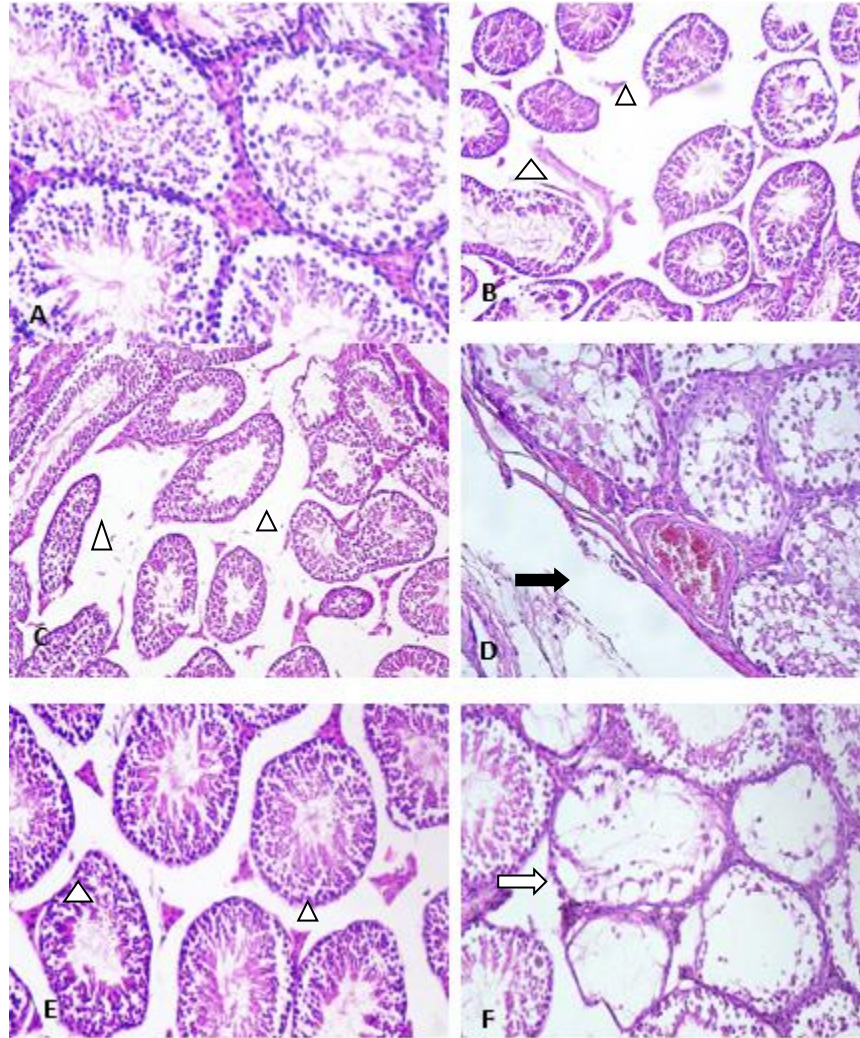


Figure 2 Transverse section of testis of the mice in the control (A), phthalate 1 (B), phthalate 2 (C), and phthalate 3 (D-F) groups (H & E staining). Black arrow represents congestion, arrow tip represents widening space, and white arrow represents vacuoles and necrosis.

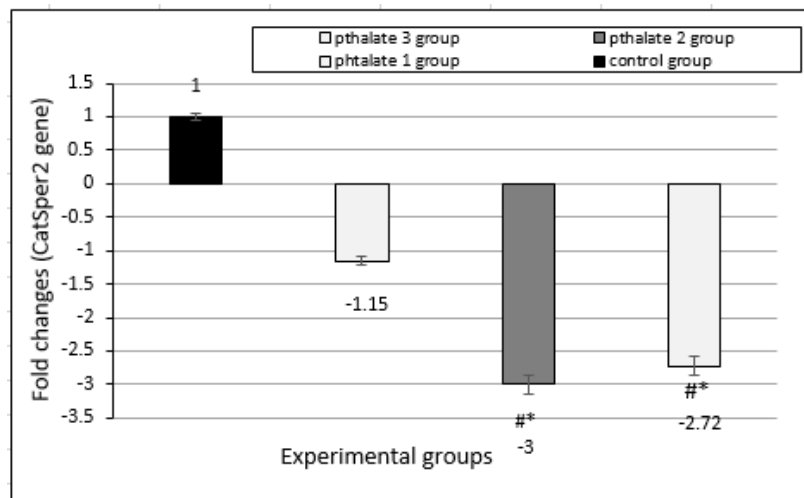


Figure 3 Expression of CatSper2 gene in testicular tissue of different groups

Data are expressed as mean \pm standard deviations. The ANOVA and post-hoc Bonferroni are used for testing statistic.

$p < 0.05$ was significantly different with the control group

$p < 0.05$ was significantly different with the phthalate 1 group

Effects of Phthalate Administration on CatSper 2 Protein Expression in the Male Mice

The results obtained regarding the intensity of CatSper protein expression are shown in Figure 4. CatSper 2 protein expression significantly decreased in the phthalate 1 ($p < 0.05$), phthalate 2, ($p < 0.01$), and phthalate 3 ($p < 0.001$) groups compared to the controls (Figure 5). As the dose of phthalate increased, the intensity of CatSper2 protein expression declined ($p < 0.001$).

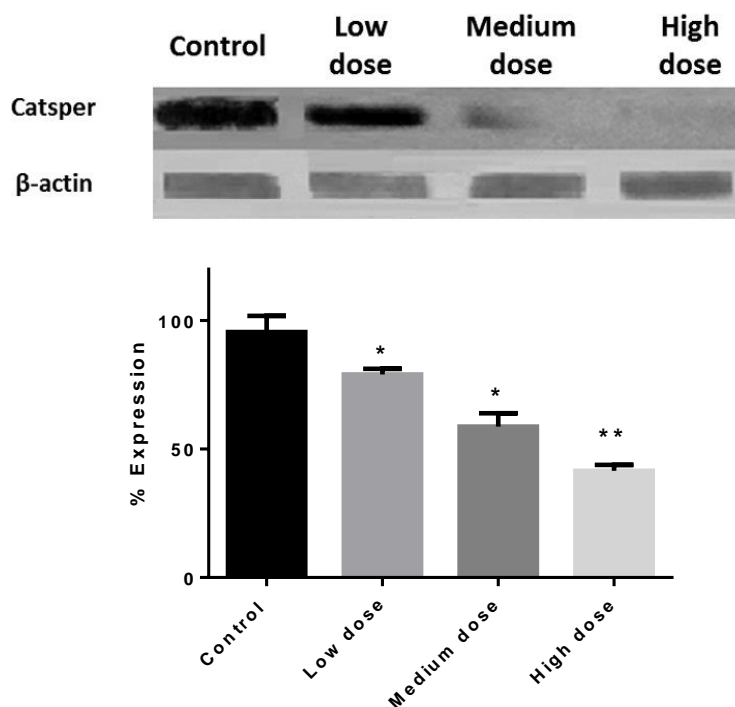


Figure 4 Expression of CatSper 2 protein in testicular tissues of different groups

Data are expressed as mean \pm standard deviations. The ANOVA and post-hoc Bonferroni are used for testing statistic. β -actin gene used as internal control. * $p < 0.05$ was significantly different with the control group; ** $p < 0.01$ was significantly different with the control group

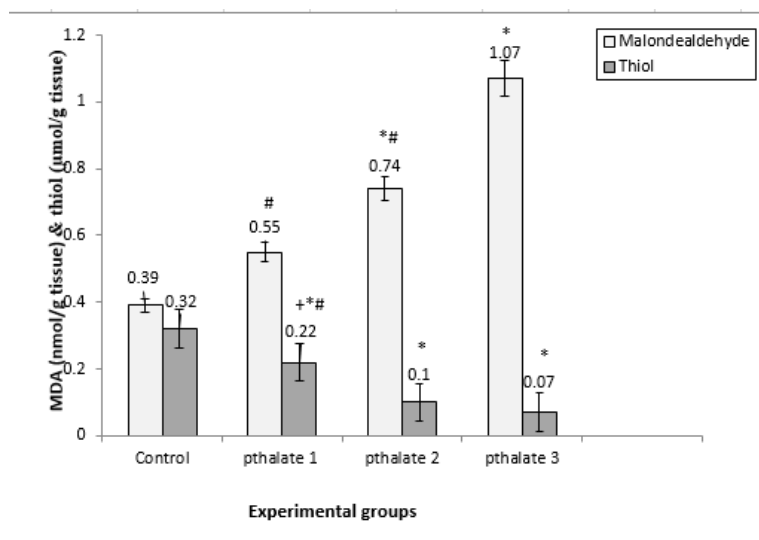


Figure 5 Comparison of MDA and thiol levels in testicular tissues in different groups

Data are expressed as mean \pm standard deviations. The ANOVA and post-hoc Bonferroni are used for testing statistic.

* $p < 0.05$ was significantly different with the control group

$p < 0.05$ was significantly different with the phthalate3 group

+ $p < 0.05$ was significantly different with the phthalate2 group

MDA Level

Figure 5 shows MDA levels in testicular tissues in different groups expressed as nmol/g. The statistical analyses showed a significant difference in the mean level of MDA between the phthalate 2 ($p < 0.001$), and phthalate 3 ($p < 0.001$) groups and the control group. In addition, a significant elevation was observed in the in MDA level of the phthalate 3 group compared to the phthalate 1 ($p < 0.001$) and phthalate 2 ($p < 0.001$) groups.

Evaluation of Thiol Levels

Figure 5 exhibits the amount of thiol in the testicular tissues of different groups expressed as $\mu\text{mol/g}$. The statistical analysis showed a significant difference in the mean thiol level between the phthalate 1 ($p = 0.04$), phthalate 2 ($p < 0.001$), and phthalate 3 ($p < 0.001$) groups and the control group. Level of thiol increased in the phthalate 1 group compared to the phthalate 2 ($p = 0.01$) and phthalate 3 ($p = 0.001$) groups.

Activity of Superoxide Dismutase and Catalase enzymes

Figure 6 displays the activity of superoxide dismutase and catalase enzyme in different groups expressed as U/g. There was no significant difference in the mean activity of catalase enzyme in different groups ($p > 0.05$). There was a significant decrease in the mean activity of superoxide dismutase enzyme in the phthalate 2 ($p < 0.001$) and phthalate 3 ($p < 0.001$) groups compared to the control group. A significant reduction was observed in the mean activity of superoxide dismutase enzyme in the phthalate 3 group compared to the phthalate 1 ($p < 0.001$) and phthalate 2 ($p = 0.008$) groups. Also, a significant decline was noted in the mean activity of superoxide dismutase enzyme in the phthalate 2 group compared to the phthalate 1 group ($p < 0.001$).

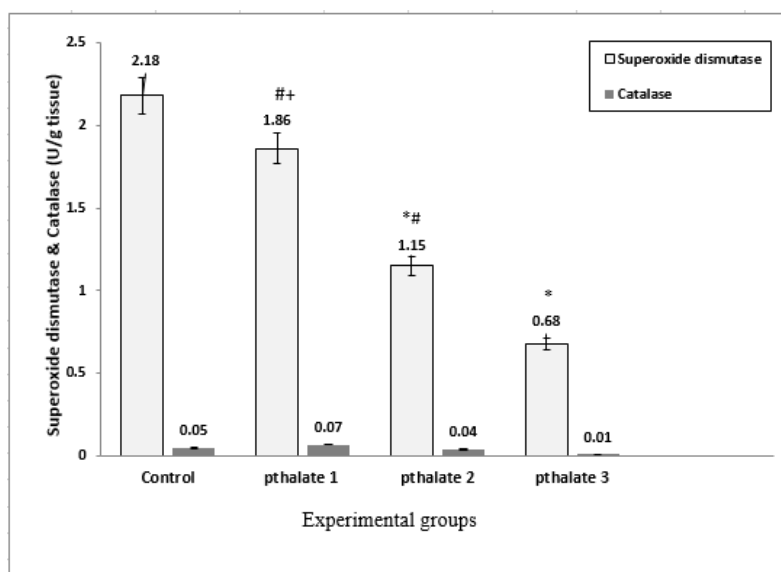


Figure 6 Comparison of superoxide dismutase and catalase enzymes in testicular tissues in different groups. Data are expressed as mean \pm standard deviations. The ANOVA and post-hoc Bonferroni are used for testing statistic.

* $p < 0.05$ was significantly different with the control group

$p < 0.05$ was significantly different with the phthalate3 group

+ $p < 0.05$ was significantly different with the phthalate2 group

4. DISCUSSION

The findings of the present study revealed that, the administration of phthalate reduced sperm quality and increased interstitial space and congestion along with incomplete spermatogenesis. Our results also showed that, the CatSper gene and protein expression decreased in the phthalate groups in a dose-dependent manner. In line with the present results indicating that phthalate administration influenced the histopathology of testes, Luo et al. reported that, administration of 2 mg/100 μl of phthalate diminished testicular weight and seminiferous tubules thickness and caused degenerative changes in testis tissue. Lee et al. in a study on mice administrated 250, 500, and 750 mg/kg of phthalate for 30 days. They observed a decrease in the in the sperm count at the doses of 500 and 750 mg and decreased testosterone levels at all the doses along with a change in the expression of Cyp19-Cyp17-PBR-SR and LDH1-Spag4 related to steroidogenesis and spermatogenesis (Lee et al., 2009). Pan et al. reported the adverse

effects of administrating 450 mg/kg of phthalate for 28 days on sperm count and motility as well as a reduction in testosterone level in testes of male mice. Moreover, phthalate administration caused down-regulation of p450-cholesterol related to testosterone synthesis (Pan et al., 2017).

Our results also showed that, the CatSper gene and protein expression decreased in the phthalate groups in a dose-dependent manner. There are limited numbers of studies on the effects of environmental factors on CatSper gene and protein expression. Consistent with our results, Wang et al reported that, the administration of bisphenol on mice caused a dose-dependent reduction in sperm parameters and CatSper gene and protein expression (Wang et al., 2016). Besides, degenerative changes were observed during spermatogenesis along with a decrease in acrosomal reaction. A greater decrease in gene expression was found at higher doses of bisphenol. In another research, 2 mg/kg of cadmium chloride and 5 mg/kg of nickel chloride administration influenced the level of prooxidant-antioxidant balance, spermatogenic arrest, and Leydig hyperplasia. In addition, a decrease was found in sperm count, motility, and normal morphology. After nickel and cadmium administration, a decrease was observed in gene expression, which was greater in cadmium group than nickel group (Zare et al., 2009).

Mohammadi et al. investigated the effects of exposure to 60 mg/kg of lead acetate and 1.25 mg/kg of mercury chloride on mice for 2 weeks. They reported that, both heavy metals reduced CatSper 1, 2 genes expression. This reduction was more in the mercury group compared to the lead group (Mohammadi et al., 2018). In agreement with these reports, in the present study, a significant down-regulation was detected in the phthalate groups. An inverse relationship was found between phthalate administration and sperm motility. Interestingly, the changes observed in CatSper2 protein expression levels were consistent with their relative mRNA level. Three possible mechanisms are assumed for the effects of phthalate administration. First, phthalate through its effect on CatSper gene and protein expression influences the spermatogenesis. Second, phthalate exposure causes an increase in apoptosis in the testes and the amount of oxidative stress influencing the male fertility. Oxidative stress decreases the sperm motility by reducing the axonemal phosphorylation. Third, phthalate can disrupt tight junctions of testicular blood barrier and prevent the synthesis of testosterone hormone; as a result it reduces sperm parameters through disruption in hormonal pathway (Zhang et al., 2008; Ge et al., 2007). To our knowledge, there is no study reported the effects of phthalate administration on CatSper gene and protein expression.

The current study had two limitations. First, there was no possibility to investigate acrosomal reaction using specific antibodies and intracellular calcium concentration of sperm through the flow-cytometry due to financial constraints. Second, we should have administered the phthalate exposure instead of intra-peritoneal injection of phthalate, which is similar to human exposure. Besides, mice testicular genomic mutational analysis is missing in the paper. Catsper is a big family and both Catsper 1 and Catsper2 have been identified with male infertility. It only analyzed Catsper2 gene and protein because of financial support. Future studies are recommended to evaluate the effects of phthalate on other members of CatSper genes family as well as other Catsper proteins expression after di(2-ethylhexyl) phthalate treatment.

5. CONCLUSION

The results of this study reflected that bis (2-ethylhexyl) phthalate reduces sperm quality especially sperm with tail defects and degenerative changes in testicular tissue and enhances oxidative stress in male mice. In addition, a dose-dependent reduction in CatSper gene and protein expression was observed.

Author's contribution

Concept and idea by: Assistant Professor (Dr.) Shabnam Mohammadi,

Method design by: Assistant Professor (Dr.) Seyed Mehdi hasanian, Farzad Rahmani, Mahsa Akbari Oryani, Farimah Beheshti

Method review by: Professor (Dr.) Mehdi Jalali & Parisa Haeri

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List of Abbreviations

Bis Phthalate: Bis (2-Ethylhexyl) Phthalate; WHO: World Health Organization; PCR: Polymerase Chain Reaction; RIP: Radioimmunoprecipitation; BSA: Bovine Serum Albumin; MDA: Measurement of Malondialdehyde; DTNB: Dithionitrobenzoic; ANOVA: Analysis of Variance

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Conflicts of Interest: The authors declare no conflict of interest.

Data and materials availability: All data associated with this study are present in the paper.

Peer-review: External peer-review was done through double-blind method.

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