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Assessment of a sub-chronic consumption of sodium benzoate (E211) on male reproductive functions in Swiss mice

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Sodium benzoate (SB) is a commonly used as a chemical food preservative. Studies on the influence of benzoates on reproductive patterns are very rare in the scientific literature. The present study investigates the effect of sub-chronic consumption of sodium benzoate on male reproductive function in Swiss mice. Thirty Swiss male mice were randomly divided into five groups of 6 mice each. Control group was given drinking water, and treated groups were given sodium benzoate at doses of 0.1, 0.25, 0.5 and 1%. After 13 weeks, we observed a significantly decrease body weight gain, sperm count, sperm motility, normal morphological sperm and serum testosterone levels as well as a significantly increased relative testes weight and impaired testes histology in 0.5 and 1% sodium benzoate treated groups. These findings suggest that excessive consumption of sodium benzoate induces impair spermatogenesis and sperm quality which affects the reproductive performance of male Swiss mice.

Keywords: Sodium benzoate, Testis, Sub-chronic toxicity, Male reproduction, Mice.

INTRODUCTION

Sodium benzoate (E211) is one of the most commonly chemical preservative (Saad et al., 2005; Mpountoukas et al., 2008) , used in a variety of foods, beverages and condiments (Zengin et al., 2011) to prevent alteration or degradation caused by microorganisms during storage (Lück, 1985). Sodium benzoate is a salt of benzoic acid, which is used to inhibit the growth of bacteria; it exhibited inhibitory activity against a wide range of fungi, yeasts, molds and bacteria (Mota et al., 2014; Alsudani, 2017).

In addition, sodium benzoate is used to treat hyperammonemia in patients with urea cycle disorders (Leonard et Morris 2002; Scaglia et al., 2004), in order to facilitate an alternative pathway of nitrogen excretion.The joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO, 1997) suggested a limit on the amount of sodium benzoate, which is 0 - 5 mg/kg. It is noted that intake estimations from several countries are averaged at 0.18–2.3 mg/kg body weight. However, individuals in China can consume up to 14 mg/kg body weight per day from diet alone (WHO, 1999). The US Food and Drug Agency (FDA) have classified sodium benzoate as “Generally Recognized as Safe” and regulated the concentration of sodium benzoate to 0.1% by weight in food products and 1% concentration in medicines (FAO, 1994; Rothschild, 1990).

However, the consumption of sodium benzoate beyond its acceptable daily intake (ADI) levels may produce toxic consequences in the exposed population. Many studies incriminated

sodium benzoate to be responsible for hyperactivity in children (McCann et al., 2007; Beezhold, Johnston and Nochta, 2014), genotoxicity (Loutsidou et al., 2012; Pongsavee 2015), teratogenicity and high mortality in zebrafish embryos (Tsay et al., 2007; Chen et al., 2009). In addition, it can also cause hepatocirrhosis (Kaboglu and Aktac, 2002), muscle damage (Michalak and Qureshi, 1995), cancer (Xing et al., 2000), and leads to the change of morphological structure of lymphocyte and membrane damage in mice (Hu et al., 2008).

Other effects are attributed to sodium benzoate, such as hypersensitivity (Brahmachari and Pahan, 2007; Maier et al., 2010), contact urticaria and contact dermatitis (Andersen, 2006; Sutton and Nixon, 2006), cytostaticity in human lymphocytes (Mpountoukas et al., 2008), inhibition of gluconeogenesis, ureagenesis (Cyr et al., 1991), and fatty acid oxidation (Kalbag and Palekar, 1988).

Nevertheless, so far there are no studies on the effect of sodium benzoate on the testicular toxicity or on the reproductive function. Thus, the purpose of this study is to show the affects of subchronic consumation of sodium benzoate on the reproductive functions in Swiss male mice.

MATERIALS AND METHODS

Chemicals

Sodium benzoate (CAS No. 532-32-1; C7H5O2Na; benzoic acid, sodium salt [E 211 (EU No. Regulation on Labeling of Foodstuffs)]; molecular weight 144.11mol/l) was purchased from Prochima Sigma Tlemcen (Algeria). Triton X-100 and gentian violet were purchased from Sigma (USA), hematoxylin and eosin stains were purchased from Merck (Germany).

Animals and treatments

Male Swiss mice weighting 23.22±0.52g purchased from Pasteur institute, Algiers, Algeria, were kept under proper conditions of ambient temperature and adequate humidity.

Mice were divided into five groups of six animals each. The first group was given drinking water as a control, the second the drinking water containing 0.1% sodium benzoate, the third the drinking water containing 0.25% sodium benzoate, the fourth the drinking water containing 0.5% sodium benzoate, and the fifth the drinking water containing 1% sodium benzoate each for 13 weeks (Chipley, 1983; Toth, 1984). Mice were fed a commercial standard diet (Local Production of Bouzareah, Algiers) and given free access to water all along the experiment. Food and water intake were measured every day and body weight weekly.

The experiments described in this study comply with the current Algerian legislation covering the protection of animals.

Reproductive performance study

After 13 weeks of experiment, groups of sodium benzoate-treated mice, six males per dose group, were mated 1:1 with untreated females for 1 week. Females were then separated and allowed to gestate to term. For females that failed to deliver a litter, this was considered as a sign of male infertility whereas litter delivery indicated male fertility.

Sacrifice of animals and collection of biological samples

After mating, the mice were fasted for 18 hours and killed by cervical dislocation. Testes and epididymis were removed immediately, cleaned of adhering tissues and weighed. Then, they were prepared for fertility evaluation. Right testis for each animal was taken for histopathological examination through light microscopy.

Sperm motility

The left epididymis was excised and placed in a warmed petri dish containing 4 ml of physiological saline solution at 37°C. The tissue was minced with scalpels for approximately 1 min and placed in a 37°C incubator for 15 min prior to determining sperm motility. The suspension was stirred; one drop was placed in a hemocytometer. Under a light microscope (400x magnification), Sperm motility was expressed as percentage of total sperm (Llobet et al., 1995; Yousef and Salama 2009).

Sperm count

The cauda epididymis and the testis were homogenized in 5 or 10 mL of a solution of 0.9% NaCl containing Triton X100 0.5% respectively. The testis and epididymis homogenates were diluted with 1.5 ml of the homogenization solution and spermatozoa were counted at 400x magnification using a Malassez hemocytometer. Five counts *per* sample were averaged (Llobet et al., 1995; Yan et al., 2013).

Sperm morphology

20 µL of suspension were spread onto a glass slide and allowed to dry at room temperature. Once air-dried, the cells were fixed in 96% ethanol for 5 min, stained with 0.5% gentian violet and rinsed with distilled water. 200 spermatozoa were analyzed *per* animal using bright field illumination at final magnification of 1000x (oil immersion). Morphological abnormalities were classified into general categories pertaining to head, mid-piece and tail morphology (Gautam et al., 2010).

Hormone assay

Blood was collected from the retro-orbital venous plexus. Blood serum was separated by centrifugation (3500 rpm for 15 min). Serum testosterone concentrations were measured by a competitive ELISA kit according to manufacturer's instructions (ab108666; Abcam).

Histological analysis

Histological examination of testis was performed. The right testis was fixed in 10% formalin-buffer. Six microns thick paraffin sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy (Optica Axiom 5000, China).

Statistical analysis

Data are expressed as mean values ± SE. Statistical analysis was performed using statistical test one way ANOVA to find significant difference between values of various parameters recorded for control and treated animals. $p<0.05$ was considered statistically significant. All statistical analysis were performed using Graph Pad Prism 5 Project software (version 5.01 2007 Graph Pad Software, San Diego, California, USA).

RESULTS

The oral administration of sodium benzoate to the Swiss male mice for 90 days exhibited behavioral and morphological changes like loss of body weight, agitation and aggressiveness.

Body weight and testes weight

Body weight gain was significantly decreased in 1% benzoate group compared with 0.1% group ($p < 0.05$), as well as in 0.5% ($p< 0.01$) and 1% ($p < 0.001$) treated groups compared to control (Table 1).

No significant changes in the absolute testes weight were recorded in all treated groups compared to the control. However, a statistically significant increase of relative testis weight was observed in 1% benzoate group compared with 0.1% group ($p < 0.05$). The same result was observed in 0.5% ($p < 0.05$) and 1% ($p < 0.01$) treated groups compared to the control (Table 1).

Reproductive performance

The result of the fertility test performed on mice Swiss male, treated with sodium benzoate is shown on Table 2. Male mating index was significantly decreased in 0.5% ($p < 0.05$) and 1% ($p < 0.001$) treated groups compared to the control values, and in 1% group compared to the 0.1% ($p < 0.01$). We also noted that females coupled with intoxicated males have a reduced offspring rate compared to females mated with control mice.

Sperm parameters

Table 3 shows the various sperm parameters in the five groups. It reveals that sperm count in testis and epididymis, motility and morphology were decreased in all treated groups compared to control. Significant decrease was observed in the testis and epididymis sperm count of 0.5% ($p < 0.01$) and 1% ($p < 0.001$) sodium benzoate treated groups compared to control group. In addition, we observed a significant decrease in sperm epididymis count between the two treated groups 0.1 and 1% ($p < 0.01$).

Also, a significant decrease in sperm motility was found in 0.5% ($p < 0.05$) and 1% ($p < 0.01$) treated groups compared with control, as well as in 1% group compared with 0.1% group ($p < 0.05$).

In contrary, significant increase was observed in the abnormal sperm rate of 0.5% ($p < 0.01$) and 1% ($p < 0.001$) sodium benzoate treated groups compared to the control group, and in 1% group compared with 0,1% group ($p < 0.01$). Morphological abnormalities involved the sperm head, mid piece and sperm tail (Fig. 1).

Testosterone levels

Serum testosterone level was decreased in all treated groups compared to the control values. However, a significant reduction in serum testosterone level was observed in groups treated with 0.5% and 1% of sodium benzoate compared to control group ($p <0.01$) (Fig. 2).

Histological Study

Histological study in the testis of control mice revealed normal structure of interstitial tissue and seminiferous tubules with dynamic spermatogenesis (Fig. 3 A).

The germ cells (spermatogonia, primary and secondary spermatocytes, spermatides and spermatozoa) and Sertoli cells within the seminiferous tubules were normal. However, testes of mice treated with sodium benzoate displayed variable degree of histopathological alterations.

We observed dilation in some seminiferous tubules with mild degenerative and necrotic changes, in some of the spermatogonial cells, and detachement germ cells from the irregular basal lamina, in testes of mice of all treated groups (Fig. 3 B, C, D and E).

The testes of mice treated with 0.5% of sodium benzoate (Fig. 3 D) showed important dilation in seminiferous tubules, loss germ cells, and decline of spermatogenesis within the individual lumen with absence of spermatozoa in the lumen of seminiferous tubules and beginning dystrophy of the interstitial spaces.

In 1% sodium benzoate treated group, we observed severe degeneration and necrosis of all the seminiferous tubules (Fig. 3 E), disruption in layers germinal epithelium and widening of the interstitial spaces with atrophy of interstitial cells around the seminiferous tubule.

Table 1: Effects of oral ingestion of sodium benzoate on body weight in adult male mice.

Groups concentrations (%)	Control	0.1	0.25	0.5	1
Initial BW (g)	22.22 ± 0.59	22.92 ± 0.84	23.9 ± 0.6	22.18 ± 1.2	24.89±0.42
Final BW (g)	47.16 ± 0.47	43.97±0.61	43.91±0.6	40.55±0.52	41.45±0.71
Body weight gain (g)	24.94±0.55	20.99±1.13	20.00±0.64	18.37±1.33**	16.7±0.71***, #
Testes weight (g)	0.233±0.012	0.234±0.51	0.264±0.005	0.256±0.01	0.272±0.01
Testes/BW ratio (%)	0.503±0.02	0.512±0.031	0.593±0.01	0.63±0.019*	0.657±0.02**, #

Results are expressed as mean ± SE (n=6). Significance of the difference was determined using One-Way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001 significance of difference versus control; # p< 0.05, significance of difference versus 0.1% group.

Table 2. Effect of sodium benzoate on reproductive performance.

Parameters	Control	0.1%	0.25%	0.5%	1%
Mating index § (%)	6/6 (100)	6/6 (100)	6/6 (100)	5/6 (83)*	4/6 (67) ***, ##
Average litter number	9,16±0.30	8.5±0.22	7.5±0.42	5.1±1.08	3.33±1.09
No. of off spring	55	51	45	31	20

§: No. of males producing a pregnant female/No. of males co housed with females;

Results are expressed as mean ± SE (n=6). Significance of the difference was determined using One-Way ANOVA. *p < 0.05 **p < 0.01, ***p < 0.001 significance of difference versus control; ##p < 0.01, significance of difference versus 0.1% group.

Table 3. Sperm parameters in male Swiss mice treated with sodium benzoate.

Groups concentrations (%)	Control	0.1	0.25	0.5	1
Count *per* testis ($x10^6$)	6.88±0.4	4.33±0.41	3.68±0.31	3.03±.42**	2.43±0.45***
Count *per* epididymis ($x10^6$)	20.7±2.59	14.26±2.34	9.45±0.9	5.91±.53**	3.76±0.52***, ##
Motility (%)	73.91±2.28	64.08±2.44	52.11±2.77	47.47±1.23*	36.57±4.78***,#
Abnormal morphology (%)	9,77±0,76	13,19±0.72	15.17±1.08	23.64±0.95**	30.49±1.27***, ##

Results are expressed as mean ± SE (n=6). Significance of the difference was determined using One-Way ANOVA. *p < 0.05 **p < 0.01, ***p < 0.001 significance of difference versus control; #p < 0.05. ##p < 0.01, significance of difference versus 0.1% group.

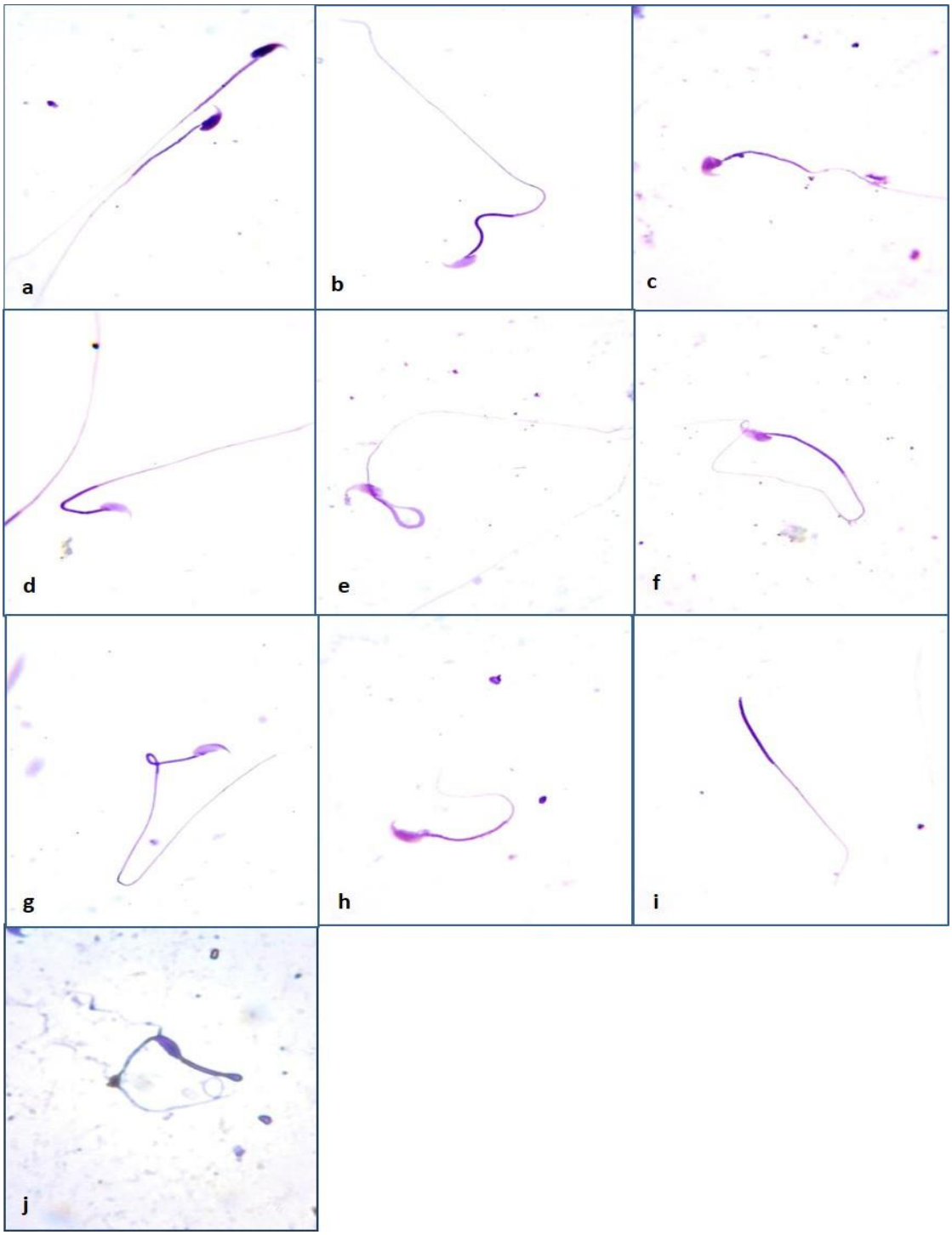

Figure 1. Gentian violet staining of sperm from mice treated with Sodium Benzoate under a light microscope with magnification of 1000x.

Abnormal mice sperm morphology classifications; a: normal sperm; b: macrocephalic sperm head; c and d: angulated mid piece; e, f and g: sperm with coiled tail, h: short, i: isolated, j: double.

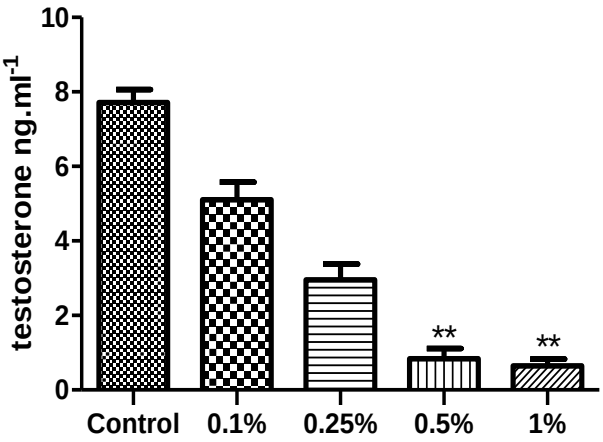

Figure 2. Change in testosterone in sera of different animal groups.
Results are expressed as mean ± SE (n=6). Significance of the difference was determined using One-Way ANOVA. **p < 0.01: significance of difference versus control.

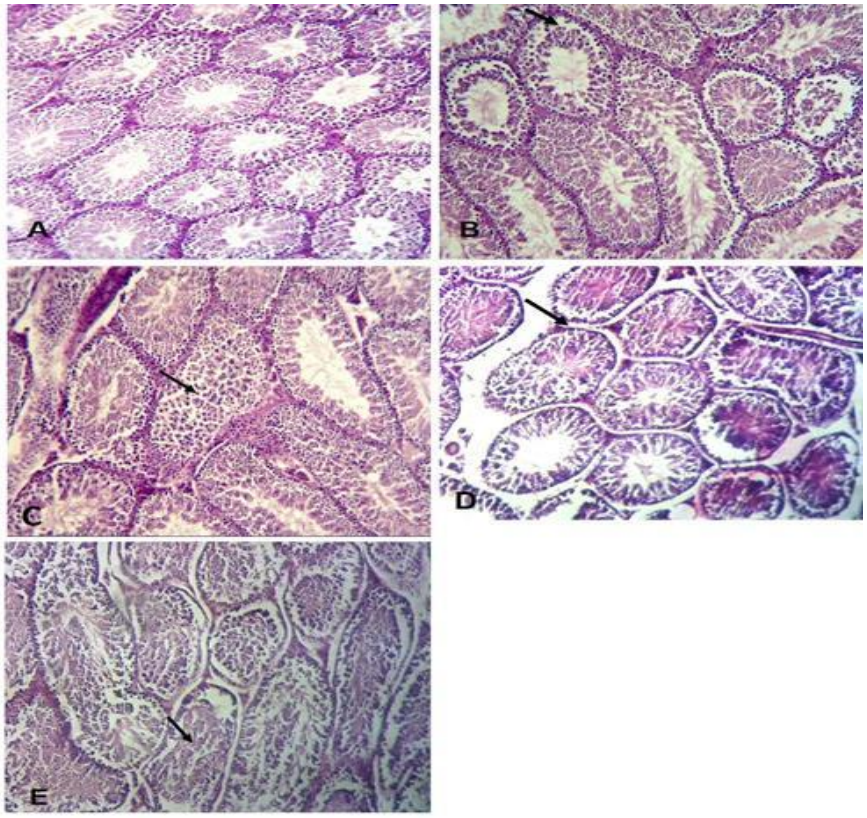

Figure 3. Light microscopy of the seminiferous tubules from mice treated with or without SB
(A) Section in testis of a control mouse showing normal testicular architecture with an orderly arrangement of germinal cells. (B) Testis of 0.1% BS treated mouse showing detached germ cells from the irregular basal and dilatation of seminiferous tubule (arrow). (C)Testis of 0.25% SB treated mouse showing a degenerated germ cells (arrow). (D and E)Testes of 0.5 and 1% SB treated mice showing degenerated interstitial tissue and degenerated germ cells (arrow).

DISCUSSION

The current study sought to determine the effect of sodium benzoate on reproductive parameters in Swiss male mice.

We observed a decrease in body weight gain, sperm count, sperm motility and serum testosterone levels. However, we showed an increased relative testes weight and abnormal sperm morphology as well as damage in testis structure.

Our study revealed a significant decrease in the body weight gain and significant increase in the relative testes weight in 0.5 and 1% sodium benzoate treated groups. The reduced weight gain may be attributed to reduced food intake (data not shown). This result is consistent with the study of Fujitani et al. (1993) who showed a reduce in average weight of the rats treated with sodium benzoate at a concentration of 2.4%, with increase in relative liver and kidney weight.

In a 90-days study with rats dosed with 0, 1, 2, 4, or 8% sodium benzoate via diet, the mortality in the highest dose group was about 50%, with reduced weight gain, increased relative weights of liver and kidneys along with histopathological changes, of the survivors animals (Deuel et al., 1954). This study conclued that sodium benzoate fed at level of 4 % in the diet have no harmful effect on rats. On the other hand, it has been demonstrated that 4% dose of sodium benzoate in diet results in growth depression within 35 days, in surviving Swiss mice (Toth, 1984).

The loss weight of the gonads, epididymis and accessory sex organs as well as reduced sperm count and epididymal sperm motility, are considered standard criteria for the characterization of toxic agents that may cause fertility problems in the treated subjects (Ban et al., 1995; Queiroz-Neto et al., 1997).

Dietary benzyl acetate, expressed as benzoic acid equivalents (JECFA, 1997), at up to 5% in the diet for 13 weeks, had no effect on the weights of the epididymis, cauda epididymis, or testis, on sperm motility or density, or on the percentage of abnormal sperm in mice of rats (US NTP, 1993). Nevertheless, we observed a significantly reduce in testicular and epididymal sperm counts, with decrease in sperm motility in 0.5 and 1% sodium benzoate treated mice. Reduction in total sperm count reflects that less sperms are produced in testes. These findings are similar to data from Fathabad et al. (2017) who showed that sodium sulfite, another commonly food preservative, affected spermatogenesis, epididymal morphometry, and sperm parameters. There can be several explanations for less sperm production such as loss of spermatogonia, arrest of cell cycle and death of intermediate stages of sperm formation.

In addition, the long-term administration of butyl paraben, at the upper-limit acceptable daily intake (10 mg/kg body weight/day), also caused the reduction of sperm counts in the testis and epididymis (Oishi, 2002a).

Previous work has found parabens to be associated with sperm DNA damage in men as measured by the percentage of DNA in comet tail (Meeker et al., 2010). Methyl and propyl paraben have been shown to affect mitochondrial activity in isolated rat hepatocytes (Nakagawa et Moldeus, 1998), which may be a mechanism of male infertility (Soni et al., 2002; Tavares et al., 2009). Furthermore, the motility of sperm relies on ATP synthesized by oxidative phosphorylation and ceases when the mitochondria become damaged or uncoupled (Jääskaläinen et al., 2003).

Presence of abnormal sperm is another useful indicator of chemical toxicity on the reproductive cells. A high consumption of foods containing preservatives, such as the “Western diet” is associated with poorer sperm count and normal sperm morphology (Liu et al., 2015).

In our present study, a statistically significant increase of sperm abnormalities number was observed in the 0.5 and 1% sodium benzoate treated mice. According to Wyrobek et al., (1983) several kinds of mutations can lead to abnormal sperm morphology. Generally, damage to the sperm cell occurs either by physiological, cytotoxic or genetic mechanism (Otitoloju et al., 2010). This investigation is in accordance with *in vitro* study conducted by Zengin et al., (2011) who showed that sodium benzoate at very high concentrations (100µg/ml), does have genotoxic effects in human lymphoblastoid cell lines. Recent studies suggest that the sodium benzoate associated with other food additives can contribute to the activation of inflammatory pathways (Raposa et al., 2016), and may have a toxicogenomic effect (Loutsidou et al., 2012).

In a four-generation study with male and female rats, no adverse effects on fertility or lactation were seen after dosing with benzoic acid at up to 1% in the diet (Kieckebusch et Lang, 1960). However, in the present study, crosses between males exposed to sodium benzoate and control male mice with healthy females, showed a significant decrease in mating index and the number of births in 0.5% and 1% treated groups. These results may indicate the inability of a male

to fertilized, because of various defects in sperm morphology and/or decrease in the numbers of sperm.

Testosterone is essential for maintaining spermatogenesis and male fertility (Oishi, 2002a; Ahangarpour et al., 2014). Chemicals can damage the structure and function of the epididymis directly or through suppressing Leydig cell testosterone production and can also reduce the number of qualitatively normal sperm entering the epididymis (De Grava et Klinefelter, 2014). In the present study, a significantly decrease of testosterone levels was observed in the 0.5 and 1% sodium benzoate treated mice. Additionally, several studies showed that rodent exposure to butylparaben (Oishi, 2001, 2002a), propylparaben (Oishi, 2002b) and butylated hydroxyanisole (Jeong et al., 2005) induced a decrease in testosterone synthesis and adversely affected the male reproductive function.

Indeed, Jana et al., (2006) suggest that decreased serum testosterone is due to inhibition of testicular steroidogenic enzymes, responsible for the synthesis of testosterone.

In the present study, the dose-levels of sodium benzoate produced adverse effect on testes. Studies on evaluating effects of chemicals on male fertility indicated that testis histopathology is most sensitive parameter for detecting any effect (Mangelsdorf et al., 2003; Dent, 2007).

Histological results revealed damage of the seminiferous tubules together with atrophy of interstitial cells (composed of cells Leydig) and inhibition of spermatogenesis in the testis of mice orally exposed to sodium benzoate to 0.25, 0.5 and 1% doses. Testicular damages are characterized by dilatation of some siminiferious tubules in all treated groups and loss of germ cells in 0.5% as well as extensive disruption in 1% treated group. This finding is in agreement with the study that indicates that sodium metabisulfite (SMB) at 260 mg/kg/day induced many histological and biochemical alterations in the testicular tissue of rats (Fathabad et al., 2017).

Leydig cells have a direct role in the regulation of spermatogenesis by producing hormones, specifically testosterone. Reduction in testosterone causes regression of the epididymal epithelium and reduces the androgen-dependent facets of sperm maturation (De Grava and Klinefelter, 2014). O'donnell et al., (1996) suggest that testosterone affects the adhesives function between round spermatids and Sertoli cells, leading to the sloughing of round spermatids for epithelium. It has been confirmed that in the absence of testosterone, progressive germ cell degeneration beguins during stage VII of the spermatogenic cycle (Sharpe, 1994).

Our findings clearly indicate that sodium benzoate has toxic effects on the testis physiology and morphology of Swiss Albino mice when administered at the high dose of 1% equivalent to 1017.31 $mg.kg^{-1}.day^{-1}$ and middle dose of 0.5% corresponding to 522.6 $mg.kg^{-1}.day^{-1}$. These doses level were in excess of the ADI of sodium benzoate (0-5 mg kg−1 body weight). In fact, no one knows exactly how much benzoate is ingested through dietary regimen, and sodium benzoate was the first chemical preservative permitted in food for human consumption in the U.S. in 1908 and continues to be used in a large number of foods (Jay, 1992). Furthermore, the international program on chemical safety assured no adverse effects in humans at doses of 647-825 mg/kg body weight per day (Nair, 2001). In China, the maximum permitted utilization of benzoates in different types of food ranges from 0.2 to 2.0 g/kg [GB2760-2014].

Because the level of chemicals in food and also the food consumption pattern are different from country to country, it is necessary in developing countries to conduct their own exposure assessment using domestic data, and compare the local exposure data with the safe intake level developed by international expert groups (Chen, 2004).

CONCLUSION

In conclusion, the present data demonstrate that sub-chronic ingestion of sodium benzoate, in drinking water is able to impair male reproductive health in male mice, at 0.5 and 1%. Above the ADI, sodium benzoate possesses adverse effects. It is very important for reproductive health to carry out surveys among the population to estimate their daily intake of additives, since processed and packaged foods have become a convenient choice for today's busy families.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

DR performed the experiments, data analysis and also wrote the manuscript. YB, MG, AH performed animal treatments and tissue collection, NM provided scientific advice, DS and OK contributed to the experimental design and to the manuscript revision. All authors read and approved the final version.

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