

Original Article

Histopathological evaluation of the Zebrafish (*Danio rerio*) testis following exposure to methyl paraben

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Abstract: Methyl paraben (MP) is widely used as a preservative in various products. It frequently enters into aquatic environment and renders potential threat to fish. The aim of this study was to evaluate reproductive toxicity of MP on zebrafish (*Danio rerio*) under laboratory conditions. Male zebrafish were exposed to four concentrations of MP (0.001, 0.01, 1, and 10 mg L⁻¹) for 21 days in semi-static condition. Changes in mean length, mean weight, gonadosomatic index (GSI) and histology of testis were studied. Treatment at 0.001 to 10 mg L⁻¹ MP had no significant effect on the survival, mean length and mean weight of fish. But, GSI decreased in a dose dependent manner and the decrease was significant in the group that received the highest dose. Histological alteration of testis consisted of general testicular atrophy, multi-nucleated gonocytes (MNGs), impaired germ cell, spermatogonial proliferation, Leydig cell hyperplasia, interstitial fibrosis and apoptosis of Sertoli cells. It was concluded that sub-chronic exposures of MP could adversely affect GSI, disrupt the histology of testis and produce estrogenic and antispermatogenic activity in male zebrafish.

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Introduction

The endocrine disruption compounds (ECDs) are a large group of chemicals which enter into the aquatic environment from manufacture of various industrial and consumer products, agriculture and food/drug processing, waste water treatment plants and human wastes. This group includes certain polychlorinated biphenyls, polyaromatic hydrocarbons, dioxins, furans, pesticides, alkylphenols, synthetic steroids, plant sterols and parabens (Hamilton et al., 2008; Jasinska et al., 2015). Some of the most potent endocrine disruptors share p-substituted phenol as a molecular structure, which includes parabens.

Parabens (esters of 4-hydroxybenzoic acid, also known as alkyl p-hydroxybenzoates), are used as preservatives in cosmetics, toiletries, pharmaceuticals and foods, and are antibacterial agents in certain toothpastes (Fig. 1). Four main parabens are in use: methyl, ethyl, propyl and butyl parabens; many products will have two or more of these chemicals as

part of a preservative system. In a survey of 215 cosmetic products, methyl paraben was detected in 98% of products (Barse et al., 2010; Oishi, 2002). Approximately 8000 tons of parabens are consumed annually around the world, and most are continuously released into the environment during their production, use, and disposal (Gao et al., 2016). Methyl paraben is one of a homologous series of parabens, used singly or in combination to exert the intended antimicrobial effects and it has been used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years (Taylor et al., 2002).

Exposure to synthetic estrogens (such as parabens) may adversely affect human health, particularly with regard to the reproductive cycle and reproductive function. Ethyl, propyl and butyl parabens have previously been shown to possess estrogen-mimicking properties in fish (Barse et al., 2010; Taylor et al., 2002; Bjerregaard et al., 2003). Recently, parabens have been shown to act as xenoestrogens, a class of

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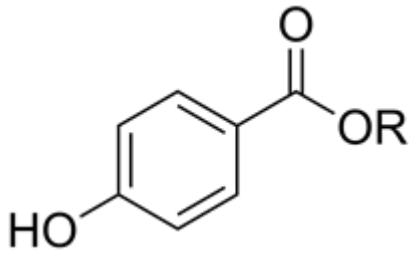


Figure 1. Chemical structure of paraben.

endocrine disruptors (EDs), whose chemical structures can be closely associated with differences in their estrogenicity. Like other xenoestrogens, parabens can also mimic the effects of physiological estrogens. They may bind to estrogen receptors (ERs), stimulate the ER-dependent response, and/or influence the expression of estrogen-responsive genes, including ER α , the progesterone receptor (PR) and pS2. The potency of parabens as estrogens appears to depend on the lengths of their alkyl side chains (Vo et al., 2010). In addition, some recent studies have reported adverse reproductive effects of parabens (Routledge et al., 1998; Boberg et al., 2010; Gonzalez-doncel et al., 2014).

The zebrafish (*Danio rerio*) is increasingly used as an ecological model species for studies of stress physiology including the effects of the exposure to different environmental chemicals (Segner, 2009). In fact, zebrafish has been found useful in EDC screening, in EDC effects assessment and in studying targets and mechanisms of EDC action. Since many of the environmental EDCs interfere with the sex steroid system of vertebrates, most EDC studies with zebrafish addressed disruption of sexual differentiation and reproduction (Yang et al., 2009).

Numerous studies have evaluated susceptibility of aquatic organisms such as fish to toxic effects of exposure to different parabens (Barse et al., 2010; Vo et al., 2010; Bjerregaard et al., 2003), in vivo studies of methyl paraben with zebrafish, which are lacking in the literature. Gonad histopathology is also a valuable tool for the assessment of endocrine-disrupting effects on fish. The molecular events evoked by hormonally active agents have an effect on the levels of cell, tissue, and organ organization and morphology, and the nature of the evoked effects is specific and depends

on the hormonal system that has been disrupted. In addition, histopathological changes, specifically those in the gonads, can be predictive of the (reproductive) fitness of the specimen under study and thus for the fitness of the population (En et al., 2003). Therefore, the aim of this study was to investigate the effects of sub-chronic exposure of methyl paraben on GSI and testis histopathology in zebrafish under laboratory conditions.

Materials and Methods

Test organisms: Adult male zebrafish, with an age of about 120 day, were obtained from a local dealer and acclimated to laboratory conditions for 2 weeks before the experiment. During the acclimation period and experiment phases, fish were given daily supplies of standard fish pellets (1% body weight / day) and inspections were conducted twice a day to discard wounded, diseased and dead fish. Fish were kept at a maximal density of 5 fish L⁻¹ in 10L tanks (27 \pm 1 $^{\circ}$ C) on a 12-h light:12-h dark photoperiod. Ion concentrations in the synthetic water were (mg L⁻¹): 6.26 K⁺, 11.5 Na⁺, 4.74 Mg²⁺, 11.6 Ca²⁺, 32.4Cl⁻, 31.0 NO₃⁻, 9.61 SO₄²⁻ and 0.45 CO₃²⁻.

Experimental design: Methyl paraben (CAS No: 99-76-3) (Fluka Chemika, Germany), HPLC grade ethanol (Merck), were purchased. Stock solution of the methyl paraben was prepared in ethanol at concentration 100 mg L⁻¹. Four groups of fish were subjected to serial dilutions of the stock solution of methyl paraben of 0.001, 0.01, 1.0 and 10.0 mg L⁻¹. In addition to natural controls (unexposed males), solvent control (ethanol) and a positive control group (17- β estradiol; 10.0 ng L⁻¹) were employed in the study. Each treatment was performed in two different tanks (20 fish each). The test was performed by the semistatic (renewal) method in which the exposure medium was exchanged every 24 hrs to maintain toxicant strength and level of dissolved oxygen as well as minimizing the level of ammonia excretion during this experiment. The exposure period lasted 3 weeks. Length and weight of the fish were recorded after the experiment. On the last day of experiment, fish were anesthetized on ice and testis were excised and

Table 1. Mean (\pm SD) values of length, weight and GSI following 21 days exposure of male zebrafish to control and methyl paraben treatments.

	Control	Solvent control	17- β estradiol	Methyl paraben (mean of all doses)
Length (mm)	37.81 \pm 4.21 ^{a*}	38 \pm 1.10 ^a	37.94 \pm 1.34 ^a	37.40 \pm 6.06 ^a
Weight (g)	36.02 \pm 8.12 ^a	35.45 \pm 6.04 ^a	35.02 \pm 2.71 ^a	36.10 \pm 1.21 ^a
GSI (%)	5.32 \pm 0.88 ^a	5.02 \pm 1.06 ^b	4.12 \pm 0.94 ^c	3.16 \pm 0.81 ^d

*Values in a column followed by different letters are significantly ($P < 0.05$) different Duncan's multiple range

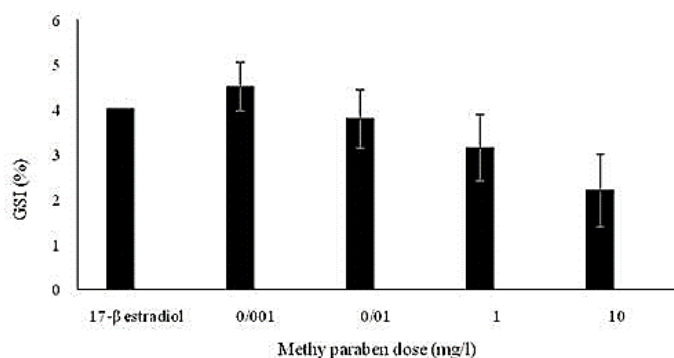


Figure 2. Changes in gonadosomatic index (GSI) in adult male zebrafish exposed to 17- β estradiol (10.0 ng L⁻¹, positive control) and different concentrations of methyl paraben. Data are given as mean (n=5) \pm standard deviation (SD)

weighed for gonadosomatic index (GSI) determination. The GSI was expressed in grams per 100 g body weight.

Histological preparation: Histological examinations were performed as described by (Khodabandeh and Abtahi, 2006; Eagderi et al., 2013). For histological studies, gonads were immediately fixed in Bouin's fluid for 24 hrs. Fixed testes were dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections were cut at 4 μ m and collected onto glass slides, stained by haematoxylin and eosin, and then examined under a light microscope. Gonads from six males were examined from each treatment.

Statistical analysis: To compare the mean length, mean weight and GSI of fish between controls and mean values of different concentrations of methyl paraben, one-way analysis of variance (ANOVA) was used. The data were tested for homogeneity of variances before ANOVA and significance was tested at a level of $P < 0.05$. Significant difference between means were tested by Duncan's multiple range test ($P < 0.05$). The statistical package SPSS, version 16, (Chicago, IL, USA) were used for data analysis.

Ethical considerations and animal rights in this

paper were considered and the study was approved by the international Agency for Protection of Experimental Animals and by the In house Animal Welfare Committee.

Results

There was no mortality during the experimental period in control and treatments. The mean length, mean weight and gonadosomatic index (GSI) of fish from different controls and methyl parabenare treated groups have been presented in Table 1. The results indicated that the mean length and mean weight of male zebrafish were not significantly different between controls and methyl paraben treatments. But GSI was significantly different between methyl paraben treatments and control groups. Changes in GSI in fish exposed to different doses of methyl paraben (0.001, 0.01, 1 and 10 mg L⁻¹) as compared to positive control (17- β estradiol; 10.0 ng L⁻¹) have been shown in Figure 2. The results showed a significant difference in GSI between control and two higher doses of methyl paraben i.e. 1.0 and 10.0 mg L⁻¹. These two doses significantly reduced GSI suggesting that methyl paraben inhibited the development of the zebrafish testes.

The histopathological results of control and methyl paraben exposed fish testis are displayed in Figure 3. Testicular sections from the control group had no visible histopathological alterations and were composed of normal, well-organized and uniform seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Fig. 3a). The arrangement of the seminiferous tubules was regular and the tubular walls were smooth. The Sertoli cells were sparsely distributed, among the spermatogenic cells (i.e. spermatogonia, spermatocytes, and spermatids) at different stages of differentiation.

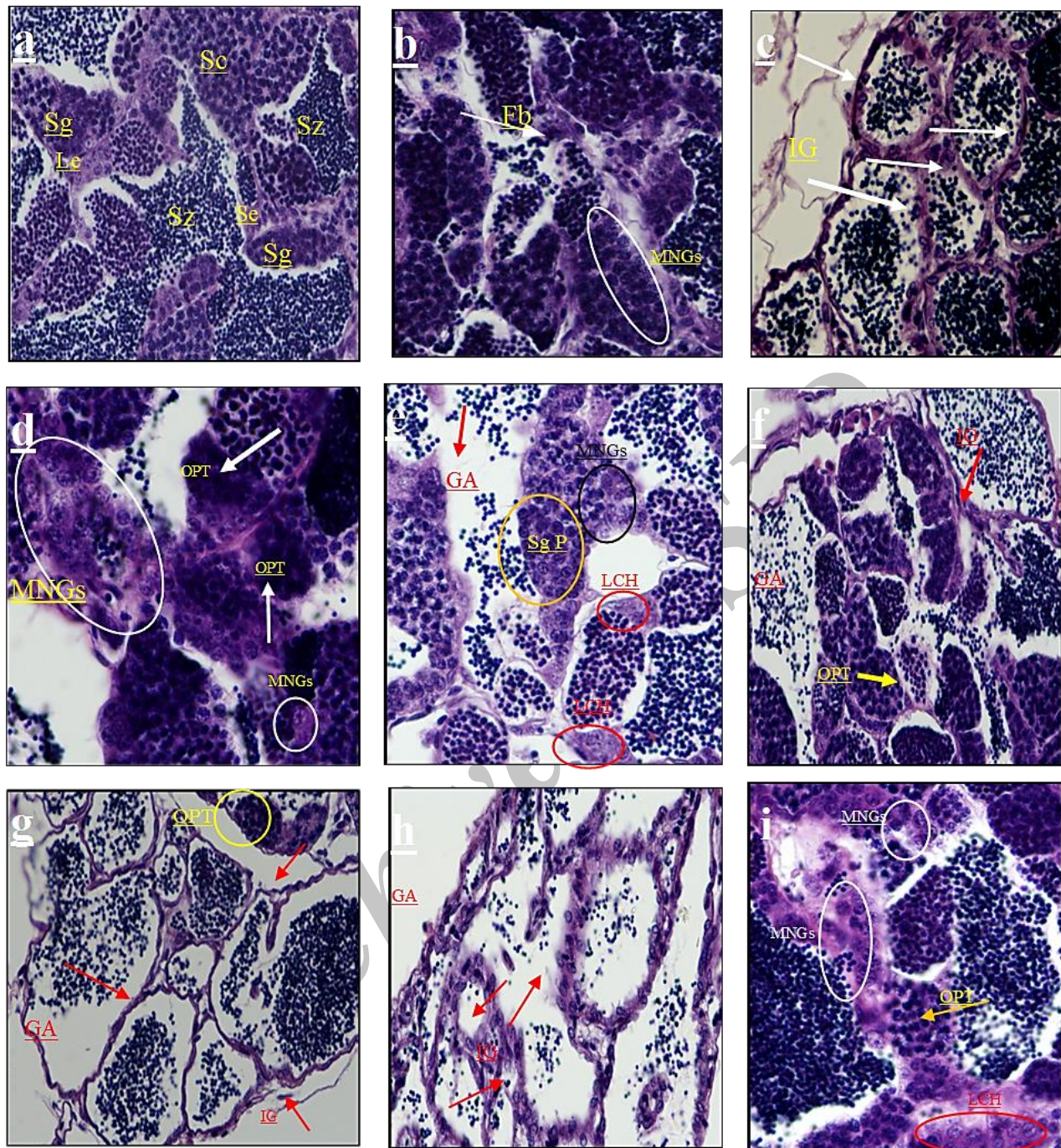


Figure 3. Testicular histopathology in zebrafish exposed to methyl paraben for 21 days (H&E, 40X). The panels include a: control group, b-c: 0.01 mg L⁻¹, d-f: 1 mg L⁻¹ and g-i: 10 mg L⁻¹. The testis of control fish indicated normal histology, while other treatments show injuries, including general atrophy (GA), multi-nucleated gonocytes (MNGs), impaired germ cell (IG), Leydig cell hyperplasia (LCH), opoptosis (OPT), spermatogonial proliferation (Sg P), interstitial fibrosis (Fb) (Sg: Spermatogonia, Sc: spermatocyte, St: spermatic, Sz: spermatozoa, Le: Leydig cell and Se: Sertoli cell).

There was no remarkable histopathological differences between the control and low dose (0.001 mg L⁻¹) of methyl paraben. Exposure to 0.001–10 mg L⁻¹ methyl paraben did not induce ova-testes condition in the male zebrafish. However, different degrees of seminiferous tubular changes were observed in higher

doses of methyl paraben. These damages to fish testis increased with increasing concentrations of methyl paraben.

Slight distortion of seminiferous tubules was recorded at the 1.0 mg L⁻¹ methyl paraben treatment group, and these changes were characterized by slight

sloughing (atrophy) of germ cells and vacuolar degeneration of spermatogenic, Sertoli cells, as well as, the reduction in number of spermatids in the testicular parenchyma (Fig. 3d-f). At higher dose (10 mg L⁻¹) of methyl paraben, the general atrophy of testis was higher as compared to control and lower doses of methyl paraben (Fig. 3g, h, i). The number of gonocytes appeared to be increased, and they were located centrally in the seminiferous tubules due to vacuolization of Sertoli cell cytoplasm. Leydig cells were found in clusters and had small cytoplasm and small irregular nuclei.

Treatments with 1.0 and 10.0 mg L⁻¹ of methyl paraben induced a significant proliferation of the spermatogonia ($P \leq 0.05$). A corresponding significant decrease in the proportion of the spermatozoa in the 1.0 and 10.0 mg L⁻¹ methyl paraben treated fish was noted ($P \leq 0.05$). Atrophic tubules with a massive reduction of germ cells layers was also observed. The small scrotal testis in the highest dose also displayed multifocal Leydig cell hyperplasia and presence of enlarged cells with enlarged or multinucleated nuclei. The Leydig cells in the areas of hyperplasia were smaller in size with less cytoplasm relative to controls and were grouped in large aggregates versus smaller clusters of cells in the control testes. However, the most severe injuries in zebrafish testis were observed in 10 mg L⁻¹.

Discussion

Gonads are frequently used in the evaluation of estrogenic potential of different endocrine disruptors in fish. Impairment of spermatogenesis by estrogenic compounds has been already reported (Taxvig et al., 2008; Boberg et al., 2010). In the present study, methyl paraben at environmentally relevant concentrations was found to interfere with the reproductive performance of adult male zebrafish.

The gonadosomatic index is a useful criterion to quantify sperm production, since spermatozoa form the majority of cells in the mature testis. The lower GSI induced by methyl paraben is probably a result of reduction in the testis size due to the loss of mature germ cells. GSI decreases have been reported in adult

male of other fish species exposed to parabens (Taxvig et al., 2008; Boberg et al., 2010).

The results of the present study also showed that in the methyl paraben treated fish histopathological changes in testes such as multi-nucleated gonocytes (MNGs) and Leydig cell hyperplasia occurred at different exposure concentrations. The cause of MNGs is unknown, but it is not due to abnormal/incomplete cell division because it occurs at ages when the germ cells are quiescent and not dividing. It seems likely that the mechanisms underlying the induction of MNGs are separate from those involved in the formation of focal dysgenetic areas, because MNGs occur throughout the testis and are equally evident in normally formed seminiferous cords as in areas destined to form dysgenetic areas (Soni et al., 2005). Multinucleated germ cells (MNGs) are of particular interest because of their theoretical potential to undergo mutations (given their abnormal DNA content) and thus the potential to give rise to testis germ cell cancer and the MNGs that are formed typically have 2-4 nuclei, but may have as many as 13 nuclei all contained within a common cytoplasm. Theoretically, MNGs could arise either from nuclear division without cytoplasmic division, or from the collapse of intercellular bridges. Given the ability of parabens to induce MNGs by a single exposure during a time when the germ cells are not proliferating, the most logical conclusion is that MNGs form the opening of intercellular bridges (Barse et al., 2010)

The abnormal clustering of Leydig cells in the testes of the methyl paraben-exposed fish appears to be due to abnormal cell migration, which also trapped Sertoli cells, and peritubular myoid cells among the Leydig cell aggregates (Taxvig et al., 2008). These data would suggest that the observation of multinuclear gonocytes in methyl paraben treated testes is due to an inappropriate initiation of cell division, whilst the observed increase in Leydig cell number may be due to a compensatory mechanism as a result of lowered testicular testosterone levels.

However, at higher exposure concentrations (1.0 and 10.0 mg L⁻¹) there was a general increase in general atrophy, fibrosis, impaired germ cell. Unlike

control fish, where the seminiferous lobule lumen was filled with spermatozoa, testis of fish exposed to MP showed altered testicular structure including atrophy, reduced spermatozoa cells and fibrotic changes. The interstitium became narrow and there were less spermatozoa. Other studies have suggested that Leydig cell hyperplasia is associated with disturbances in paracrine relationships between Sertoli and Leydig cells due to germ cell loss and testicular atrophy. Adverse effects on testis structure of mature male common carp (*Cyprinus carpio*) exposed to 90-1,000 4-tert-pentyl phenol (p-alkylphenol) have been reported (Gimeno et al., 1998). These include inhibition of spermatogenesis, disappearance of spermatozoa and spermatogenic cyst and higher incidence of pathological alterations such as fibrosis, vacuolation, and atrophy of germinal epithelium. In the present study, the reduced gonadosomatic index (GSI) and testicular damages were observed in fish exposed to methyl paraben. Several *in vitro* and *in vivo* studies have confirmed that parabens are weak estrogenic compounds (Routledge et al., 1998; Soni et al., 2005; Tayloret al., 2002).

Estrogens may act either directly on the mature testis to regulate androgen production, reducing the basal testosterone and 11-ketotestosterone levels as in E2 treated goldfish (*Carassius auratus*) (Gimeno et al., 1998), or indirectly on gonadotropin secretion. Little is known on how (pseudo-)estrogens, including methyl parabens induce the regression of the testes. Based on the data available from fish models, it is suggested that exposure of fish to pseudo-estrogens decreases the ability of the testestosterone to synthesize androgens by inhibiting genes involved in steroidogenesis (*StAR* and *cyp17 α 1*) and suppressing GnRH-regulated FSH and LH levels (Scholz and Mayer, 2008; Van der Oost et al., 2003; Silva et al., 2012). Steroid hormone levels were not assessed in the present study. But, it is known that estradiol regulates the proliferation of spermatogonia in teleosts (Bhatia et al., 2014). We suggest that slower transformation of spermatogonia to the spermatocyte, spermatid and spermatozoa stage after treatment with methyl paraben is a multicausal condition involving hormonal

imbalance and testicular damages. The interstitial fibrosis observed in the testes of parabens-exposed fish is in line with previous studies reporting testicular fibrosis after exposure to estrogens in male common carp (Barse et al., 2010).

The localisation or expression levels of the gene for ER α in the interstitial tissue of the testes was not measured in the present study. We suggest that an increase in estrogenicity in the micro-environment of the testicular cells after treatment of fish with parabens could have caused these anomalies. In addition, we observed apoptotic cells in the testes of the paraben-treated fish. This is in agreement with the mammalian studies using parabens (Scialli, 2011). However, the intracellular mechanisms of testicular histopathological changes with the possible involvement of (EDCs) are not known and further investigation is needed. As demonstrated in mammalian studies, the development of abnormal germ cells could also be due to abnormal interactions between the germ cells and the Sertoli cells resulting in multinucleated gonocytes (Clewell et al., 2013; Gray et al., 2001). The multinucleated cells could have resulted decreasing in the proportion of spermatocytes (Fisher et al., 2003).

It is concluded that methyl paraben can cause reproductive toxicity to male zebrafish. Exposure to subacute concentrations of methyl paraben for 3 weeks can cause irreparable damages on testes. Estrogenic and antispermatogenic activity of methyl paraben on fish, as observed in the present study, is of great concern because parabens are widely used as preservatives in topical cosmetic preparations and find their ways to aquatic ecosystem.

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چکیده فارسی

ارزیابی آسیب شناسی بافت بیضه ماهی گورخری (*Danio rerio*) پس از مواجهه با متیل پارابن

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چکیده:

متیل پارابن به‌عنوان نگهدارنده کاربرد وسیعی در تولید محصولات مختلف دارد. ورود مکرر این آلاینده به محیط‌های آبی، تهدید بالقوه‌ای برای آبزیان محسوب می‌گردد. هدف از این مطالعه بررسی سمیت تولید مثلی متیل پارابن بر تغییرات هیستوپاتولوژی بیضه ماهی گورخری (*Danio rerio*) در شرایط آزمایشگاهی است. در این مطالعه تغییرات هیستوپاتولوژی بیضه و شاخص گنادی در ماهی گورخری پس از مواجهه ۲۱ روزه با غلظت‌های ۰/۰۰۱، ۰/۰۱، ۱ و ۱۰ میلی‌گرم بر لیتر متیل پارابن در شرایط نیمه‌استاتیک بررسی شد. نتایج نشان داد که مواجهه با متیل پارابن در هیچ‌یک از تیمارها منجر به بروز تاثیر معنی‌داری در زنده‌مانی و فاکتور k نشد. شاخص گنادی به‌طور معنی‌داری با افزایش غلظت متیل پارابن کاهش یافت. تغییرات بافت‌شناسی بیضه شامل ایجاد آتروفی عمومی بیضه، تشکیل گونوسیت‌های چند هسته‌ای، تخریب سلول‌های زایشی، پرولیفراسیون اسپرماتوگونیای هایپرپلازی سلول‌های لیدیگ، فیبروز بینابینی و مرگ برنامه‌ریزی شده سلول‌های سرتولی بود. این نتایج نشان داد که مواجهه نیمه مزمن با متیل پارابن می‌تواند تاثیرات مضر بر کاهش شاخص گنادی و تخریب بافت شناسی بیضه به‌عنوان مهم‌ترین اندام تولید مثلی ماهی نر داشته باشد. متیل پارابن با خاصیت استروژنی منجر به بروز تاثیرات غیر قابل برگشتی در سیستم تولید مثلی ماهی نر می‌شود.

کلمات کلیدی: ماهی گورخری، آسیب‌های بافتی، پارابن‌ها، سمیت تولید مثلی.