Received: 5 December 2017 | Revised: 22 December 2017 | Accepted: 27 December 2017

DOI: 10.1002/jbt.22036

WILEY

Methylparaben induces malformations and alterations on apoptosis, oxidant–antioxidant status, *ccnd1* and *myca* expressions in zebrafish embryos

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Abstract

Methylparabens (MP) are widely used as preservatives in cosmetics, pharmacy, and food industry. Although acute toxicity studies in animals indicated that parabens are not significantly toxic, the effects of chronic exposure under sublethal doses are still unknown and the number of related studies is limited. Our aim was to evaluate the effects of MP on the development of zebrafish embryos focusing on development, locomotor activity, oxidant–antioxidant status, apoptosis, and *ccnd1* and *myca* expressions. The expressions of *ccnd1* and *myca* were determined by RT-PCR. Lipid peroxidation (LPO), nitric oxide (NO), and glutathione-S-transferase (GST) activities were determined spectrophotometrically. Apoptosis was determined using acridine orange staining. Locomotor activity was measured using touch-evoked movement test. MP exposure increased malformations, LPO, apoptosis, *ccnd1* and *myca* expressions, and decreased GST activities and NO levels compared with the control group. Our findings will lead to further understanding of the mechanism of MP toxicity, and merit further research.

KEYWORDS

apoptosis, *ccnd1*, methylparaben, *myca*, oxidant–antioxidant status

1 | INTRODUCTION

Endocrine-disrupting compounds (EDC) have gained popularity as a popular subject in human health. EDCs are widespread in the aquatic environment and can cause alterations in development, physiological homeostasis, and health of vertebrates by acting in estrogenic, antiestrogenic, androgenic, or antiandrogenic ways.[1] Zebrafish, *Danio rerio*, has been suggested as a model to identify targets as well as modes of EDC action.[2]

Methyl ester of *p*-hydroxybenzoic acid (parabens) are widely used as preservatives in cosmetics, pharmacy, and food. Acute toxicity studies in animals indicated that parabens are not significantly toxic through various routes of administration and subchronic and chronic oral studies indicated that parabens are practically nontoxic.[3] Despite all these, the effects of chronic exposure under sublethal doses are still unknown and the number of studies on the effects of methylparaben (MP) in literature is limited.

Several alkyl parabens have been shown to exert binding affinities to estrogen receptors and MP has been shown to affect FSH levels, folliculogenesis, and cause histopathological effects.[4–6] On the other hand, exposure to MP from early embryonic stage is a major concern and it has been shown that parabens exist as high concentration in maternal blood, plasma, and amniotic fluid.[7]

Myc, a transcription factor that regulates the expression of cellular genes, is well known for its participation in many malignant conversions. Cyclin D1 (Ccnd1) activates cyclin dependent kinases CDK4 and CDK6 and drives cell proliferation.[8] Analyzing apoptosis and oxidative stress markers is also important to evaluate the relative toxicity of EDCs. Because of its small size and ease of reproduction and breeding, zebrafish is one of the most commonly used fish species in xenoestrogenic potential testing in embryonic stage.[9] The aim of this study was to evaluate the effects of MP exposure on the development of zebrafish embryo focusing on the apoptosis, oxidant–antioxidant parameters, locomotor activity, and *myca* and *ccnd1* expressions.

2 | MATERIAL AND METHODS

2.1 | Chemicals

MP (CAS no: 99-76-3) was purchased from SUPELCO (Bellefonte, PA) and acetone (67-64-1) was purchased from Merck (Darmstadt, Germany). They were all analytical grade with the highest purity available.

2.2 | Maintenance of zebrafish and embryo collection

Wild-type AB/AB strain zebrafish were maintained in disease-free conditions. Zebrafish were housed in an aquarium rack system (ZebTEC, Tecniplast, Italy) at 27 ± 1°C under a light/dark cycle of 14/10 h. They were fed commercial flake fish food complemented with live brine shrimp twice a day. All experiments were fulfilled using reverse osmosis water supplemented with 0.018 mg/L sea salt (Instant Ocean™, USA). Fertilized embryos were collected following natural spawning, cultured, and staged by developmental time and morphological criteria under the stereomicroscope (Zeiss Discovery V8, Germany) as described previously.[10]

2.3 | Embryo exposure

For the embryo toxicity tests, stock solutions of MP were prepared by dissolving in 0.1 mL/L acetone. Range-finding experiments were applied initially to find out the lethal concentration that cause 50% mortality (LC50) in the zebrafish embryos and environmentally relevant concentrations of MP that affect development were determined as 50 mg/L. Acetone was used as the solvent control whereas embryo medium was used as the blank control. Embryos were exposed to MP solutions in well plates for 5 days and they were observed under the stereomicroscope. All exposure solutions were replaced with fresh solutions each day. Developmental parameters were monitored and documented daily under a stereomicroscope (Zeiss Discovery V8, Germany). The images of malformations were captured and the percentage of abnormal embryos was counted every 24 h during the exposure period (4–72 hpf). Individual malformations such as axial malformations, pericardial edema, and yolk sac edema were listed. Embryonic mortality and hatching rate were evaluated every 24 h. The hatching rate is a ratio of hatching embryos to the living embryos in each well. Delays in development were confirmed by comparing with the control embryos. Embryo staging was conducted as explained before,[10] and the pectoral fin, yolk sac, anal pore, and swim bladder were used as the indicators of development.

2.4 | Behavioral analysis

Locomotor activity in vertebrates, including swimming, relies upon neural networks in the brain and spinal cord.[11] The first movements are observed in the embryos precisely at 17 hpf and consisted of alternating side-to-side contractions of the tail, much like a metronome.[12] At 72 hpf, touch-evoked movement test was performed as described previously.[13] The test was performed on MP exposed (n = 80) and control group (n = 72).

2.5 | Biochemical assays

For the biochemical analyses zebrafish embryos at 72 hpf were used. They were prepared as replicate pools of 72 hpf zebrafish (n = 5, 100 individuals per pool). For each pool, 100 embryos were homogenized in 1 mL PBS, followed by centrifuging briefly. The supernatant was used for the determination of biochemical parameters.

2.6 | Total protein determination

Total protein level was determined by the method of Lowry et al.[14] Briefly, alkaline proteins are reacted with copper ions and then reduced by Folin reactive. The absorbance of the product was evaluated at 500 nm by a spectrophotometer and calculated to express the results of the parameters per protein.

2.7 | Lipid peroxidation determination

The method of Yagi[15] was used to determine malondialdehyde (MDA) level, an end product of lipid peroxidation (LPO), as thiobarbituric acid reactive substances. The extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used and LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein.

2.8 | Nitric oxide determination

Nitric oxide (NO) was determined by the method of Miranda et al.,[16] which is based on reducing nitrate to nitrite by vanadium (III) chloride. In an acidic media, nitrite and sulfonylamide reacts with N-(1-Naphtyl) ethylenediamine dihydrochloride and complex diazonium compound was formed. The colored complex was measured at 540 nm by a spectrophotometer and results were expressed as nmol NO/mg protein.

2.9 | Glutathione-S-transferase determination

The activity of glutathion-S-transferase (GST) was determined based on the spectrophotometric evaluation of the absorbance at 340 nm of the product formed by GSH and 1-chloro-2,4-dinitro-benzenin (CDNB) conjugation.[17]

2.10 | Reverse transcription (cDNA synthesis) and quantitative real-time PCR

RNA was isolated from the embryos using RNeasy Mini Kit and QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 mg of total RNA using RT2 Profiler PCR Arrays (Qiagen). PCRs were performed using the DNA Master SYBR Green kit (Qiagen). Primers were obtained from Qiagen (Catalog number of *ccnd1*: PP00106A-200; *myca*: PPZ00447A-200; *β-actin*: PPZ00526A). The expressions of *ccnd1* and *myca* were evaluated by quantitative reverse transcription PCR (RT-PCR) using the Qiagen Rotor-Gene. *β-Actin* was used as the housekeeping gene. Relative transcript levels were calculated using the ΔΔCT method by normalizing the values with the housekeeping gene.[18]

2.11 | *In vivo* cell death assay

Cellular death was detected in live embryos using acridine orange (AO) staining, which is a nucleic acid selective metachromatic dye that connects with DNA and RNA by intercalation or electrostatic attractions.[19] AO does not stain normal cells but selectively stains necrotic or late apoptotic cells with disturbed plasma membrane

permeability. For this method, live embryos were immersed in 5 mg/mL AO for 10 min at room temperature and then washed with E3 medium. Embryos were anesthetized with tricaine for 3 min before examination, and they were visualized and imaged for less than 1 min. Apoptotic cells were identified with a fluorescence microscope (Zeiss V16 Axio Zoom with 546 nm filter).

3 | RESULTS

Data analyses did not indicate any obvious difference between the solvent control and blank control for the indexes investigated in this study; therefore, the test data of the blank control were given for the following sections.

3.1 | Mortality rate and morphological abnormalities of the embryos exposed to methylparaben

Representative images of the zebrafish embryos at 72 hpf are given in Figure 1. Control group embryo exhibited normal growth and development (Figure 1A), whereas tail defect (Figure 1B), pericardial edema (Figures 1C and 1D), and pigmentation defect (Figures 1B and 1D) were observed in the MP group. The percentages of the malformations are given in Figure 1E. MP exposures significantly increased tail defect, pericardial edema, and pigmentation defect compared with the control group. The mortality rates significantly increased in the MP exposed embryos compared with the control group (Figure 2A).

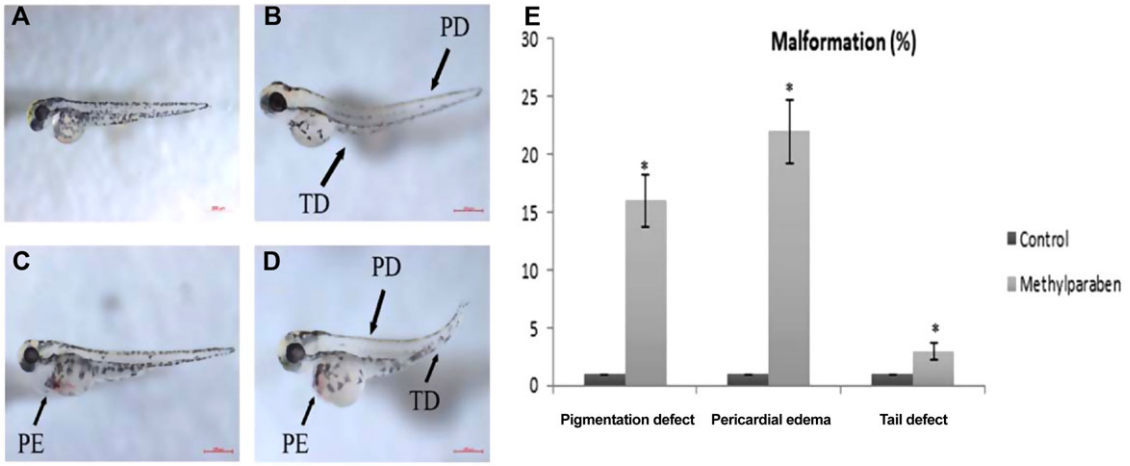

FIGURE 1 (A) Morphology of control embryo at 72 hpf; B–D: morphologies of MP exposed embryos. E: Percentages of cumulative 72 hpf summary of individual morphologic abnormalities observed in zebrafish embryos exposed to MP. TD, tail defect; PE, pericardial edema; PD, pigmentation defect. *Indicates a statistical difference between control and the exposure groups ($P < 0.05$). Data are expressed as means ± S.D. from three independent experiments. Morphology of the embryos were monitored and documented under a stereomicroscope (Zeiss Discovery V8, Germany)

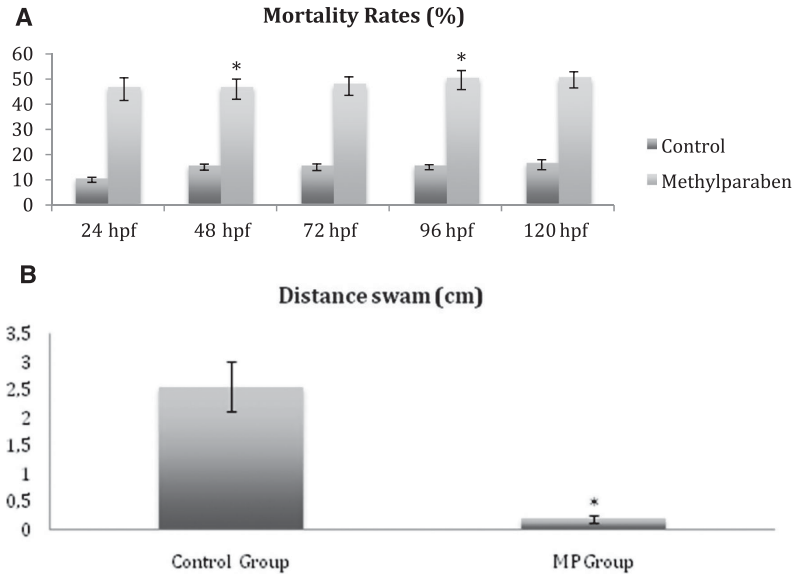

FIGURE 2 (A) Mortality rates of zebrafish embryos exposed to methylparaben for 120 hpf. Data are expressed as mean ± S.D. from the three independent experiments, *$P < 0.05$, significantly different from the control group. (B) Total distance swam by the embryos in the control ($n = 72$) and MP group ($n = 80$). *$P < 0.05$, significantly different from the control group)

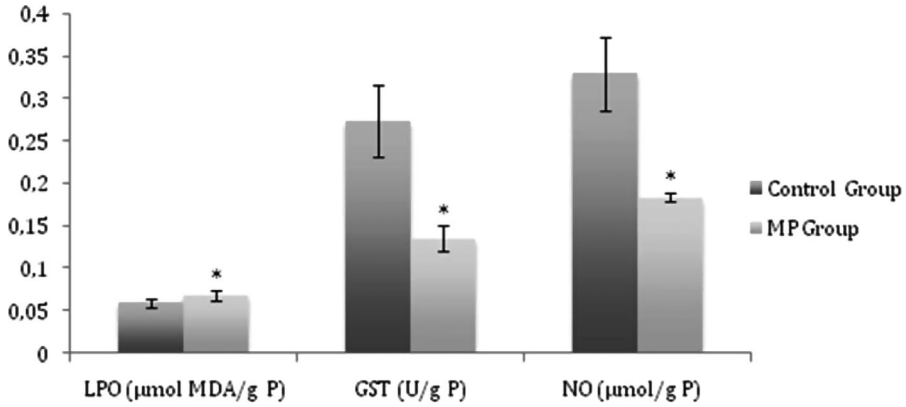

FIGURE 3 LPO levels, GST activities and NO levels of the embryos in the MP and control group. Replicate pools of 72 hpf zebrafish ($n = 5$, 100 individuals per pool) were used. Values are given as mean ± S.D. *$P < 0.05$ significantly different from the control group. LPO, lipid peroxidation; GST, glutathione S-transferase; NO, nitric oxide

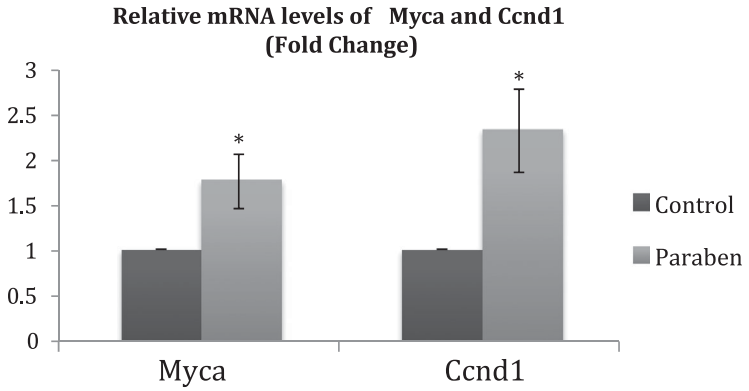

FIGURE 4 Bar graph presentation of fold change of *myca* and *ccnd1* transcript quantified by RT-PCR. All RT-PCR results are normalized to *β-actin*, the house keeping gene and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± S.D. Significant difference is indicated by asterisk, and *P* value < 0.05 was considered as significant

3.2 | Behavioral assay results

Results of the behavioral assay showed a strong inhibition in the locomotor activity of the MP exposed embryos when compared with the control group. The total distance swam is given in Figure 2B. MP exposed embryos swam 0.5 cm and below, whereas the majority of the control group embryos swam 3.5 cm and over.

3.3 | Biochemical assays results

Spectrophotometric analyzes showed significant reductions in the GST activities and NO levels in the MP group. On the other hand, LPO process enhanced in the MP group as evidenced by slight but significant increase in MDA levels (Figure 3).

3.4 | RT-PCR analysis results

The expressions of *ccnd1* and *myca* are given as fold change of transcript quantified by RT-PCR. RT-PCR results were normalized to *β-actin*, the housekeeping gene, and expressed as change from their respective controls. The average values obtained from the three experiments are given in Figure 4. In the MP, increased expressions of *ccdn1* and *myca* were found compared with the control group.

3.5 | *In vivo* cell death assay results

In vivo cell death results of AO staining are presented as representative images of whole embryo cell death determined using AO staining at 72 h in the control and MP exposed embryos. Images were detected by a fluorescence microscope (Figure 5).

4 | DISCUSSION

In this study, MP increased mortality rates and induced pericardial edema, pigmentation, and tail defect, in zebrafish embryos. Similar to our results, Dambal et al.[20] reported pericardial edema in MP-exposed embryos. MP exposure increased the mRNA levels of *myca* and *ccdn1*. Overexpression of *myca* has been associated with increased cellular proliferation and *myca* has been referred as a "primary oncogene" because of these effects.[21] *ccnd1* is referred as a proto-oncogene and its overexpression is frequently observed in tumor cells.[22] Free radicals and oxidants are produced either from normal cell metabolisms or from external sources. Their accumulation in the body leads to a phenomenon called oxidative stress, which plays a major role in the development of chronic and degenerative

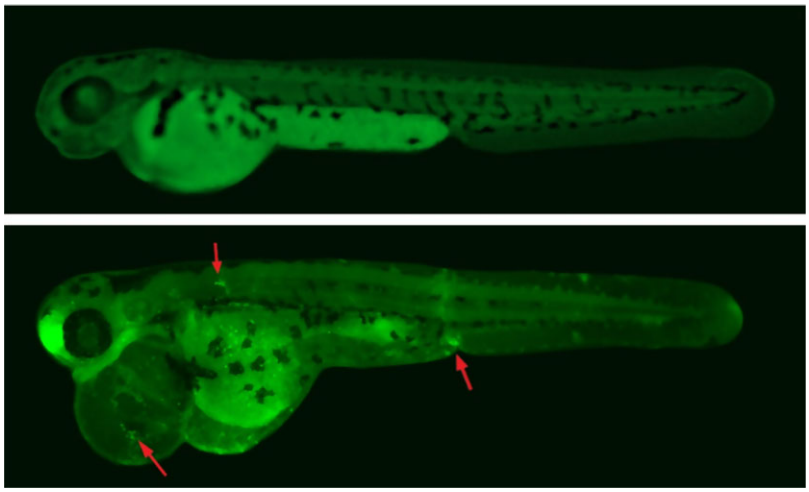

FIGURE 5 Representative images of whole embryo cell death determined by using acridin orange staining at 72 hpf in the control, and MP exposed embryos. Images were detected by fluorescence microscope. Arrow heads indicate apoptotic cells

diseases such as cancer, autoimmune disorders, and aging. Free radicals are scavenged by enzymatic mechanisms including GST to prevent oxidative damages *in vivo*. LPO is an autocatalytic process that causes peroxidative tissue damage.[23] In our study, MP exposure increased MDA, which is used as an index of LPO and decreased GST activity. Our results are in consistent with the other studies that suggested parabens could impair antioxidant capacity. Chen et al.[24] showed the potential additive toxicity of MP on lifespan and preadult development period for *Drosophila melanogaster* and suggested that parabens may increase levels of oxidative damages on macromolecules leading to gradual disruption of organelle function to promote senescence.

NO affects cellular decisions of life and death through apoptotic pathways. Both proapoptotic and antiapoptotic actions of NO have been reported.[25] In our study, NO decreased, whereas apoptosis increased in the MP exposed embryos, which support the antiapoptotic actions of NO. The studies that evaluated the effects of paraben exposure on locomotor activity are limited. Prolonged exposure to ethyl paraben has been reported to decrease locomotion behavior in nematode *Caenorhabditis elegans*. In the same study, locomotion behavior defects were partially recovered in progeny, and higher concentrations of ethyl paraben caused more significant defects.[26] Consistent with these reports, our results revealed decreased locomotor activity in the MP exposed embryos. In conclusion, MP disturbed the oxidant–antioxidant balance, decreased locomotor activity, increased *myca* and *ccnd1* expressions, and apoptosis. We believe our findings will lead to further understanding of the mechanism of MP toxicity and merit further research.

ACKNOWLEDGEMENTS

This project was supported by Marmara University Scientific Research and Project Commision, Project No: SAG-E-120314-0056. Our special thanks are for the Vice Rector of Marmara University Prof. Dr. Mehmet Akalin and his team in the Department of Textile Engineering as it would have been impossible to carry out these long-term research projects without their support.

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How to cite this article: Ateş PS, Ünal İ, Üstündağ ÜV, Alturfan AA, Yiğitbaşı T, Emekli-Alturfan E. Methylparaben induces malformations and alterations on apoptosis, oxidant-antioxidant status, *ccnd1* and *myca* expressions in zebrafish embryos. *J Biochem Mol Toxicol* 2018;32:e22036. https://doi.org/10.1002/jbt.22036