Clinical and Experimental Health Sciences

Effects of Water Samples in Polyethylene Terephthalate Bottles Stored at Different Conditions on Zebrafish Embryos with Relevance to Endocrine Disrupting Chemical Migration and Adenomatous Polyposis Coli Tumor Suppressor Gene

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ABSTRACT

Objective: Polyethylene terephthalate (PET) is a material that is most commonly used for production of clear plastic bottles. Adenomatous polyposis coli (APC) and β-catenin have been related with cancer. Aim was to investigate the effects of PET bottled water samples that were exposed to sunlight and hot water on zebrafish embryos. Moreover the effects of these water samples on APC knockdown zebrafish embryos were also evaluated.
Methods: Phthalate concentrations in water samples were determined using ELISA. Immunohistochemical method and RT-PCR were used to analyse the expressions of proliferative cell nuclear antigen (PCNA), β catenin, Wnt 3a and Gsk3β. Biochemical parameters were measured using spectrophotometric methods. Vitellogenin concentrations were measured using ELISA and apoptotic cells were evaluated by Acridine Orange staining.
Results: Increased PCNA, β-catenin, Wnt 3a, Gsk3β expressions, vitellogenin, nitric oxide, apoptosis and impaired oxidant-antioxidant balance were observed in the exposure groups with these increases being more profound in APC knockdown groups.
Conclusion: APC knockdown embryos were more prone to the deleterious effects of water samples used in this study.
Keywords: Polyethylene terephthalate, phthalates, Wnt/β-catenin signaling, adenomatous polyposis coli

1. INTRODUCTION

The issue on the potential effects of endocrine disrupting chemicals (EDCs) on public health revealed the need for new research into the mechanisms of their effects in case of exposure. EDCs are defined as synthetic or natural molecules in the environment, they can impair endocrine functions and they are suspected carcinogens. EDCs can be detected in different products such as bottles, canned waters, storage boxes as well as treated waste water and they can be categorized as pesticides, plasticizers, industrial side products, pharmaceuticals, flameretardants, phytoestrogens, or heavy metals. EDC exposure during development is a serious and major health concern and may lead to permanent or long-lasting defects (1-3).

Polyethylene terephthalate (PET) is a widely used material for the production of clear plastic bottles to sell water. They are also used to produce soda beverages, sports drinks, vinegar containers and for cosmetic products packaging. On the other hand the potential of plastic materials from packagings to migrate EDCs into foods and beverages has been a neglected isssue for long (4). Phthalates are a group of chemicals that are used to provide flexibility and durability to plastics and chemically they are the diesters of 1,2-benzenedicarboxylic acid, known as phthalic acid. Phthalates have been related with different adverse outcomes including adiposity and insulin resistance (5,6), anogenital distance decrease (7) and alterations in sex hormone levels (8). Other consequences of phthalates have been reviewed by Hauser and Calafat (9). Being as the most commonly used plasticizer di-[2-ethylhexyl]-phthalate (DEHP) has been reported to lead to reproductive and developmental toxicity (10). Migration is defined as leaching of chemicals from food packaging into food. Although this is systematically checked by market authorizations storage conditions and the effects of acidic or alkaline foodstuffs, UV light, and heat may degrade polymers. Leaching of monomers as a result of this process is also known as “release”(11).

Adenomatous polyposis coli (*APC)* gene produces the APC protein which plays critical roles in many cellular processes. The roles of APC protein include its tumor suppressor function, control on cell division, cell attachments and movements. The APC protein works with beta-catenin through Wnt/β-catenin pathway in association with other proteins (12). Mutations in the *APC* gene has been shown to lead to uncontrolled proliferation in intestinal epithelial cells and are related with the earliest colorectal carcinogenesis stages (13). On

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the other hand although the role of Wnt/β-catenin signaling in embryonic development and tissue homeostasis has been evaluated in some studies, the relationship between abnormalities in Wnt ligands and tumorigenicity has not yet been clarified (14).

In recent years zebrafish embryo has become a popular model due to its external and rapid development, small size, high level of fecundity and optical transparency (15). In the current study we tested the hypothesis that *APC* knockdown zebrafish embryos are more susceptive to the deletious effects of water samples that had been heated in PET bottles to simulate the release of chemicals due to storage conditions. Accordingly expressions of Wnt/β-catenin pathway proteins, vitellogenin levels, apoptosis and oxidant-antioxidant status of zebrafish embryos have been evaluated.

2. METHODS

2.1. Maintenance of zebrafish

Wild type AB/AB Strain were maintained in apparently disease-free conditions, kept in the aquarium rack system (Zebtec, Tecniplast, Italy) at 27 ± 1 °C under a light/dark cycle of 14/10 h. Zebrafish were fed twice a day with commercial flake fish food supplemented with live Artemia. Reverse osmosis water that was added 0.018 mg L^{-1} Instant Ocean™ salt was used for all experiments. After natural spawnings, embryos that were fertilized were collected cultured, and staged by developmental time and morphological criteria as described previously (16). This study was approved by the Marmara University Animal Experiments Local Ethics Committee (138.2013.mar; 28.04.2014).

2.2. Preparation of water samples

Commonly used bottled water samples were randomly selected from supermarkets in Istanbul, Turkey. The first group consisted of PET bottles that were kept under sunlight for 30 days in July. The second group consisted of PET bottles that were filled with 100 ^{0}C boiled water and allowed to cool in them. Water samples from these bottles were used for the exposure groups in petri dishes (volume: 40ml). Embryos were added to each dish and placed into the incubation chamber and examined for 120 hours.

2.3. Embryo exposures

For the exposure experiments, embryos were exposed to the water samples in well plates for 120 hours after fertilization (hpf). In order to evaluate development, mortality and hatching parameters the group were prepared as three replicate wells. Each group contained 20 embryos. The exposure solutions were prepared every day as fresh solutions. When the exposure period ended, the embryos were washed with embryo medium for several times and they were allowed to develop until 120 hpf. A stereomicroscope (Zeiss Discovery V8, Hilden, Germany) was used for the detection of developmental parameters. Malformation images were recorded. The rates of mortality and hatching were determined for every 24 h. The hatching rate is defined as the ratio of hatching embryos to the living embryos in a well.

2.3. Phthalates Analyses in Water Samples

Phthalates were measured using the phthalates Elisa kit (Abraxis Phthalates ELISA, Microtiter Plate, Katalog No: 530050, Railroad Drive, Warminster,USA) which is a direct competitive ELISA method that is based on the recognition of total phthalates by antibodies. The concentrations of phthalates in the samples are evaluated by interpolation using the standard curve constructed with each run. Using the absorbances at 450 nm, a dose-response curve was obtained from known concentrations of phthalates standards and the BPA concentration in the samples (n=7) were calculated using the absorbances obtained from the standard curve.

2.4. Morpholino Microinjections

Morpholino oligonucleotides were maintained from Gene Tools LLC. APC and (5'-TAGCATACTCTACCTGTGCTCTTCG-3') the control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') were dissolved in 1 mM in 1× Danieau buffer. 0.5 mM morpholino was injected into wild type embryos that were at one – four cell stages for the microinjections (17,18).

2.5. Expression Analyses

2.5.1. Whole Mount Immunohistochemistry

For whole-mount immunohistochemical expression of proliferative cell nuclear antigen (PCNA), β-catenin and Wnt 3a zebrafish embryos were fixed. Pronase (2.0 mg/ml, in E3 medium; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) was used to dechorionate embryos for 3 to 5 min and then they were rinsed five times in E3 medium. They were incubated for 1 hour in 4% paraformaldehyde and Anti-PCNA antibody, Anti-β-Catenin antibody, Anti-Wnt 3a antibody (abcam ab28472; abcam ab6302 and abcam ab29 respectively) were used as primary antibodies.

2.5.2. Reverse Transcription (cDNA synthesis) and Quantitative Real-Time PCR

Rneasy Mini Kit and Qiacube (Qiagen, Hilden, Germany) were used for the isolation of RNA from the embryos according to the manufacturer's instructions. After that single-stranded cDNA was produced from 1 µg of total RNA using RT^2 Profiler PCR Arrays (Qiagen). DNA Master SYBR Green kit (Qiagen) was used for PCRs. The expression of *cyclin D1*, *c-myc, β actin*, *wnt 3a* and *gsk3β* were evaluated by quantitative RT-PCR using the Qiagen Rotor Gene-Q Light Cycler instrument (Qiagen, Hilden, Germany). The average values were calculated based on the results of three experiments. DDCT method was used

normalizing the values with the house keeping gene *β actin* (19).

2.6. Determination of Vitellogenin levels

Vitellogenin levels were determined at the end of 120 hours in zebrafish embryos by the Zebrafish Vitellogenin Elisa Kit (Biosense, Prod No: V01008402,Michigan, USA). For whole body homogenates, 50 embryos were homogenized in 500 µL PBS. Then whole body homogenates were 1:500 diluted (5 µL homogenate and 2495 µL dilution buffer) according to the manufacturer's instructions. Each group was prepared as five replicates. This assay is based on the use of specific binding between antibodies and vitellogenin to measure vitellogenin in the samples.

2.6.1. Biochemical Assays

Zebrafish embryos at 72 hpf were used (n=5, 100 individuals per pool). The embryos were then homogenized in 1ml PBS, which was followed by centrifugation. The forming supernatant was used for the biochemical assays.

2.6.2. Total Protein Determination

Total protein concentrations were measured according to the method of Lowry (20). For this method, alkaline proteins were reacted with copper ions and then they were reduced by the Folin reactive. A spectrophotometer was used to evaluate the absorbance of the product at 500 nm. The results were calculated and expressed as levels per protein.

2.6.3. Lipid Peroxidation Determination

Malondialdehyde (MDA) is the end product of lipid peroxidation (LPO) and the method of Yagi (21) was used to determine MDA concentrations. The MDA results were presented as nmol MDA/mg protein using the extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

2.6.4. Nitric Oxide Determination

Nitric oxide (NO) levels were assessed using the method based on reducing nitrate to nitrite by vanadium (III) chloride (22). In this method, nitrite and sulfonylamide reacts with N-(1-Naphtyl) ethylenediamine dihydrochloride in an acidic media and as a result diazonium compound is produced. The absorbance of the colored complex was measured at 540 nm using spectrophotometer and results were calculated and presented as nmol NO/mg protein.

2.6.5. Glutathione-S-transferase Determination

Glutathion-s-transferase (GST) activity was evaluated using the method based on GSH and 1-chloro-2,4-dinitro-benzenin (CDNB) conjugation and the absorbance of their product at 340 nm (23).

2.6.6. Determination of Apoptosis

Apoptosis of live embryos was determined at 72 hpf using acridine orange (Sigma, Darmstadt, Germany) staining method (24). Accordingly, live embryos that were immersed for 10 min at room temperature, in 5 µg/ml acridine orange a nucleic acid-selective metachromatic stain. After that embryos were washed in E3 medium. Then the embryos were anesthetized using Tricaine for 3 min. Embryos were visualized and imaged for less than 1 minute, apoptotic cells were determined using fluorescence microscope (Zeiss V16 Axio Zoom microscope-546 nm filter, USA).

2.6.7. Statistical analysis

One-way Anova with post-hoc Tukey's Multiple Comparision Test was used to analyse the differences between normally distributed data (Shapiro–Wilk normality test), using Graph Pad 6, A p value of ≤0.05 was considered as significant.

3. RESULTS

In order to examine the hypothesis that *APC* knockdown embryos were more prone to the possible deleterious effects of chemicals that leach from PET bottles and DEHP, we aimed to to knock down the function of APC and evaluate the effects of exposure to the water samples kept in PET bottles on both control and *APC* knock down embryos. To achieve this, a morpholino that is splice blocking specific for *APC* that targets the splicing of *APC* between exon 15 and intron 15 was designed as previously shown (25). At the one – or two-cell stage this APC morpholino was injected this into wild type zebrafish embryos. The intron-retained RNA transcript was produced as shown by the expression levels of the reported β-catenin target genes, *c-myc* (26) and *cyclin D1* (27) that are expected to be up-regulated following *APC* knockdown. We observed the malformations in the *APC* morphant embryos such as pericardial edema, tail and pigmentation defects (Figure 1A). Accordingly increased expressions of c-*myc* and *cyclin D1* were confirmed by quantitative RT-PCR (Figure 1B). Immunohistochemical results revealed the elevated cellular β-catenin staining in the neural tube, notochord and eye region which indicates clonal loss of APC function in the MO group. The expression of PCNA was evaluated as a proliferation marker and in neural tube and eye intense staining revealed elevated expression in the MO group. MO injected embryos also presented more intense Wnt 3a staining in eye, brain, neural tube, notochord, somites, dorsal, caudal and anal fins (Figure 1C).

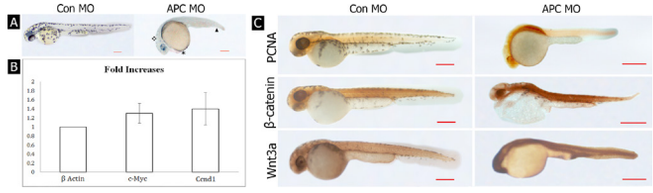

Figure 1. A) *Phenotypes of Control Morpholino (Con MO) and APC Morpholino (APC MO) *pericardial edema, ▲tail defect, □ ❖ pigmentation defect* ***B)*** *Quantitative RT-PCRwith primers specific for ϐ-actin, c-Myc, and Ccnd1 was performed on cDNAs obtained from embryos injected with control or APC morpholinos, expressed as fold increases.* ***C)*** *PCNA, ϐ-catenin and Wnt 3a expressions in Con MO and APC MO group; Con: Control, APC: Adenomatous polyposis coli, PCNA: Proliferative cell nuclear antigen*

The mortality rate increased and hatching rate decreased significantly in the APC morphant embryos compared with the Con MO group. Significant increases in mortality rates and decreases in hatching rates were observed in the 30d Sun PET and PET 100 °C groups when compared with the Con MO group. On the other hand, mortality rates increased and hatching rates decreased in both MO injected exposure groups when compared with their respective uninjected controls (Figure 2).

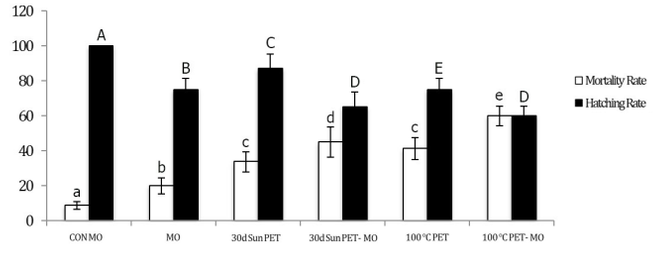

Figure 2. *Mortality rates and hatching rates of embryos, n=20, The average values were obtained from three experiments. Data presented are mean ± SD, Different letters within columns indicate statistically significant differences in the mortality rate (lower case) or hatching rate (upper case) as determined by One-way Anova followed by post-hoc Tukey's Multiple Comparision.*

Immunohistochemical analysis revealed increased intensity of PCNA in neural tube, brain and eye; β-catenin in notochord and somites, Wnt 3a in eye, brain and notochord in the 30 days sun exposed; PCNA in eye, neural tube, notochord and somites, β-catenin in eye, brain, neural tube, Wnt 3a in eye, brain, notochord and somites in the 100 ^{0}C boiled water filled PET bottles especially in the MO injected groups (Figure 3A).

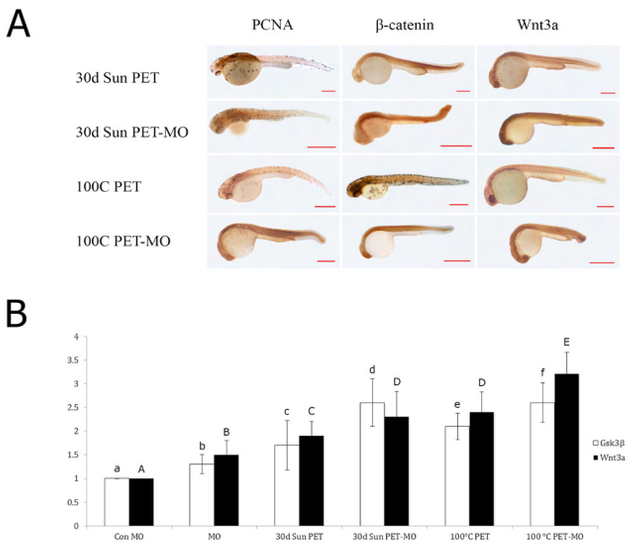

Figure 3. A) *Immunohistochemical analysis of the expression of PCNA, ϐ-catenin, Wnt 3a, Control Morpholino images are given in Figure 1.* ***B)*** *Quantitative expression of wnt 3a and gsk3ϐ expressed as fold increases in 30 days direct sun light exposed PET group (30d Sun PET), 100°C boiled water filled PET group (100^{0}C PET), and APC morpholino embryos corresponding to the exposure groups (MO). Increased expressions were observed in the exposure groups but yet increases more evident in the APC morphant embryos. Data presented are mean ± SD. Different letters within columns indicate statistically significant differences in the expressions of Gsk3ϐ (lower case) or Wnt 3a (upper case) as determined by One-way Anova followed by post-hoc Tukey's Multiple Comparision. Con: Control, MO: Morpholino, PET: Polyethylene terephthalate*

The *wnt3a* and *gsk3ϐ* expressions were found to be elevated given as fold increases with the *ϐ-actin* gene being used for normalization. Significant increases were observed in *wnt3a* and *gsk3ϐ* expressions in the MO group when compared the Con MO group. The expressions of *wnt3a* and *gsk3ϐ* in MO injected 30 d Sun PET and PET 100 °C exposed embryos increased significantly when compared with their uninjected controls (Figure 3B).

Results of total phthalates analyses in the water samples are given as the mean concentration of three replicates in Table 1. Phthalate levels in the control PET bottle was below the detection limits of the assay used in this study. On the other hand, total phthalate levels increased to the detection limit of the assay in the exposure groups, 100^0 C boiled water filled (9,63±1,3 µg/L) and 30 days direct sunlight exposed PET bottles (8,45±1,28 µg/L) (Table 1). The concentrations of vitellogenin, nitric oxide and lipid peroxidation increased significantly whereas glutathione S-transferase decreased in the exposure groups when compared with the control group (Table 2). According to the acridine orange staining results there were considerable numbers of apoptotic cells in the exposure groups mainly in the head and eye region (Figure 4).

Table 1. *Total phthalates concentrations in drinking water samples in PET and PC bottles*

Total Phytalates Concentration in PET Bottles (ng/ml)	
Control PET bottle	ND
30 days direct sunlight exposed PET bottle	8,45±1,28
100^{0}C boiled water filled PET bottle	9,63±1,3

Data presented are mean ± SD. ND: Not detected, PET: Polyethylene terephthalate

Table 2. *Vitellogenin, nitric oxide, lipid peroxidation and glutathione-S transferase levels of the zebrafish embryos*

	Vitellogenin (ng/mL)	NO (nmol/mg P)	GST (U/g P)	LPO (µmol MDA/g P)
Control	59,8±5,7	13,44±1,9	0,41±0,022	0,028±0,002
30d PET	92,6±6,3^a	21,34±3,6^b	0,23±0,01^a	0,035±0,002^d
100° PET Group	108,8±9,4^a	24,76±3,72^c	0,23±0,012^a	0,032±0,003^e

Data presented are mean ± SD. NO: Nitric oxide; GST: Glutathione-S transferase; LPO: Lipid peroxidation. Significant differences from the control group are indicated by letters, ^{a}p<0,0001; ^{b}p=0,0046; ^{c}p=0,0003; ^{d}p=0,0004; ^{e}p=0,021

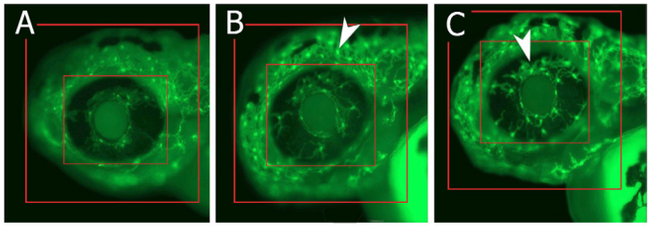

Figure 4. *Apoptotic cells were observed in the head and retina region of the zebrafish embryos.* ***A)*** *Control Group,* ***B)*** *30 d Sun Exposed PET,* ***C)*** *100 °C heated PET Group. Small square: eye region; Big square:head region, PET: Polyethylene terephthalate*

4. DISCUSSION

Use of plastic materials increased for bottled water production and PET is one of the most used polymer. But chemical migration of plasticisers and additives to water (28,29) became a major health concern. Phthalates are used to improve flexibility and phthalate migration has been shown before (30-31).

Quantitative phthalate determination is challenging as phthalates do not persist in outdoor environment. Gas chromatography mass spectrometry is the most used technique but in the present study ELISA was used. Phthalate levels were below the detection limit for control PET bottled water samples but increased in exposure groups. Increased DEHP concentrations due to poor storage conditions have been reported before (31-33).

DEHP is a potential carcinogen and toxic to reproductive organs, kidneys and liver (7,34-37). Zebrafish (*Danio rerio*) is used for studying the effects of environmental toxins (38). To examine the hypothesis that APC knockdown embryos were more prone to the effects of chemicals that leach from PET bottles we aimed to knock down APC function. Mutations in the Wnt pathway are related with birth defects, cancer and other diseases (39). Genetic mutations in APC and β-catenin way lead to activation of canonical signaling as the protein product of *APC* gene is a key component of the β-catenin destruction complex (40).

The expressions of *wnt 3a* and g*sk3β* increased significantly in the 30 days sunlight exposed and 100 ^{0}C boiled water filled PET bottle groups and increases were more profound in the *APC* knockdown groups. Mutations in APC result in the accumulation of β-catenin and stimulate proliferation genes like *c-myc* (26). In our study PCNA was evaluated as a proliferation marker and increased expressions were observed in the APC morphant exposure group. β-catenin's abnormal expression causes various diseases including cancer (41). β-catenin expression increased in exposure groups especially in *APC* knockdown groups. Acridine orange staining showed that apoptotic cells mainly accumulated in the head region which indicates possible EDC induced impairments in this region through the aryl hydrocarbon receptor (AhR) (42). Oxidant-antioxidant balance was impaired and NO levels increased in the exposure groups. There are conflicting roles of NO in proapoptotic pathways (43-45). Increased *gsk3β* expressions may be related with apoptosis through the inhibition of prosurvival transcription factors, and activation of proapoptotic transcription factors (46,47).

Vitellogenin genes are expressed in an estrogen-dependent manner therefore only mature females are able to produce vitellogenin in larger quantities. On the other hand estrogenic molecules trigger vitellogenin synthesis in males and larvae as well (48-50). In this study vitellogenin levels increased in exposure groups. DEHP's estrogenic activity is still unclear (50,51). We have previously shown that DEHP (2,5 µg/L) did not increase vitellogenin levels in zebrafish embryos (52). Increased vitellogenin levels in exposure groups may be due to higher concentration of phalates in water samples or other chemicals that may have migrated into water from PET bottles.

5. CONCLUSION

The results of this study demonstrate that poor storage conditions increase phthalate concentrations in PET bottled water samples through photolysis (53,54) and *APC* knockdown embryos were more prone to the deleterious effects of these water samples. Therefore although the determined phthalate concentrations are not yet a thread for human health, their striking effects on zebrafish embryo should be taken into account and appropriate storage conditions should be promoted for public health.

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