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RESEARCH ARTICLE

Effects of Low Doses of Bisphenol A on Primordial Germ Cells in Zebrafish (*Danio rerio*) Embryos and Larvae [1] [2]

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Summary

Primordial germ cells are the precursors of gametes in sexually reproducing organisms. The migration and homing process of primordial germ cells are prone to environmental effects and the endocrine system hormones. One of the environmental pollutants which humans are exposed to is bisfenol A (BPA). BPA which is an endocrine disrupter generally used for making plastics harder. In this study we investigated the effects of low doses of BPA (4 mg/L and 8 mg/L) on primordial germ cells at the zebrafish embryos and larvae. Whole mount in situ hybridization for germ cell marker gene vasa showed that upon exposure to BPA, the primordial germ cell number increased and these cells ectopically localized outside the normal germ cell niche in zebrafish. Migration of primordial germ cells into ectopic locations and morphological changes in these cells were also proved by histological studies. Additionally, results of acridine orange staining to detect apoptotic cells showed that low doses of BPA did not increase apoptosis. Results of the present study showed that low doses of BPA exposure increased the number of primordial germ cells and induced ectopic primordial germ cell localization in the zebrafish embryos.

Keywords: *Zebrafish, Primordial germ cell, Bisphenol A*

Düşük Dozlardaki Bisfenol A'nın Zebra Balığı (*Danio rerio*) Embriyo ve Larvalarında Primordiyal Germ Hücreleri Üzerine Etkisi

Özet

Primordiyal germ hücreleri, eşeyli üreyen canlılarda gametlerin öncül hücreleridir. Endokrin sistem hormonları ve çevresel etkenler, primordiyal germ hücrelerinin göçünü etkilemektedir. İnsanların maruz kaldığı çevresel kirleticilerden biri de bisfenol A (BPA)'dır. Endokrin sistemde hasar oluşturan biri olan BPA, genellikle plastik sertleştirme işleminde kullanılmaktadır. Bu çalışmada zebra balığı embriyo ve larvalarında düşük dozlardaki (4 mg/L and 8 mg/L) BPA'nın etkileri incelenmiştir. Bir germ hücresi belirteci gen olan vasa ile yapılan whole mount in situ hibridizasyon deneyleri sonucunda BPA'nın primordiyal germ hücre sayısında artışa sebep olduğu ve bu hücrelerin göç yolu dışındaki (ektopik) bölgelerde bulunduğu tespit edilmiştir. Primordiyal germ hücrelerinin ektopik bölgelere göçü ve bu hücrelerdeki morfolojik değişiklikler histolojik çalışmalarla da doğrulanmıştır. Buna ek olarak, düşük dozlarıyla yapılan boyamalarda apoptozisin tespitinde kullanılan akridin turuncusu boyaması sonucunda BPA'nın apoptozisi artırmadığı saptanmıştır. Bu çalışma sonuçları, düşük dozlardaki BPA'nın primordiyal germ hücre sayısını artırdığını ve bu hücrelerin ektopik bölgelere göç etmesine yol açtığını ortaya koymaktadır.

Anahtar sözcükler: *Zebra Balığı, Primordiyal germ hücresi, Bisfenol A*

INTRODUCTION

Primordial germ cells (PGCs) constitute an embryonic cell type that migrate to gonadal precursors and form the gametes. Thus PGCs are the cells that provide the permanence of the species and are crucial for the development of a new organism in the next generation [1]. In many organisms PGCs are specified during early

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embryonic development and they contain germ granules [2-7].

PGC migration must be finely regulated as it follows a complex pathway through various developing tissues [8]. The ultimate location of PGCs in zebrafish is between the endodermal cells the yolk sac which are close to allantois, at early stages of the embryonic development [9]. They migrate into dorsal mesentery and reaches in the gonad primordia with amoeboid movements. The pathway which PGCs follows defined as "gonadal pathway" [10-13]. PGC migration in zebrafish takes place during the first 24 h of the embryonic development [14-16].

In zebrafish, germ line lineage can be traced throughout embryogenesis by means of transcripts of *vasa* [17] which is a gene that is first identified in *Drosophila melanogaster* as a maternal determinant of germ cell formation [18]. In zebrafish, *vasa* is a molecular marker for PGC [19,20]. Vasa protein can be detected in the germline cells in early embryos, because that it specifically located into polar granules, which are located where the germ cells are specified. PGCs are specified at four positions along the margin of the blastodisc and start migrating dorsally during gastrulation [16]. PGCs move towards the intermediate targets around somites 1-3 at 10.5 h post fertilization (hpf), and subsequently to the final target region at the level of somites 8-10 at 13 hpf. At 24 hpf, PGCs localize to the junction between the yolk ball and yolk extension in the gonadal region, forming compact clusters [16].

Several chemicals or environmental pollutants can cause cellular damage in PGCs which has ramifications in different diseases/disorders in organisms [21,22]. Therefore, investigating the migratory behaviour and regulations of PGCs is an intriguing biological question. Additionally, human are exposed to many of the environmental toxicants everyday. Information obtained from experiments on the reproductive systems of the experimental animals can provide important findings about the impact of humans.

Endocrine disrupting chemicals (EDCs) are compounds in the external environment that modulate the physiology of the endocrine system and often cause health disorders [23]. Bisphenol A (2 bis(4-hydroxiphenyl) propane, BPA) is one of the most potent endocrine disrupting chemicals which mimics estrogen *in vivo* and *in vitro* [24] and therefore is classified as xenoestrogen. BPA is widely used in industry for making plastics harder. It's found in polycarbonate plastics (baby feeding bottles, carboys) [25] and inside of epoxy coated cans [26]. With the increasing usage of these materials in daily life, humans are exposed to BPA in an escalating manner, which could cause endocrine disorders. High concentrations of estrogens stimulate PGC growth *in vitro* through the somatic cells of the gonadal ridges [27]. BPA acts by several different mechanisms to perturb the early stages of oogenesis and also affects primordial germ cells, by influencing mitotic proliferation and the timing of meiotic entry [28].

Duan and Zhu [29] investigated the toxicity of BPA on the growth of zebrafish embryos and found the median embryo lethal concentration (LC50) for 24 h as 16.36±0.40 mg/L. They also mentioned that all embryos died at 25 mg/L dose at 24 hpf. So in the present study, doses were chosen according to LC50 value of bisphenol A on zebrafish embryos. We carried out zebrafish embryos 4 mg/L and 8 mg/L BPA and investigated the effects on primordial germ cells of the zebrafish embryos. In 24 hpf zebrafish embryos we determined the location of PGCs with whole mount in situ experiments. With histological analysis we observed the migration pathway of PGCs for two weeks.

MATERIAL and METHODS

This study was approved by Kocaeli University Animal Researches Ethics Committee (HADYEK- 5/3 - 2011).

Material

All animal experiments and procedures were carried out in accordance with the recommendations and official guidelines for animal handling and research. The temperature and humidity were kept at 28.5°C and 61%, respectively. Sexually matured zebrafish were fed daily with *Artemia* sp. and Tetra- Min© Hauptfutter (Tetra Werke, Germany) under standardized conditions (20-L glass aquaria, 28±1°C, light/dark cycle = 14 h/10 h) and after egg laying embryos were collected immediately. Under a stereo microscope, fertilized and unfertilized eggs were separated. Fertilized embryos were transfered into special small embryo aquariums.

Methods

BPA Exposure

BPA, 2 bis(4-hydroxiphenyl), (Sigma Aldrich, CAS No: 50-05-7) was dissolved in 1% dimethylsulfoxide (DMSO) to generate desired concentrations for treatment of the embryos. Two control and 2 experimental groups were used as: control (untreated), solvent control group (1% DMSO), 4 mg/L BPA and 8 mg/L BPA. DMSO and BPA were applied from 1 h post fertilization until sacrificing the embryos.

Whole Mount In Situ Hybridization

24-hpf-old embryos from control and BPA treatment groups were collected and fixed in 4% paraformaldehyde (PFA, Sigma) overnight at 4°C. After removing chorion, the embryos were washed in methanol series and stored in 100% MeOH at -20°C. Digoxigenin-labelled antisense RNA probes were generated for vasa gene as described Miyake et al.[30]. Whole mount *in situ hybridizations* with chromogenic substrates Nitro Blue Tetrazolium (NBT)/5-Bromo4-Chloro3-Indoyl phosphate (BCIP) were on the 24-hpf-embryos performed as previously described [31,32].

Acridine Orange Staining

After removing the chorion of the zebrafish embryos 5 µg/L acridine orange (Sigma) was added into the embryo rearing medium. Embryos were incubated in acridine orange solution for 30 min. They were anaesthetised with 0.04% of 3-aminobenzoic acid methyl ester (MESAB) and imaged under dissecting fluorescent microscope.

Histology

Embryos and larvae (0-2 weeks old) were fixed in Bouin's fixative for 8 h. After fixation, tissue was dehydrated in ascending concentrations of ethanol, equilibrated in xylene. The tissues were then embedded in paraffin wax and cut into 5-7 µm sections on a microtome. The sections were mounted on glass slides and stained with haematoxylin and eosin, toluidine blue and Best's carmine before examination under a light microscope.

Statistical Analysis

Statistical analysis were performed by using IBM SPSS Statistics 5.0 programme. Quantitative data was given as mean ± SD. T test were used for analyses and $P<0.05$ was considered as statistical significance value. For statistical analysis, 7 samples were used in each group (n=7).

RESULTS

Results of Expression of vasa Gene in Primordial Germ Cells

Vasa gene is a germ cell marker [19,20]. At embryonic stages of development, *vasa* transcripts are concentrated in an electron-dense structure, the putative zebrafish germ plasm [20,33].

The location and number of PGCs were detected at 24 h post fertilization (*Fig. 1* and *Fig. 2*). In each group, 7 embryos were tested. PGCs that migrate the correct and ectopic locations were counted. In control (untreated) and DMSO groups PGCs migrate to the target region correctly (*Fig. 1-a* and *1-b*). When compared the 4 mg/L BPA and 8 mg/L BPA groups with control ones, there was an increase in the number of PGCs *(Fig. 2)* and a significant portion of germ cells were at ectopic locations (*Fig. 1-c* and *1-d*). It was observed that 1% DMSO and BPA treatment increased the PGCs numbers in comparison to the untreated group. t test results showed that DMSO and BPA treatment increased the total PGC number *(Table 1)*. The number of PGCs increased in BPA treated group *(Table 2)*. BPA treatment also increased ectopic PGCs *(Table 3)*. Our data suggest that BPA caused an increase in the number

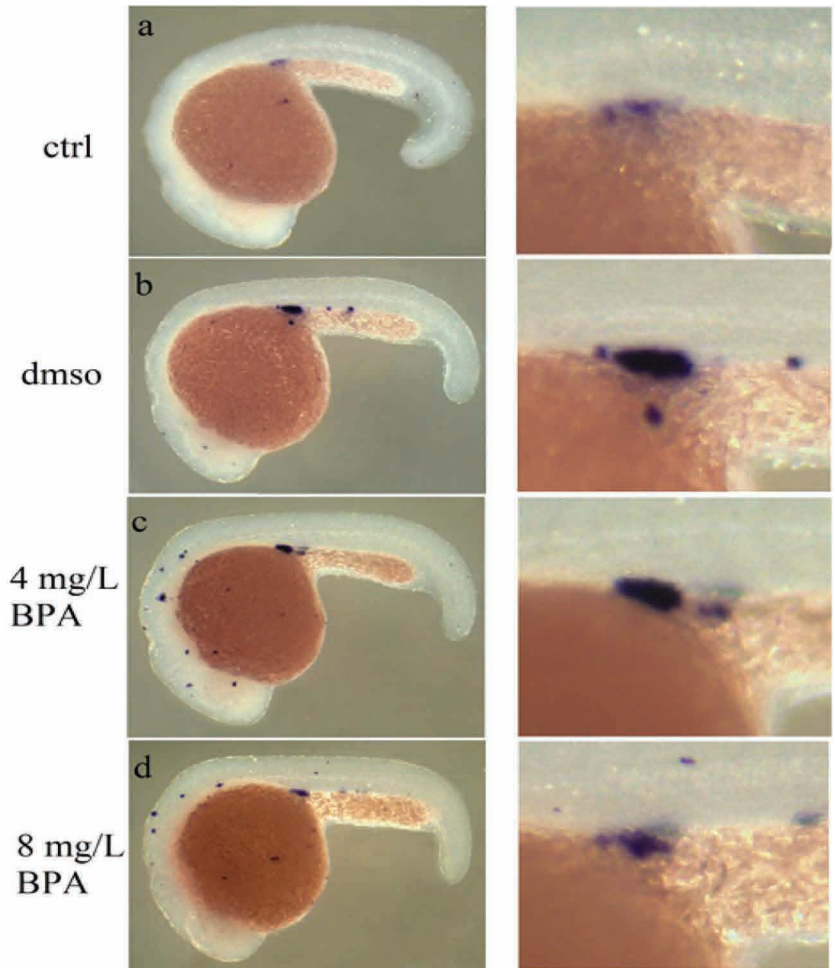


Fig 1. Whole mount *in situ* hybridization results of 24 hpf old zebrafish embryos. Magnifications at the right side show PGCs which locate the right direction. **a)** Untreated (ctrl) group, PGCs located to the right direction, **b)** 1% DMSO (solvent control) group, PGCs located to the right direction but there are few ectopic cells, ***c)*** 4 mg/L BPA treated group, most of PGCs are ectopic, **d)** 8 mg/L BPA treated group, most of PGCs are ectopic

Şekil 1. 24 saatlik zebra balığı embriyolarında whole mount *in situ* hibridizasyon sonuçları. Sağ taraftaki büyütülmüş kısımlar doğru bölgede bulunan primordiyal germ hücrelerini (PGH) göstermektedir. **a)** Kontrol grubu, PGH doğru bölgede bulunmaktadır, **b)** Çözücü kontrol (%1 DMSO) grubu, PGH doğru bölgede bulunmaktadır fakat birkaç hücre ektopik bölgede bulunmaktadır, **c)** 4 mg/L BPA uygulanmış grup, pek çok PGH ektopik bölgelerdedir, **d)** 8 mg/L BPA uygulaması yapılmış grup, pek çok PGH ektopik bölgededir

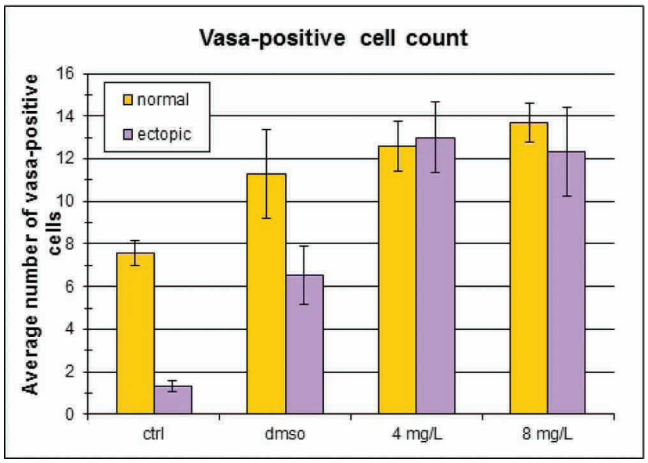


Fig 2. General comparement of control and BPA treatment groups. When compare with control group, as a result of BPA treatment more vasa-positive cells (PGCs) were detected at ectopic and right locations (n=7)

Şekil 2. Kontrol ve BPA gruplarının genel karşılaştırması. Kontrol grubu ile karşılaştırıldığında BPA uygulaması sonucunda vasa pozitif hücreler (PGH) doğru ve ektopik bölgelerde tespit edilmişlerdir (n=7)

Table 1. *Total (ectopic + gonadal region) PGC frequency at 24 hpf zebrafish embryos*

Tablo 1. *24 saatlik zebra balığı embriyolarındaki toplam (ektopik + gonadal bölgedeki) PGH sıklığı*

Group	Total PGCs Frequency			
	Average		Standart Deviation	*
Control (untreated) (n=7)	4.43	±	0.94	a
1% DMSO (n=7)	9.00	±	1.45	b
4 mg/L BPA (n=7)	13.50	±	0.81	c
8 mg/L BPA (n=7)	13.71	±	0.98	c

** There are significant difference between samples that are shown with different letters (P<0.05)*

Table 2. *PGC frequency at gonadal region at 24 hpf zebrafish embryos*

Tablo 2. *24 saatlik zebra balığı embriyolarındaki gonadal bölgedeki PGH sıklığı*

Group	PGCs Frequency at Gonadal Location			
	Average		Standart Deviation	*
Control (untreated) (n=7)	7.57	±	0.65	a
1% DMSO (n=7)	11.29	±	2.22	a,b
4 mg/L BPA (n=7)	12.57	±	1.25	b
8 mg/L BPA (n=7)	13.71	±	0.99	b

** There are significant difference between samples that are shown with different letters (P<0.05)*

of PGCs, although all of these cells cannot migrate into the prospective primordia and remained at ectopic locations.

Results of Acridine Orange Staining

In the second part of the study we investigated the effects of BPA on the apoptosis frequnecy in zebrafish embryos. Acridine orange is a nucleic acid fluorochrome which emits fluorescence when it intercalates into the DNA [34]. BPA treatment did not change apoptosis frequency and the frequency was in normal range that normally prevails during the embryonic development *(Fig. 3)* of zebrafish embryos.

Table 3. *PGC frequency at ectopic locations at 24 hpf zebrafish embryos*

Tablo 3. *24 saatlik zebra balığı embriyolarındaki ektopik bölgelerdeki PGH sıklığı*

Group	PGCs Frequency at Ectopic Locations			
	Average		Standart Deviation	*
Control (untreated) (n=7)	1.29	±	0.29	a
1% DMSO (n=7)	6.71	±	1.57	b
4 mg/L BPA (n=7)	14.43	±	1.00	c
8 mg/L BPA (n=7)	13.71	±	1.77	c

** There are significant difference between samples that are shown with different letters (P<0.05)*

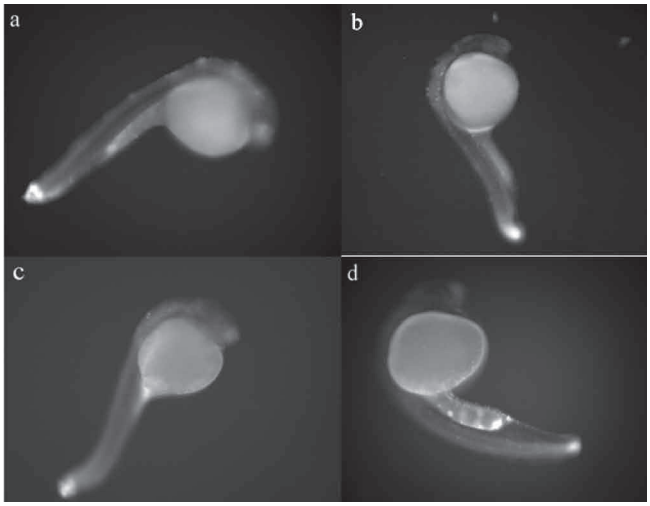


Fig 3. Acridine orange staining results **a)** Control group **b)** Solvent control group (1% DMSO) **c)** 4 mg/L BPA treated group **d)** 8 mg/L BPA treated group. When compare BPA treatment groups with control ones, it was not seen any differences between the groups

Şekil 3. Akridin turuncusu boyama sonuçları **a)** Kontrol grubu **b)** Çözücü kontrol grubu (%1 DMSO) **c)** 4 mg/L BPA uygulaması yapılmış grup **d)** 8 mg/L BPA uygulaması yapılmış grup. Kontrol grupları BPA uygulaması yapılmış gruplarla karşılaştırıldığında gruplar arasında bir fark görülmemiştir

Histological Studies

Morphology of primordial germ cells were detected with histological methods during 2 weeks. The embryos of the 8 mg/L BPA group could survived 7 days maximally. PGCs which are bigger than somatic cells and have large nucleus, nuage material and glycogen granules were detected by H&E, toluidine blue and Best's carmine stains. It was found that results of histological studies were supported by whole mount *in situ* results. In DMSO and BPA treated groups, increase in the number of PGCs which located at ectopic regions were detected.

At 3 day post fertilization (dpf) embryos, PGCs were observed at the dorsal part of yolk sac in untreated group. PGC structure was normal *(Fig. 4-a)*. Location of PGCs were shown with arrows *(Fig. 4-b,c,d)*. In BPA treated groups, decrease at glycogen granules in PGCs were observed *(Fig. 4-c,d)*.

At 7 dpf prelarvae, PGCs were observed near muscle tissue *(Fig. 5-a,b,c,d)*. In untreated and DMSO group, the shapes of migrating PGCs were ameboid (shown with star) *(Fig. 5-a,b)*. In BPA treated groups, most of PGCs did not have ameboid shape *(Fig. 5-c,d)*.

At 14 dpf larvae, in untreated and DMSO groups, PGCs were observed at gonadal region *(Fig. 6-a,b)*. PGCs could not migrate gonadal region at 4 mg/L BPA treated group. In this group, PGCs were observed near somites *(Fig. 6-c)*. Decrease at nuage material of PGCs were also detected in this group *(Fig. 6-c,d)*.

DISCUSSION

We conducted this study to evaluate the effects of BPA on primordial germ cells of zebrafish (*Danio rerio*) embryos because very little is known about the effects of BPA on primordial germ cells in vertebrates. There are not any study the effects of BPA on primordial germ cells. so, our study is pioneer about this subject.

Avcı et al.[35] determined the effects of nonylphenol on growth parameters and antioxidant defense system

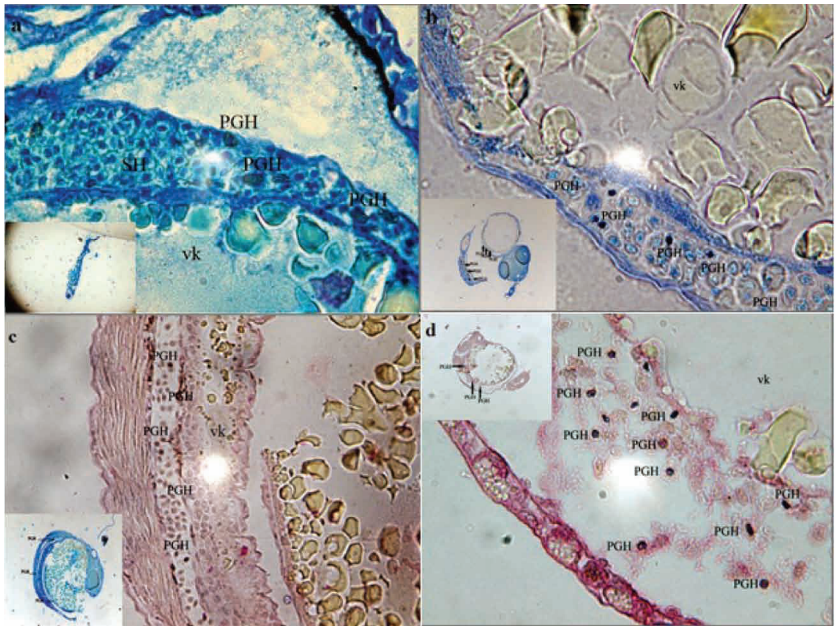
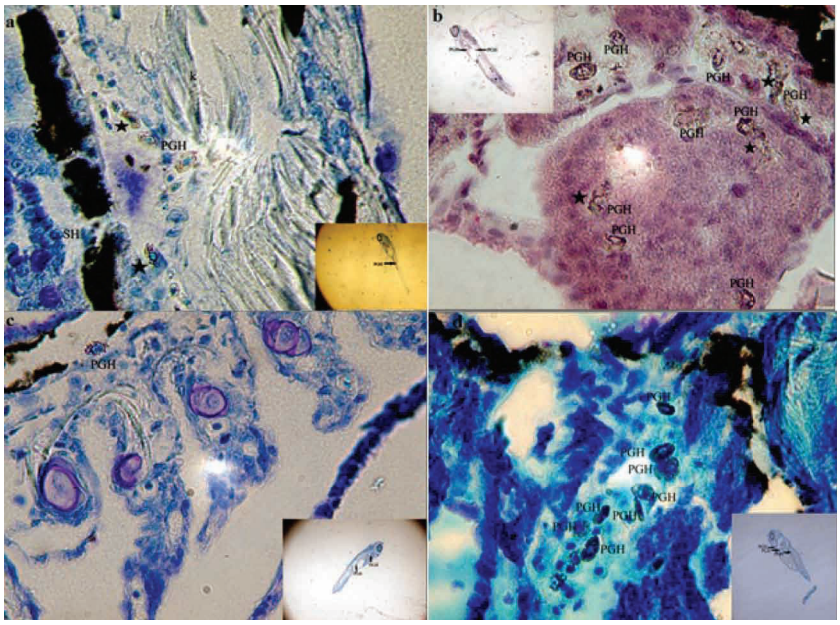


Fig 4. Histologic sections of 3 dpf embryos. Location of PGCs were shown with arrows. **PGH:** primordial germ cell, **SH:** somatic cell, **vk:** yolk sac, **a)** Untreated group, Toluidine blue staining (x4), (x100), **b)** Solvent control group (1% DMSO), Toluidine blue staining (x10), (x100), **c)** 4 mg/L BPA treated group, Toluidine blue and Best carmine staining (x10), (x100), **d)** 8 mg/L BPA treated group, Best carmine staining (x10), (x100)

Şekil 4. 3 günlük embriyoların histolojik kesiti. PGH bulunduğu yerler oklar ile gösterilmiştir. **PGH:** Primordiyal germ hücresi, **SH:** somatik hücre, **vk:** vitellüs kesesi, **a)** Kontrol grubu, Toluidine mavisi boyaması (x4), (x100), **b)** Çözücü kontrol grubu (%1 DMSO), Toluidine mavisi boyaması (x10), (x100), **c)** 4 mg/L BPA uygulaması yapılmış grup, Toluidine mavisi ve Best Carmine boyaması (x10), (x100), **d)** 8 mg/L BPA uygulaması yapılmış grup, Best carmine boyaması (x10), (x100)

Fig 5. Histologic sections of 7 dpf prelarvae. Location of PGCs were shown with arrows. Migrating PGCs were mentioned with star. **PGH:** primordial germ cell, **SH:** somatic cell, **k:** muscle tissue, **a)** Untreated group, Toluidine blue staining (x10), (x100), **b)** Solvent control group (1% DMSO), Best carmine staining (x10), (x100), **c)** 4 mg/L BPA treated group, PGC at ectopic region, Toluidine blue staining (x10), (x100), **d)** 8 mg/L BPA treated group, Toluidine blue staining (x10), (x100)

Şekil 5. 7 günlük prelarvaların histolojik kesiti. PGH bulunduğu yerler oklar ile gösterilmiştir. Göç eden PGH yıldız ile gösterilmiştir. **PGH:** Primordiyal germ hücresi, **SH:** somatik hücre, **k:** kas dokusu. **a)** Kontrol grubu, Toluidine mavisi boyaması (x10), (x100), **b)** Çözücü kontrol grubu (%1 DMSO), Best carmine boyaması (x10), (x100), **c)** 4 mg/L BPA uygulaması yapılmış grup, ektopik bölgedeki PGH, Toluidine mavisi boyaması (x10), (x100) **d)** 8 mg/L BPA uygulaması yapılmış grup, Toluidine mavisi boyaması (x10), (x100)



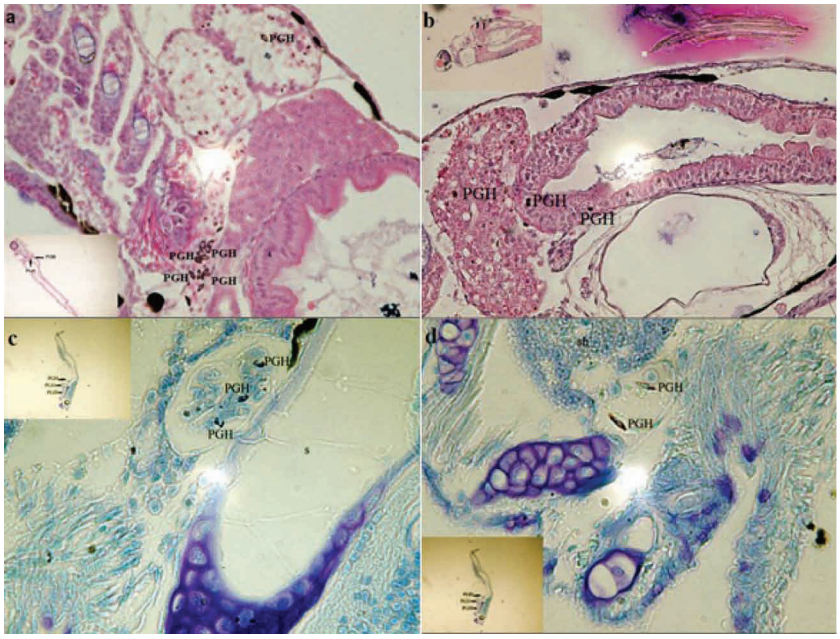


Fig 6. Histologic sections of 14 dpf larvae. Location of PGCs were shown with arrows. **PGH:** primordial germ cell, **sh:** somatic cell, **s:** somites, **a)** Untreated group H&E staining (x10), (x40), **b)** Solvent control group (1% DMSO), H&E staining (x20), (x40), **c)** 4 mg/L BPA treated group, Toluidine blue staining (x10), (x100), **d)** 4 mg/L BPA treated group, PGC at ectopic region, Toluidine blue staining (x10), (x100)

Şekil 6. 14 günlük larvaların histolojik kesiti. PGH bulunduğu yerler oklar ile gösterilmiştir. **PGH:** Primordiyal germ hücresi, **sh:** somatik hücre, **s:** somitler, **a)** Kontrol grubu, H&E boyaması (x10), (x100), **b)** Çözücü kontrol grubu (%1 DMSO), H&E boyaması (x20), (x40), **c)** 4 mg/L BPA uygulaması yapılmış grup, Toluidine mavisi boyaması (x10), (x100), **d)** 4 mg/L BPA uygulaması yapılmış grup, ektopik bölgedeki PGH, Toluidine mavisi boyaması (x10), (x100)

Japanese quails *(Coturnix japonica)* and they found that nonylphenol has dramatic effects on egg production rate and anti-oxidant system in Japanese quails *(Coturnix japonica)*. In addition, Willey and Krone [21] investigated effects of endosulfan and nonyphenol on the primordial germ cell population in pre-larval zebrafish embryos during the first day of development. In this study, vasa antisense RNA probe was used for whole mount in situ hybridization. As a result of the study, they proved that endosulfan treatment cause decrease the number of PGCs in 5th and 6th somites, increase the number of PGCS in 7th and 8th somites. Between 9th and 13th somites any change in the number of PGCs didn't observed. In nonylphenol treated group they found decrease in the number of PGCs between 6th and 7th somites. As a result, these chemicals cause alternations in the distrubition of PGCs along the posterior and anterior axis. In our study, we proved that BPA treatment cause ectopic localization of PGCs. Besides, it cause increase in the number of total PGCs.

Lawson et al.[28] studied the effects of low doses of BPA on gene expression in the fetal mouse ovary and they suggested that BPA acts to down-regulate mitotic cell-cycle genes, raising the possibility that fetal BPA exposure may act to limit expansion of the primordial germ cell population. On contrary, we found an increase in the number of PGC population after BPA exposure in zebrafish embryos.

Doitsidou et al.[36] found that SDF-1a chemokine is the guidance for primordial germ cell attraction. In our study, it's obvious that BPA treatment cause an increment at the number of ectopic primordial germ cells. This result can be interpreted with 2 ways. First, BPA can inhibit SDF-1a chemokine signal mechanism. Second, BPA treatment increses primordial germ cell production and the ectopic cells that can be seen from figures are migrating cells to the targets.

In some references it's mentioned that BPA induces apoptosis at rodents. Li et al.[6] gave high doses of BPA to mice and they used TUNNEL assay, immunohistochemistry and western blotting techniques. As a result they found that high doses of BPA exposure induces apoptosis and upregulation of fas/fasl and caspase-3 expression in testes of mice. Similarly, Wang et al.[37] found that BPA induced germ cell apoptosis in testes. These studies were done at tissues of adult individuals. On contrary in our study we studied with embryos and we could not see any apoptotic effects of low doses of BPA on zebrafish embryos. Similarly to our results, Oka et al.[38] studied the effects of BPA on apoptosis in central neural cells during early development of Xenopus and they also couldn't observe apoptotic effects in early stages.

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