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Histopathological and apoptotic examination of zebrafish (*Danio rerio*) gonads exposed to triclosan

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Abstract: Triclosan, produced as a broad-spectrum antibiotic in the early 1960s, is generally used as a preservative in personal care products, fabrics, plastic products such as kitchenware and toys. As a result of the high demand for triclosan, this chemical threatens the aquatic ecosystem by contaminating wastewater sources. Environmental pollutants affect the reproductive potential of fish, one of the most critical aquatic organisms. This study aimed to investigate the histopathological and apoptotic effects of triclosan in zebrafish gonads. Fish were exposed to sublethal concentrations of triclosan for 5 days, and general histological methods were applied. Histological sections were examined under a light microscope after staining with hematoxylin and eosin and toluidine blue. Triclosan exposure caused deterioration in ovarian tissue, such as shrinkage in the ooplasm, accumulation of proteinaceous fluid in the interstitial tissue, morphological changes of oocyte and the zona radiata. In testicular tissue, triclosan exposure caused fusion in seminiferous tubules, hypertrophy in spermatogenic and Leydig cells, edema in seminiferous tubules, and karyorrhexis in spermatogenic cells. The TUNEL assay was used for the determination of apoptotic cells. Brown-colored apoptotic cells were visualized under the light microscope. TUNEL positive cells were observed in all exposure groups. Triclosan administration was found to cause apoptosis in zebrafish gonads. These findings indicate that triclosan potentially affects fish reproduction, and that its judicious disposal is essential for protecting the environment and maintaining the reproductive potential of fish.

Keywords: triclosan, testis, ovary, TUNEL assay; zebrafish

INTRODUCTION

Bisphenols are a class of compounds that have antimicrobial activity. Triclosan (TCS; 5-chloro-2-[2,4-dichloro-phenoxy]-phenol) is a bisphenol, often used as a preservative and broad-spectrum antimicrobial agent in many personal care products such as antibacterial soaps, deodorants, toothpaste, and hand lotions and creams [1, 2]. TCS is found in the structure of fabrics, such as surgical scrubs, surgical drapes, and in many plastics such as toys, toothbrush handles, cutting boards, pizza-cutters, etc. [1]. Due to the wide range of usage, TCS participates in the aquatic ecosystem in many ways, it has been detected in soil, surface and drinking water, sewage treatment plant effluents [3-5], and it persists in the environment through bioaccumulation [5].

Due to the use of TCS as a biocide, its toxicity has been investigated in many aquatic organisms [2, 6]. Its median lethal concentration (LC_{50}) was found to be between 270-602 μg/L in many fish such as *Danio rerio* (zebrafish), *Lepomis macrochirus*, *Oncorhynchus mykiss*, *Oryzias latipes* (medaka) and *Pimephales promelas* [7], and the 96-h LC_{50} value in zebrafish was 0.34 mg/L [8]. Studies indicate that TCS has endocrine-disrupting effects in multiple species [9-13]. In addition, *in vitro* and *in vivo* studies revealed that TCS exposure induced apoptosis in rat and porcine embryos [14-15]. TCS also induces apoptosis in Burkitt lymphoma and human lung cancer cells [16-17]. TCS exposure has been reported to have pro-apoptotic effects in the central nervous system cells of zebrafish [18].

Zebrafish (*Danio rerio*) is a vertebrate animal model widely used in many areas, from biological and genetic stages of development to human diseases. It is especially preferred in environmental toxicology studies because it is easy to maintain in the laboratory,

has a short life cycle and is resistant to variable environmental conditions. Zebrafish, which is at the forefront of toxicology studies, is used extensively as a tool in the qualitative or quantitative screening of toxins in water samples to investigate the mechanisms of environmental toxins such as endocrine-disrupting chemicals, heavy metals, and related diseases [19, 20].

Gonads are the primary reproductive organs responsible for producing germ cells such as oocytes and sperm. They are considered endocrine glands because they secrete hormones. The effects of TCS on the reproduction of non-target organisms are still not known. Because of the apoptotic and endocrine-disrupting effects of TCS, it is of great importance to investigate its effects on gonads. Therefore, this study aimed to examine the apoptotic and histopathological effects of TCS in zebrafish gonads.

MATERIALS AND METHODS

Test chemical

TCS, triclosan (CAS No: 3380-34-5), was purchased from Sigma Aldrich (Germany).

Animal husbandry and experimental design

Adult zebrafish were obtained from the Sakarya University Aquaculture Laboratory, Esentepe, Sakarya, Turkey. They were raised in dechlorinated tap water and maintained as follows: a 12 h light/12 h dark photoperiod, 28±1°C temperature, 7.0±0.5 pH, 6.0 mg/L dissolved oxygen. The fish were fed with an artificial diet TetraMin© Haupt-futter (Tetra Werke, Germany) twice a day. The experimental setup was created as described [8, 21]. Adult zebrafish of similar length and age (3±1 cm, 1 year old) were randomly selected and divided into 4 groups, 1 control and 3 exposure groups. Each group had 10 zebrafish (5 male and 5 female). Fish in the exposure groups were treated with 34, 85 and 170 µg/L TCS, respectively, for 5 days. Sublethal concentrations were determined based on the 96-h LC_{50} value. A stock solution was prepared by dissolving TCS in acetone, and the exposure concentrations were diluted from this solution. The control solvent contained 250 µl/L of acetone, the highest solvent concentration in the TCS stock solution.

Histopathological evaluation

At the end of the exposure period, zebrafish were anesthetized with 250 mg/L tricaine methanesulfonate (MS-222; Sigma Aldrich). Gonads were dissected and kept in Bouin's fixative for 1 day to ensure fixation. Standard histological procedures were applied. The tissues were cleared with xylene after passing through an ascending series of ethyl alcohol concentrations. After the tissues were embedded in paraffin, sections of 5-µm thickness were taken using a microtome (Leica RM2125RT). Sections were stained with hematoxylin and eosin (H&E) and toluidine blue (TBO). Slides were examined under a compound microscope (Leica DM 500) and monitored with a Leica MC170 HD camera.

Semiquantitative scoring

Semiquantitative scoring was performed as described [22]. Three individuals were randomly selected from each group and ten slides were investigated per individual. The histopathological changes were categorized as none, mild (25% of sections), moderate (25-50% of sections) and severe (>50% of sections).

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay

TUNEL is an established method for detecting DNA fragments in apoptotic cells. TUNEL analysis was performed with S7100 ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA). Testis and ovarian tissues were fixed with 10% neutral formaldehyde and sections were prepared using routine histological methods. After deparaffinization and rehydration, the sections were treated with 20 µg/mL proteinase K (Sigma Aldrich) for 15 min at room temperature. An anti-digoxigenin peroxidase conjugate was added to the samples after treatment with TdT enzyme. Phosphate buffered saline (PBS) was added to the sections in the negative control instead of the TdT enzyme. After the apoptotic cells were labeled with 3,3'-diaminobenzidine (DAB), sections were counterstained with methyl green. TUNEL-positive cells were examined under a light microscope.

RESULTS

Semiquantitative scoring

The results of this study indicate that TCS produced histopathological changes in the testicles and ovaries of zebrafish. The histopathological lesions observed according to the exposure concentrations are given in Table 1 and are described by semiquantitative scoring as mild, moderate and severe.

Histopathological results

Normal ovarian (Fig. 1A and 1B) and testicular (Fig. 1C and 1D) architecture was observed in control group samples. In the ovarian tissue, all developmental stages of oocytes were clearly observed. A large number of small-sized perinucleolar oocytes with multiple nucleoli within the germinal vesicle was observed. Cortical alveolar oocytes were distinguished by being larger than perinucleolar oocytes and by the cortical alveoli (yolk vesicles) contained within them. Perifollicular layers such as the zona radiata and follicular epithelium were noted in these oocytes. Vitellogenic oocytes, which are larger than cortical alveolar oocytes, are characterized by the initiation of accumulation of yolk granules, which are eosinophilic and vitellogenic granules in the center. In mature oocytes, which are the most developed oocytes, vitellogenesis is greatly increased and the nucleus migrates to the periphery and begins to disappear. At this stage, the size of the oocyte has reached its maximum (Fig. 1A and B). In testicular tissue, spermatogenic cells were located in the seminiferous tubules, and Leydig cells in interstitial tissue were visualized. Pyramid-shaped Sertoli cells were detected in the basement region of the seminiferous tubules. Spermatogonia, the largest spermatogenic cells, were distinguished from other cells by their large size and pale nuclei. A large number of primary and secondary spermatocytes were easily observed under the light microscope, as they were medium-sized, with dark nuclei, and were observed in one of the three stages of meiosis (pachytene, leptotene, zygotene). Spermatids, which were formed from spermatocytes as a result of the second meiosis, were distinguished from other cells by their small size, dense nuclei and a small amount of eosinophilic cytoplasm. Sperm cells, on the other hand, were visualized as the smallest-sized cells with round nuclei and minimal or no cytoplasm (Fig. 1C and 1D).

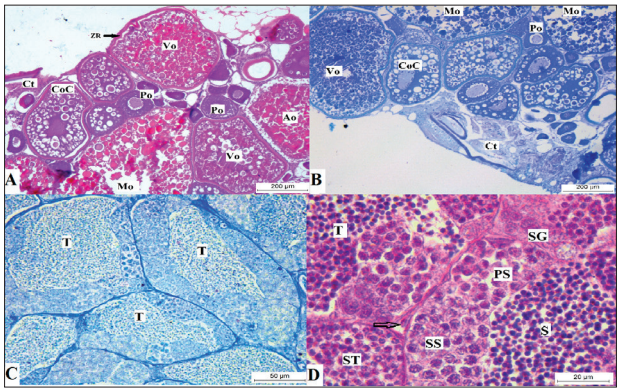

Fig. 1. Gonadal histology in the control group. **A, B –** General view of ovarian tissue and different oocyte stages. **C –** Seminiferous tubules of testicular tissue, **D –** Spermatogenic cells in the seminiferous tubules. Po – perinucleolar oocyte, CoC – cortical alveolar stage oocyte, Vo – vitellogenic oocyte, Mo – mature oocyte, Ao –atretic oocyte, Ct – interstitial tissue, ZR – zona radiata, T – seminiferous tubule, SG – spermatogonia, PS – primary spermatocyte, SS – secondary spermatocyte, ST – spermatids, S – sperm cells, Arrow – Leydig cell. A, D – H&E stain; B, C – TBO stain.

Table 1. Semiquantitative scoring of gonads of zebrafish exposed to 34, 85 and 170 µg/L of TCS.

Organ	Histopathological Lesions	Control	34 µg/L	85 µg/L	170 µg/L
Ovary	Proteinaceous fluid accumulation	-	+	++	+++
	Shrinkage of ooplasm	-	+	++	++
	Dysmorphic oocytes	-	++	++	+++
	Fusion of cortical alveoli	-	+	+	+
	Thickness of zona radiata	-	-	+	+
	Hyperplastic perifollicular cells	-	-	-	++
Testis	Fusion of seminiferous tubules	-	++	+++	+++
	Hypertrophic spermatogenic cells	-	+++	+++	+++
	Edema in seminiferous tubule	-	+	++	+++
	Karyorrhexis in spermatogenic cells	-	+++	+++	+++
	Hypertrophic Leydig cell	-	+	+	++
	Vascular congestion and interstitial fibrosis	-	-	-	+

Histopathological lesions were scored to the to their severity (-: none, +: mild, ++: moderate, +++: severe)

In the 34 µg/L TCS exposure group, histopathological changes

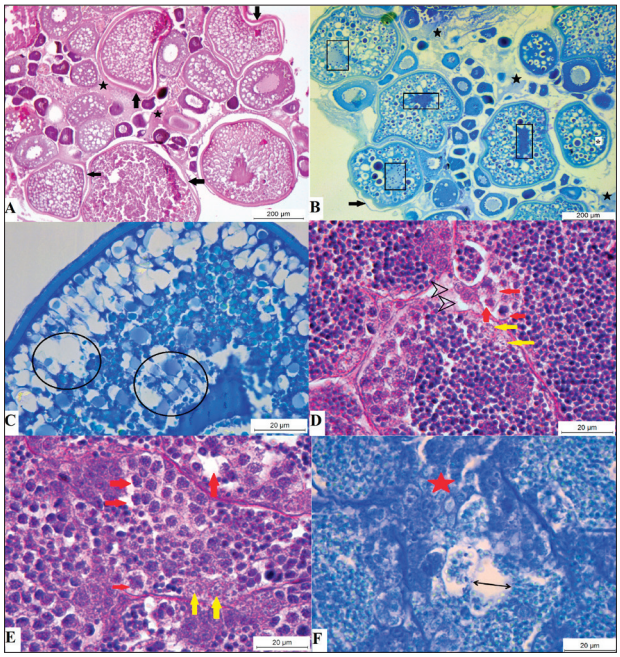

Fig. 2. Gonad histology in the group exposed to 34 µg/L TCS, **A, B –** General view of ovarian tissue. Black star – proteinaceous fluid formation in interstitial tissue, black arrow – openings at perifollicular layers, asterisks – shrinkage of ooplasm, rectangle – disorganization of nucleoli at cortical alveolar stage oocytes. **C –** Fusion of cortical alveoli in vitellogenic oocyte (shown with a circle), **D, E –** Spermatogenic cells in seminiferous tubules; arrowhead: hypertrophic Leydig cells, red arrow – hypertrophic spermatocytes, yellow arrow – karyorrhexis in spermatogonia. **F –** fusion of seminiferous tubules (shown with a red star); double headed arrow – edema. A, D, E – H&E stain; B, C, F – TBO stain.

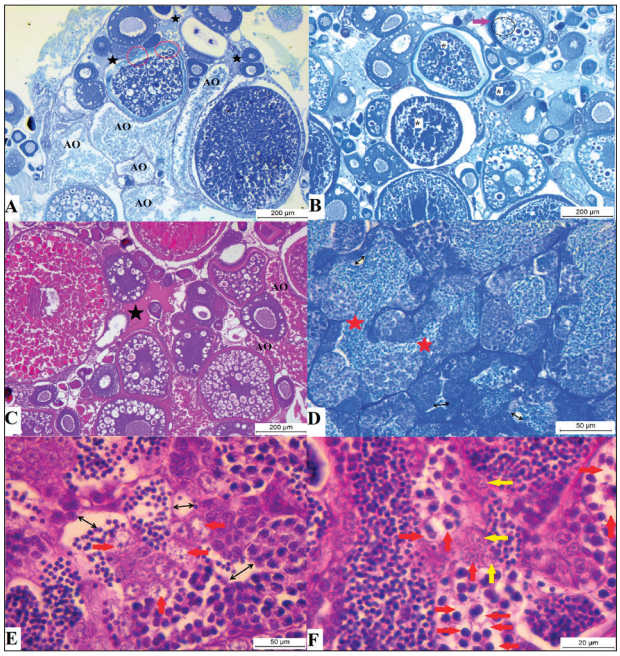

Fig. 3. Gonad histology in the group exposed to 85 µg/L of TCS, **A, B –** General view of ovarian tissue. **C –** Accumulation of proteinaceous fluid in interstitial tissue; AO – atretic oocyte, red circle – fluctuation of the zona radiata, black circle – fusion in cortical alveoli, black star – proteinaceous fluid formation, asterisks – shrinkage of ooplasm, purple arrow – thickness of the zona radiata. **D –** General view of testicular tissue. **E –** Morphological deformation in seminiferous tubule. **F –** Spermatogenic cells in seminiferous tubule; red star – fusion of seminiferous tubules, double-headed arrow – edema, red arrow – hypertrophic spermatogenic cells, yellow arrow – karyorrhexis in spermatogenic cells. Staining: A, B, D – TBO stain; C, E, F – H&E stain.

were observed in ovarian tissue. Proteinaceous fluid formation in interstitial tissue was noted (Fig. 2A and B). Shrinkage of the ooplasm (Fig. 2B) and deterioration of the form of oocytes were detected. Openings at perifollicular layers were also visualized (Fig. 2A and 2B). Disturbances in nucleolus organization (Fig. 2B) and fusion of cortical alveoli were detected in cortical alveolar oocytes (Fig. 2C). In testicular tissue, hypertrophic Leydig cells were visualized between seminiferous tubules. In addition to atrophy in spermatogonia, karyorrhexis was observed in spermatogenic cells (Fig. 2D); hypertrophic primary and secondary spermatocytes were detected (Fig. 2D and 2E). Deteriorations in seminiferous tubule morphology were also noted (Fig. 2E). Fusion was observed in some seminiferous tubules (Fig. 2F). Disorder of spermatogenic cells (Fig 2E) and edema between cell clusters (Fig. 2F) in the seminiferous tubule were detected.

In the 85 µg/L TCS group, an increase in the number of atretic oocytes was detected in the ovarian tissue (Fig. 3A). Fluctuations (Fig. 3A, 3C) and an increase in the thickness of zona radiata (Fig. 3B) were observed. Fusion in the cortical alveoli was also observed in this group. Shrinkage of the ooplasm of vitellogenic and mature oocytes was noted (Fig. 3B). Accumulation of proteinaceous fluid observed in the interstitial tissue was higher compared to the 34 µg/L TCS group (Fig. 3A and C). In testicular tissue there was a relative increase in the proportion of sperm cells to other spermatogenic cell types in the seminiferous tubule. The fusion of seminiferous tubules was monitored (Fig. 3D), and deterioration in the morphology of some seminiferous tubules was observed (Fig. 3E). Edema was evident between spermatogenic cells in the seminiferous tubules (Fig. 3D and 3E). Hypertrophic spermatogonia and secondary spermatocytes were

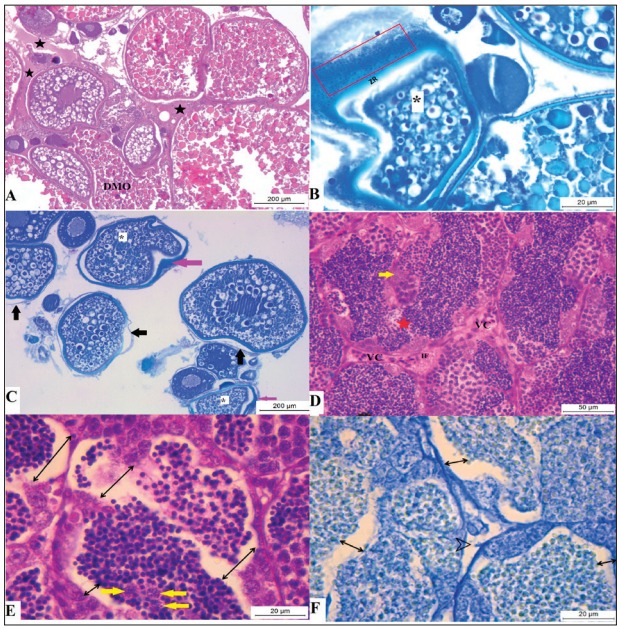

Fig. 4. Gonad histology in the group exposed to 170 µg/L of TCS, **A –** General view of ovarian tissue. **B, C –** Shrinkage of ooplasm and dysmorphic oocytes; black star – proteinaceous fluid formation, DMO – dysmorphic mature oocyte, asterisks – shrinkage of ooplasm, ZR – zona radiata, red rectangle – hyperplasia in perifollicular cells, purple arrow – thickness of zona radiata. **D –** General view of testicular tissue. **E, F –** Spermatogenic cells in seminiferous tissue; red star – fusion of seminiferous tubules, VC – vascular congestion, IF – interstitial fibrosis, double-headed arrow – edema, red arrow – hypertrophic spermatogenic cells, yellow arrow – karyorrhexis in spermatogenic cells, arrowhead – hypertrophic Leydig cell. Staining: A, D, E – H&E stain; B, C, F – TBO stain.

observed (Fig. 3E and 3F). The presence of karyorrhexis in many spermatogenic cells suggested that these cells might be apoptotic (Fig. 3F).

In the 170 µg/L TCS group, in the ovarian tissue proteinaceous fluid accumulation in the interstitial tissue was severe compared to all other groups (Fig. 4A). Increased oocyte atresia was marked. Besides cortical alveolar oocytes (Fig. 4B and C), dysmorphism was observed in mature oocytes as well (Fig 4A). Similarly, shrinkage of the ooplasm (Fig. 4B and C) and opening of the perifollicular layers (Fig. 4C) were observed. Deterioration (Fig. 4B) and thickening (Fig. 4C) of the zona radiata structure were detected. Hyperplasia was noticed in the perifollicular cells of some oocytes (Fig. 4B). In testicular tissue, an increase in the proportions of sperm and spermatids to other spermatogenic cells was apparent. Vascular congestion and interstitial fibrosis were observed between tubules (Fig. 4D). Edema was detected among the spermatogenic cell groups in the seminiferous tubules (Fig. 4E and F). Like the other groups, karyorrhexis was observed in this group as well (Fig. 4D and E). Hypertrophic Leydig cells were noted between seminiferous tubules (Fig. 4F).

TUNEL assay

According to the TUNEL assay, brown-colored TUNEL positive cells were observed under the light microscope. In negative control samples of gonads, no reaction was detected (Figs. 5A, 6A). Apoptotic cells were not found in the ovarian tissue of the control group (Fig. 5B). In the 34 µg/L TCS group, brown-colored TUNEL positive cells in the interstitial area were observed (Fig. 5C). It was concluded that apoptosis was more severe in the groups that received 85 and 170 ug/L TCS. TUNEL positive cells were visualized in primary oocytes and in the follicular epithelium of various oocytes, as well as cells in the interstitial tissue (Fig. 5D and E). Apoptosis was not found in spermatogenic cells in testicular samples belonging to the control group (Fig. 6B). In the group exposed to 34 µg/L TCS, TUNEL positive Sertoli cells, spermatocytes and a few sperm cells were detected (Fig. 6C). In the 85 ug/L TCS group, some spermatocytes and Leydig cells were found to be TUNEL positive, and apoptotic sperm-cell clusters were noted (Fig 6D). In the 170 ug/L group, many apoptotic sperm clusters were seen in the seminiferous tubules due to the increased proportion of sperm (Fig. 6E).

DISCUSSION

Endocrine disruptors are chemicals that exhibit their effects by mimicking, blocking or interfering with hormones. Due to the increasing use of personal care products and antiseptics, researchers are paying attention to the toxicity of TCS, an endocrine-disrupting compound found in water sources, its ability to accumulate in adipose tissue and its potential for bioaccumulation [23]. Studies have revealed that exposure to TCS causes changes in thyroid hormone levels [24-25]. On the other hand, the number of studies on the reproductive endocrine-disrupting effects of TCS is

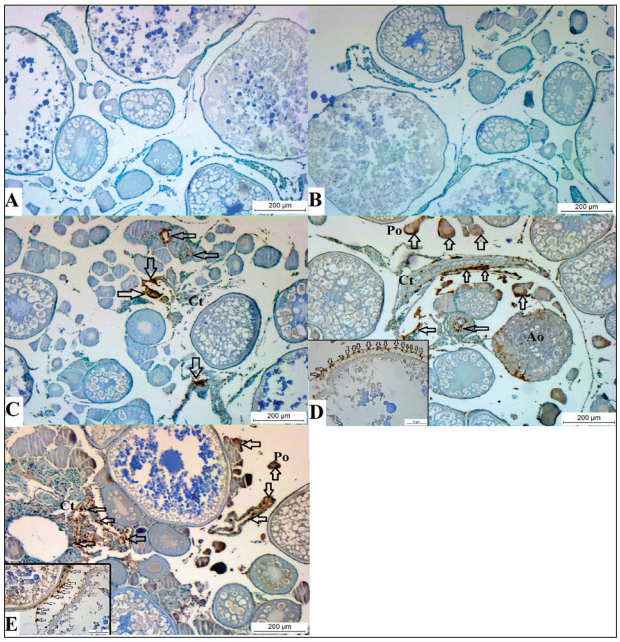

Fig 5. Analysis of apoptosis in zebrafish ovary by the TUNEL assay. Apoptotic (TUNEL positive) cells are seen in brown. **A** – Negative control; **B** – control; **C** – 34 µg/L TCS exposed group; **D** – 85 µg/L TCS exposed group; **E** – 170 µg/L TCS exposed group. Arrow – TUNEL positive cell, Ct – interstitial tissue, Po – perinucleolar oocyte, Ao – atretic oocyte

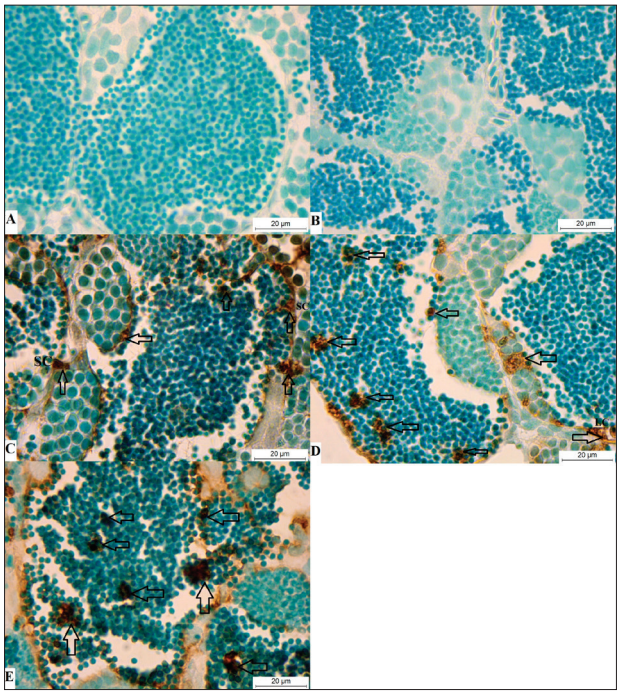

Fig 6. Analysis of apoptosis in zebrafish testis by the TUNEL assay. Apoptotic (TUNEL positive) cells are seen in brown. **A** – Negative control; **B** – control, **C** – 34 µg/L TCS exposed group; **D** – 85 µg/L TCS exposed group; **E** – 170 µg/L TCS exposed group. Arrow – TUNEL positive cell, SC – Sertoli cell, LC – Leydig cell.

limited. Considering the potential for TCS to enter the aquatic ecosystem via different routes, this study fills an essential gap in the literature.

The effects of TCS exposure on the expression of antioxidant (*SOD, GPx1a, CAT, sMT-B,* and *MT-2*) and apoptosis-related genes (*Bax, p53*) in zebrafish ovarian tissue were investigated [26], and it was shown that the antioxidant-related genes in the ovary were significantly downregulated and apoptotic-related genes were significantly upregulated in groups exposed to TCS. The authors stated that TCS exposure caused oxidative damage in the ovaries and induced reactive oxygen species (ROS)-dependent apoptosis. Similarly, histopathological study and TUNEL assay results showed that TCS exposure causes apoptosis in the gonads. Although it has been stated that TCS has an estrogenic effect [12], apoptosis detected in both testicular and ovarian tissues as a result of TUNEL analysis may have occurred due to increased ROS formation.

Histopathology is an essential and valuable tool for identifying endocrine disruptors in marine and freshwater environments [27]. In a study examining the histopathological effects of bisphenol A in zebrafish ovary, it was determined that exposure to bisphenol A inhibits oogenesis by causing an increase in the number of atretic oocytes and degeneration in developing oocytes [28]. TCS also causes similar histopathological effects in zebrafish ovaries. It can be said that bisphenol A and TCS have similar reproductive endocrine-disrupting effects. The similarity of the results may be because both substances are bisphenol derivatives and xenoestrogens.

Oral exposure of adult zebrafish to 2,4,6-tribromophenol, which is another endocrine-disruptor, affects gonad morphology and inhibits reproduction by causing an increase in the number of atretic oocytes and decreased vitellogenesis in female individuals [29]. The histopathological examination in this study showed an increase in the number of atretic oocytes but no decrease in vitellogenesis in ovarian tissues after TCS exposure. In another study, the histopathologic effects of polychlorinated biphenyls (PCBs) on the reproduction ability of zebrafish were investigated; a decrease in the number of maturing follicles and an increase

in atretic follicles in zebrafish ovary exposed to PCBs were found [30]. Based on these findings, it can be said that endocrine-disrupting chemicals cause an increase in the number of atretic oocytes in the ovarian tissue.

Histopathological effects of endocrine-disrupting chemicals have been reported not only in ovarian tissue but also in testicular tissue. Di(2-ethylhexyl) phthalate (DEHP) causes changes in the proportion of germ cells at certain stages of spermatogenesis, including a decrease in the sperm and an increase in the spermatocyte ratio [31]. Unlike the histopathological effects of DEHP, an increase in sperm ratio and significant hypertrophy and karyorrhexis in spermatogenic cells were observed as a result of TCS exposure. In *Cyprinus carpio,* the endocrine-disrupting effects of 4-nonylphenol on plasma vitellogenin, reproductive system and histology were investigated. Irregularly-shaped oocytes with rupture of the follicular layer, an increase in the number of atretic oocytes in the ovary and degeneration in Leydig cells, as well as atrophy and cellular vacuolization in spermatogenic cells were described [32]. Similar to the effects of nonylphenol, histopathological lesions such as deterioration in oocyte morphology, an increase in the number of atretic oocytes, hypertrophy in Leydig cells and morphological changes in spermatogenic cells were reported in this study as a result of TCS exposure.

Herein it was shown that TCS, which has an endocrine-disrupting effect, produces histopathological changes and activates apoptosis in the reproductive organs of zebrafish, causing degeneration of germ cells and potentially negatively affecting reproduction. Therefore, judicious disposal of these chemicals is crucial for protecting the environment and maintaining the reproductive potential of aquatic organisms.

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