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Histological effects of linear alkyl benzene sulfonic acid exposure on primordial germ cell migration and gonad formation in zebrafish (*Danio rerio*)

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Abstract: The use of detergents has become widespread and is growing. The increase in population and use of detergent threatens the life of organisms in the ecosystem. In this study, the histological effects of linear alkyl benzene sulphonic acid on primordial germ cell (PGC) migration and gonad formation in zebrafish were investigated after application of 0.25 0.5 and 1 mg/L of linear alkyl benzene sulphonic acid to embryos. Samples of embryos and larvae were collected for 60 days and the effects of linear alkyl benzene sulphonic acid on PGC migration and gonad development were examined by histological procedures. The localization and migration pathway of PGCs and structures of the gonads were inspected by Periodic Acid Schiff (PAS), alkaline phosphatase, toluidine blue and Best's carmine staining. Primordial germ cells were observed in ectopic regions and delayed gonadal development and histopathological changes in gonad structures were observed. We concluded that linear alkyl benzene sulfonic acid exposure has a negative effect on the reproduction system of zebrafish.

Keywords: linear alkyl benzene sulphonic acid; surfactant; primordial germ cell; zebrafish; gonad

INTRODUCTION

Detergents are effective cleaning products in the form of powders, granules, gels or liquids that contain one or more anionic surfactants. They are common contaminants in aquatic ecosystems, and pollution caused by detergents has increased significantly. Active ingredients of detergents can form foam at concentrations above 0.5 mg/L. The resulting foam layer closes the water surface and prevents oxygen ingress, which can cause a sudden decrease in oxygen in the aquatic ecosystem [1]; this poses a serious threat to aquatic organisms and directly affects the aquatic ecosystem. In addition, as detergent waste contains phosphate, it produces toxic effects in aquatic organisms and also causes eutrophication in aquatic ecosystems. Detergents also have dermatological, teratogenic and biochemical effects, and produce histological damage in fish, mice, pigs and humans [2-12].

Detergents are generally comprised of 4 components: surfactants, bleaches, structural agents and other excipients. Surfactants consist of hydrophilic and hydrophobic moieties. The hydrophobic part reduces surface tension in aqueous solutions and provides cleaning and foam formation, whereas the hydrophilic part provides water solubility. One of the most important anionic surfactants of detergents is linear alkyl benzene sulfonic acid or linear alkyl benzene sulfonate, also called sodium dodecyl benzene sulphonate (designated by three acronyms, LABSA, LABS or LAS). LAS accounts for approximately 40% of all surfactants [13]. As one of the main raw materials of the synthetic detergent industry, LAS is found in high concentrations in liquid, gel or powder detergents, in both industrial and domestic cleaning products. It is also found in powder detergents, liquid soaps and gel detergents used in laundry and dishwashers [14]. LAS, which is one of the most important environmental pollutants, enters the ecosystem as a result of industrial or domestic use. LAS undoubtedly affects the growth and reproduction of fish.

Primordial germ cells (PGCs) are pluripotent embryonic stem cells that migrate into gonadal ridges to form gametes. In the beginning of embryonic development, PGCs are located in a different region from the gonadal ridges and occur between endodermal

cells in the early stages of development. In response to signals from gonadal ridges, they migrate through the dorsal mesentery with ameboidal movement, to the gonadal ridges. This pathway of primordial germ cells is unique in each vertebrate [15-17].

PGCs are morphologically different from somatic cells. Their round-oval or pear-like shapes differ from other somatic cells with large cell volumes (10-20 μm in diameter), large nuclei (6-10 μm) and nuage material (electron-dense granules) [18-19]. These cells contain alkaline phosphatase activity, which is an indicator enzyme of peripheral cytoplasm [20]. Therefore, they react differently from somatic cells, especially with alkaline phosphatase stain (which is a histological stain specific to primordial germ cells), and because they contain glycogen molecules in their cytoplasm, they exhibit specific staining with histological dyes such as Best's carmine and PAS [21].

The mechanism underlying gender formation in vertebrate organisms, the differentiation of gonads to testes and ovaries, depends on genetic factors; environmental factors also play an important role [22-24]. Temperature is one of the most important environmental factors affecting gender determination in zebrafish. During the embryonic period, it was found that male sex was determined in zebrafish grown at elevated temperatures (33-37°C) or after exposure to raised temperature during the gonad differentiation phase [25-30]. Low temperatures such as 22-23°C cause a delay in gonad formation but do not affect gender differentiation [29-30]. In zebrafish embryos grown in a hypoxic environment, changes in testosterone and estrogen concentrations disrupted PGC migration and male sex determination [31-33]. Another example is provided by endocrine-disrupting chemicals, which can cause gender change in zebrafish and lead to gender disparity, leading to gender imbalance in the population [24].

During the first stage of gonadal development, an undifferentiated gonad is formed in zebrafish. This structure then differentiates into mature testes and ovaries [34-37]. Gonad differentiation in zebrafish generally begins on the 25th day of development and is completed on the 60th day [25, 34]. In this study, we investigated the effects of LAS on PGC and gonad formation in zebrafish. The localization of PGCs and gonadal structures in zebrafish were examined for 60 days by histological methods.

MATERIALS AND METHODS

Chemicals

LAS (CAS No: 25155-30-0) was obtained from Sigma Aldrich (St. Louis, MO, USA).

Experimental design and embryo collection

All experiments were performed in three replicates. Twenty-four adult zebrafish (8 females and 16 males) were used for embryo production. Adult zebrafish individuals were obtained from Sakarya University Aquaculture Lab, Esentepe, Turkey. They were kept in 20-L spawning aquariums with dechlorinated tap water and were maintained under standardized laboratory conditions (28.5±1°C, a 14 h light/10 h dark photoperiod, pH 7.0±0.5 and 6.0 mg/L dissolved oxygen). Adult zebrafish individuals were fed *Artemia sp.* and TetraMin© Hauptfutter (Tetra Werke, Melle, Germany) twice a day. It was observed that the fish laid eggs right after the morning light was turned on. After the embryos were collected, the embryos that developed normally were separated from the dead ones under the stereomicroscope.

Toxicity tests

In the blastula stage, embryos were placed in 24-well cell culture dishes (one embryo in each well) and toxicity tests were performed according to OECD standards [38]. In the zebrafish embryos exposed to LAS, LC_{50} values were determined as 5.769±0.791 for 24 h, 4.769±0.759 for 48 h, 2.306±1.037 for 96 h and 2.095±0.955 mg/L for 120 h. LAS was applied to zebrafish embryos at concentrations of 0.25, 0.5 and 1 mg/L to perform toxicity tests according to the LC_{50} values determined for 24 h, 48 h, 96 h and 120 h.

Histological analysis

Samples from control and experimental groups (1 mg/L, 0.5 mg/L and 0.25 mg/L LAS) were collected for 60 days. The embryos and larvae were fixed in Bouin's fixative for 24 h. After the samples were dehydrated in ascending series of ethanol concentrations, they were cleared with xylene. Immediately after the application of xylene, samples were transferred to liquid paraffin overnight at 58°C. On the next day, the embryos or

larvae were embedded in paraffin blocks according to the section plane and were sectioned transversely at 5 µm thickness with a Leica (Germany) microtome. Slides were stained with PAS, alkaline phosphatase, toluidine blue and Best's carmine, which specifically stain PGCs, and examined under a light microscope.

RESULTS

Primordial germ cells were determined from the first day post fertilization (dpf) and migration routes were observed in both control and exposure groups. Glycogen grains, nuage material and alkaline phosphatase activity in spindle-shaped PGCs were clearly visualized under the light microscope. On the first day of development, PGCs were located in the dorsal aspect of the yolk sac (Fig. 1A). Then, as the embryo developed, these cells migrated from the gill (Fig. 1B), heart (Fig. 1C), liver (Fig. 1D), esophagus (Fig. 1E) and small intestine (Fig. 1F) by amoboidal movement, the characteristic movement of primordial germ cells. The migrating cells had an amorphous shape. The cells finally migrated into the gonadal ridges.

When compared to the control group, PGCs were detected in the exposure groups outside the normal migration route. These cells, referred to as ectopic PGCs, were found in regions such as the tail (Fig. 2A), muscle (Fig. 2B), eye (Fig. 2C), brain (Fig. 2D), kidney (Fig. 2E) and fin (Fig. 2F).

The PGCs reached the gonad ridges from the 25 dpf. When the gonadal structure was examined, the PGCs first differentiated into cyst-like cells, and then they developed and formed gonial cells (Fig. 3A). As the gonad continued to develop, type 1 and type 2 germ cells were also detected. The type 1 germ cell was found to have a basophilic cytoplasm, a large nucleus and several nucleoli. The type 2 germ cell was larger

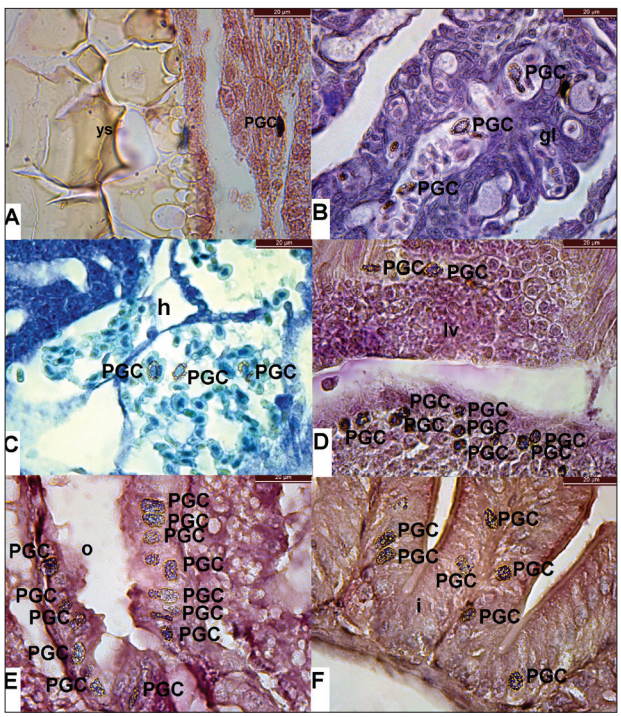

Fig. 1. Migration of PGCs in control and treated groups. **A** – 1 dpf, control group Best's carmine staining; **B** – 0.5 mg/L LAS exposure group, 42 dpf, PAS staining; **C** – 0.25 mg/L LAS exposure group, 16 dpf, toluidine blue staining; **D** – 0.5 mg/L LAS exposure group, 16 dpf, PAS staining; **E** – 1 mg/L LAS exposure group, 33 dpf, Best's carmine staining; **F** – 0.25 mg/L LAS exposure group, 33 dpf, Best's carmine staining. PGC: primordial germ cells, ys: yolk sac, gl: gill, h: heart, lv: liver, o: oesophagus, i: intestine, bar: 20 µm.

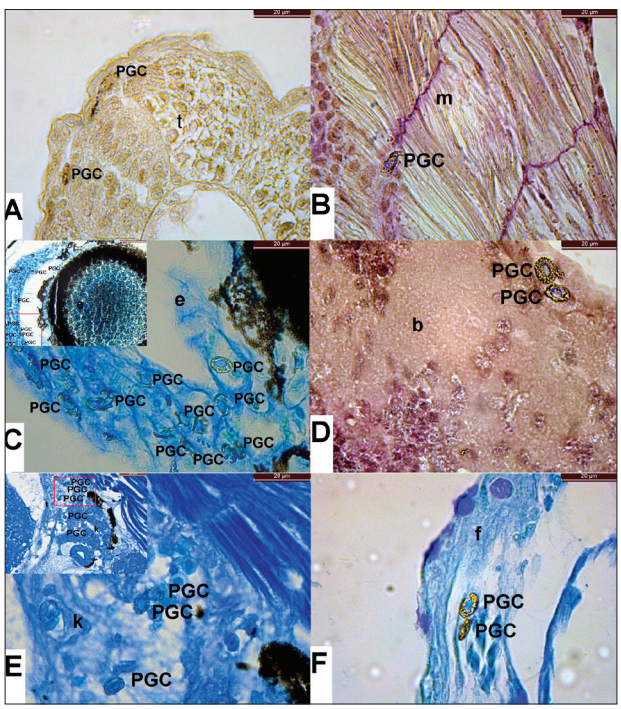

Fig. 2. Ectopic PGCs in the treated group. **A** – 0.25 mg/L LAS exposure group, 1 dpf, alkaline phosphatase staining; **B** – 0.5 mg/L LAS exposure group, 18 dpf, Best's carmine staining; **C** – 1 mg/L LAS exposure group, 33 dpf, toluidine blue staining; **D** – 0.25 mg/L LAS exposure group, 33 dpf, Best's carmine staining; **E** – 0.5 mg/L LAS exposure group, 42 dpf, toluidine blue staining; **F** – 1 mg/L LAS exposure group, 42 dpf, toluidine blue staining. PGC: Primordial germ cells, t: tail, m: muscle, e: eye, b: brain, k: kidney, f: fin, bar: 20 µm.

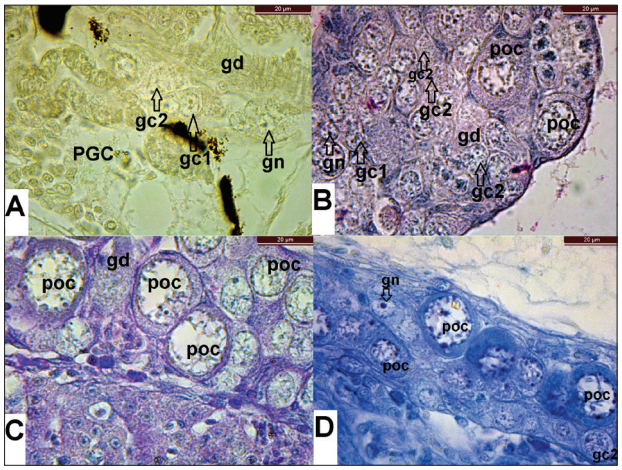

Fig. 3. Gonadal formation in the control group. **A** – 33 dpf, alkaline phosphatase staining; **B** – 42 dpf, PAS staining; **C** – 60 dpf, PAS staining; **D** – 60 dpf, toluidine blue staining. PGC: primordial germ cell, gd: gonad, gn: gonial cell, gc1: type 1 germ cell, gc2: type 2 germ cell, poc: perinucleolar oocyte, bar: 20 μm.

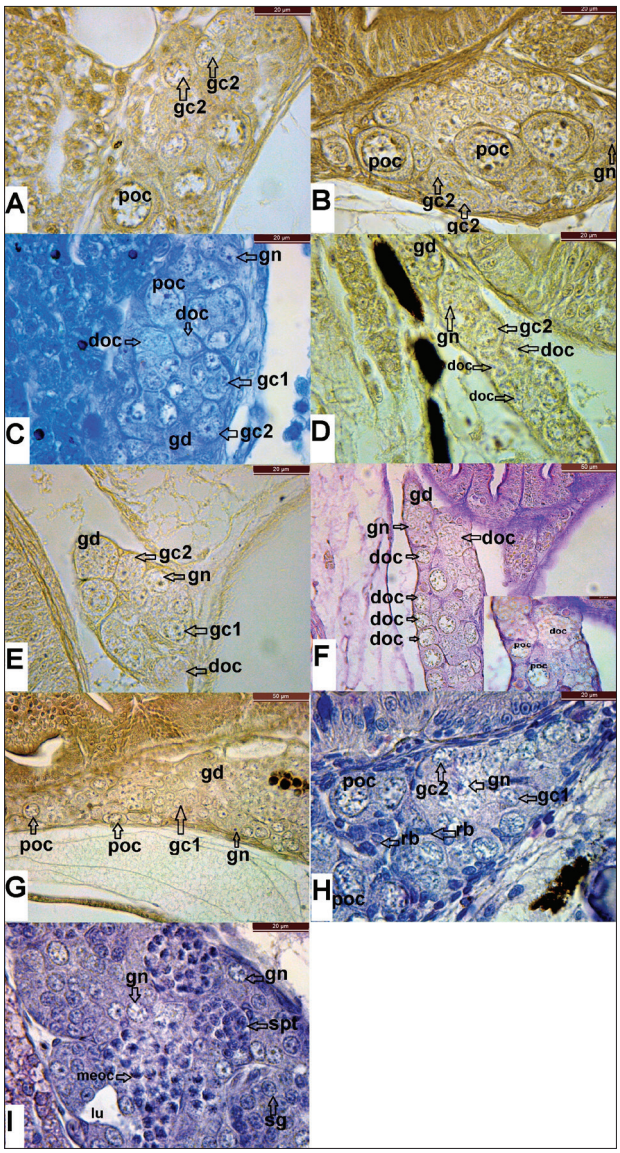

Fig. 4. Gonadal formation in the treated group. **A** – 0.25 mg/L LAS exposed group, 33 dpf, alkaline phosphatase staining; **B** – 0.25 mg/L LAS exposed group, 42 dpf, alkaline phosphatase staining; **C** – 0.25 mg/L LAS exposed group, 60 dpf, toluidine blue staining; **D** – 0.5 mg/L LAS exposed group, 33 dpf, alkaline phosphatase staining; **E** – 0.5 mg/L LAS exposed group, 42 dpf, alkaline phosphatase staining; **F** – 0.55 mg/L LAS exposed group, 60 dpf, PAS staining, **G** – 1 mg/L LAS exposed group, 33 dpf, alkaline phosphatase staining; **H** – 1 mg/L LAS exposed group, 42 dpf PAS staining; **I** – 1 mg/L LAS exposed group, 60 dpf PAS staining. gd: gonad, gn: gonial cell, gc1: type 1 germ cell, gc2: type 2 germ cell, poc: perinucleolar oocyte, doc: degenerated oocyte, rb: residual bodies, meoc: meiotic oocytes, sg: spermatogonium, spt: spermatocytes, lu:lumen; bar: A-E, H, I: 20 μm, F and G: 50 μm.

than the type 1 germ cell and contained spindle fibers in the nucleoplasm (Fig. 3B). These cells differentiated into perinucleolar oocytes (Fig. 3C and D). After this point, the gonads started to differentiate in the direction of either ovaries or testes.

As in the control group, mitotic and meiotic activities started in the cells in the treated groups. Gonial cells, type 1 and 2 germ cells and perinucleolar oocytes were also visualized (Fig. 4A, B and G). However, when the gonad structures in the treated groups were examined, the presence of degenerated oocytes was observed (Fig. 4C, D, E, F). In parallel with the increase in concentration of LAS, it was determined that the number of degenerated oocytes increased. In all experimental groups, a delay in gonadal development and differentiation was observed (Fig. 4C, F, I). In the 1-mg/L LAS exposure group, residual bodies were seen at the gonadal structure. It was found that the gonad generally differentiated to testis (Fig. 4H).

DISCUSSION

In this study, the histopathological effects of LAS, one of the most frequently used surfactants, were detected in the embryonic stem cells to gonadal formation stage in zebrafish embryos and larvae. The pathway of PGCs, which plays a key role in gonad formation, was

monitored for 60 days with specific staining methods such as PAS, Best's Carmin, toluidine blue and alkaline phosphatase.

PGC morphology can be viewed by light microscopy from the early stages of development. These cells also differ from other cells in terms of the morphology of their nuclei. These large cells with a large nucleus can be easily distinguished from round and small somatic cells by their oval cell shape. Nagai et al. [18] examined the histology of PGCs in zebrafish and determined PGCs at the gastrulation stage in histological sections. The authors observed that eosinophilic granules were present in the cytoplasm of these cells and the granules were located around the nucleus.

There is a limited number of studies investigating the effects of various chemicals on the migration of PGCs in zebrafish. In the study conducted by Yön and Yüce [39], one of the commonly used pesticides, deltamethrin, was investigated in zebrafish on the morphology and migration of PGCs. Deltamethrin was introduced into the environment of embryos at different doses and PGCs were examined by hematoxylin and eosin (H&E), PAS, toluidine blue and alkaline phosphatase stainings for 15 days. Degeneration and decrease in the number of PGCs were detected after deltamethrin exposure. In addition, a decrease in the number of PGCs, deterioration of mitochondria and other organelles were observed. During later days of development, nuage material could not be observed in the PGCs in the experimental groups. Akbulut et al. [40] also investigated the effects of bisphenol A, which is a well-known endocrine disruptor, on the PGCs of zebrafish embryos and larvae by histological methods. It was determined that bisphenol A application affected the migration of PGCs. Using whole-mount *in situ* hybridization analysis, PGCs were detected in ectopic regions. These results were confirmed by histological studies. PGCs that deviated from the migration path were detected in ectopic regions. In addition, it was observed that bisphenol A exposure increased the number of PGCs. Acridine orange staining used to detect apoptotic cells revealed that bisphenol A did not cause an increase in cell death. In the present study, LAS exposure, similar to bisphenol A, was shown to induce the migration of PGCs into ectopic regions in zebrafish embryos and larvae. The presence of PGCs in ectopic regions was observed in histological sections. LAS application was found to affect the migration mechanism of PGCs as bisphenol A.

In the study by Willey and Krone [41], the effects of endosulfan and nonylphenol (endocrine disruptors) on the distribution and migration of PGCs in zebrafish were investigated. As a result of the endosulfan exposure, the number of PGCs decreased in the 5th, 6th and 9th somites, while the number of PGCs increased in the 7th and 8th somites. After nonylphenol exposure, a decrease in the number of PGCs was observed in the 6th and 7th somites, while the number of PGCs increased in the 8th somites. At 24 h post fertilization, the highest number of PGCs was detected in the 7th somite. In conclusion, exposure to endosulfan and nonylphenol in zebrafish embryos was found to cause changes in the distribution of PGCs that affected gonad morphology. It can be said that LAS produced effects similar to those produced by endosulfan and nonylphenol in terms of causing a change in PGC distribution. In our study, unlike Willey and Krone's [41], the migration of PGCs was followed for 60 days. As a result of LAS exposure, it was determined that PGCs were detached from the migration route and migrated to different regions instead of the gonadal ridges.

In a study on the effects of endocrine disruptors or pesticides on gonad differentiation, Corvi et al. [42] investigated the effect of chronic exposure to atrazine (ATZ) and 17α-estradiol (E2) on sex development in zebrafish. Compared to the control group, the gonad structure appeared as an "ovary" in E2-treated fish. It was also observed that ATZ application had no effect on gender development. In a different study, Tanka and Grizzle [43] examined the effects of nonylphenol (NP) on gonad differentiation of hermaphrodite fish *Rivulus marmoratus*. It was found that exposure to NP inhibited oogenesis. In another study investigating the effects of 17α-ethinylestradiol (EE) and NP on gametogenesis in zebrafish, larvae were administered NP (≥100 μg/L) and EE (≥1 ng/L) from day 2 to day 60 of development [44]. NP and EE exposure was found to inhibit gametogenesis in both males and females. Only the oogonium and previtellogenic oocytes were detected in the ovary structure. The number of spermatogonia was higher in the seminiferous tubules as a result of NP and EE application, but no sperm formation was observed. As other studies, we conclude that LAS exposure inhibited gametogenesis and delayed gender differentiation in zebrafish.

CONCLUSION

Detergents are the most commonly used chemicals, and as a result of their widespread use, there is significant release of detergents into the ecosystem, which affects aquatic organisms. This study shows that even very low doses of detergents cause adverse effects on fish reproduction. LAS application caused the separation of PGCs from the migration route and delayed gonadal development. Thus, damage of PGCs and changes in migration affected gonad formation. LAS exposure slowed gonad development and caused histopathological changes in the gonad structure.

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Author contributions: NDY defined the research idea and created the research design. CA performed the experiments and wrote the paper.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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