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Histological analysis of acute toxicity of 2,4-diclorophenoxy acetic acid in ovary of zebrafish

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Plant growth regulators are the chemicals that are found in plants and produced synthetically. In agricultural applications, plant hormones are used in minor quantities for fixing the problems. In our research, we studied the effects of 2,4 diclorophenoxyacetic acid (2,4D), which is an auxin used in agricultural applications. Auxins are the group that are used most popularly in plant growth regulators. In our study, different doses of 2,4 diclorophenoxyacetic acid are given to zebrafish, and ovarium tissues are observed histomorphologically. We generated one control and three experiment groups from the stock solution. The experiment was carried out in 20 liter capacity complete glass aquarium at $24 \pm 1^{\circ}C$ water temperature. After five days of application, fishes were dissected. Histomorphological changes of the ovarium were investigated under a light microscope. A decrease in the number of oocytes in zebrafish ovarium was observed when compared with the control group. Many deformed and underdeveloped oocytes were detected. An increase in the number of atretic oocytes was observed. It was deduced that acute doses of 2,4 diclorophenoxyacetic acid decelerates oogenesis in fishes.

Keywords: 2,4 diclorophenoxyacetic acid; ovarium; zebrafish; histopathology

Introduction

The application of pesticides to manage pests in land and water management has posed potential health hazards to wildlife and humans. Acute toxicity of phenoxyacetic acid derivatives measured as the LD50 dose varies between 100 and 1200 mg/kg body mass for various species of experimental animals (Hayes and Laws 1991; Wafa et al. 2011). Ninety-six-hour LD50 value of 2,4-D for zebrafish is >1000 mg/L (Nufarm Material Safety Datasheet 2011). Although phenoxy-herbicides are relatively slightly toxic, their ubiquitous distribution and prolonged exposure may impose a substantial health risk on subjects living in rural areas. Among these 2,4-D is the most widely used herbicide in the world (Wauchope et al. 1992; Karasu Benli et al. 2007) and is a common herbicide used around the home and garden, on golf courses, ball fields, parks, and in agriculture and forestry. Agricultural uses include pasture land, wheat, corn, soybeans, barley, rice, oats, and sugar cane. Among the herbicides used in Turkey that are potentially harmful to humans are phenoxy compounds such as 2,4-D [(2,4-dichlorophenoxy)acetic acid]; 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid]; MCPA [(4-chloro-2-methylphenoxy)acetic acid]; and their respective esters (Vural 1996).

2,4-Dichlorophenoxyacetic acid functions by maintaining high levels of the plant hormone auxin, resulting in overstimulation of plant growth and ultimately death. Aquatic toxicity of 2,4-D on non-target organisms is either incomplete or lacking. Although some formulations of 2,4-D were reported highly toxic to fish; others were less so (Karasu Benli et al. 2007).

The teratogenic, neurotoxic, immunosuppressive, cytotoxic, and hepatoxic effects of 2,4-D have been well documented (Blakley et al. 1989; Charles et al. 2001; Madrigal-Bujadar et al. 2001; Osaki et al. 2001; Tuschl and Schwab 2003). There are studies in literature concerning the accumulation of 2,4-D, its derivatives, and other agricultural chemicals in tissues (Koziollek et al. 1996). Oruc and Uner (2000) studied the combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes for clarifying the mode of action of these chemicals.

The zebrafish *(Danio rerio)* is a member of the Cyprinidae family and is native to India and Pakistan. It is widely used as model organisms, especially in developmental biology. The zebrafish model is becoming more and more popular because it is easy to produce. In the laboratory, zebrafish can be stimulated to breed throughout the year, and the development from the fertilized egg to the reproducing stage takes only about 3–4 months. Their short generation time of three months makes them an ideal candidate for genetic and developmental studies, and their susceptibility to mutagens, carcinogens, teratogens, and toxins makes them ideal as environmental models.

A model organism should offer technical and practical advantages for studying principal biological

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processes, effects, and mechanisms. In addition, it needs to have traits that can be generalized, that is, the model organism has to be a representative for a larger group of organisms. Both arguments come true for the zebrafish – it is a species convenient and cost-effective to work with from a technical and methodological point of view, and it provides conceptual insights into many aspects of vertebrate biology, genetics, toxicology, and disease (Segner 2009). Whereas information on many aspects of the biology and ecology of zebrafish in the field is surprisingly limited (Spence et al. 2008), considerable knowledge exists with respect to optimum breeding and maintenance conditions in the laboratory (Westerfield 2000). The principal advantages of the zebrafish model discussed above make this species also a suitable model for toxicological purposes. Zebrafish have been used not only as a general vertebrate toxicity model (Hill et al. 2005), but also as an ecotoxicological test species to determine the effects of chemicals on fish survival, growth, and reproduction.

This study was conducted to determine the acute toxicity of 2,4-D in ovary of zebrafish, the most widely used herbicide in the world, on a widely distributed, important invertebrate in many aquatic systems. Histological examination of such tissue following exposure to sublethal doses of 2,4-D appears to be somewhat obsolete. Therefore, it was felt necessary to investigate the ovarian histopathology that may occur in zebrafish following varying exposure periods to sublethal doses of 2,4-D.

Materials and methods

Chemicals

2,4-Dichlorophenoxyacetic acid (2,4-D) is a common systemic pesticide/herbicide used in the control of broadleaf weeds. It is the most widely used herbicide in the world. 2,4-D is a synthetic auxin (plant hormone), and as such it is often used in laboratories. It is sold in various formulations under a wide variety of brand names. 2,4-D can be found in lawn herbicide mixtures such as "Weed B Gon MAX", "PAR III", "Trillion", and "Tri-Kil". In aquatic environments, microorganisms readily degrade 2,4-D, and breakdown by sunlight is not a major reason for loss. Rates of breakdown increase with increased nutrients, sediment load, and dissolved organic carbon. Under oxygenated conditions, the half-life can be short, in the order of one week to several weeks.

In our study we created one control and three experimental groups ($n = 30$) (Group I: 0.1 ppm 2,4-diclorophenoxy acetic acid; Group II: 0.5 ppm 2,4-diclorophenoxy acetic acid; Group III: 1 ppm 2,4-diclorophenoxy acetic acid; and Group IV: control group).

Animal

The zebrafish (*Danio rerio*) is a small fish about 6 cm in length, characterized by a series of five pigmented stripes running the entire length of each side of its body. The zebrafish's hardiness makes them excellent stress test subjects, as they can survive fairly severe environmental changes without succumbing, surviving long enough to show developmental defects. Finally, zebrafish are easy and inexpensive to raise, requiring only filtered water, and a minimal investment in fish food, making them an ideal animal model for research laboratories with limited funding. All of these characteristics have contributed to making zebrafish the model of choice in this study.

Zebrafish were raised in a computer-controlled incubation chamber, and ideal breeding conditions were maintained to ensure a maximum yield. The zebrafish received 14 hours of daylight and 10 hours of darkness every night. The temperature and humidity were kept at 28.5°C and 61%, respectively.

Experimental analyses

A static toxicity bioassay was performed according to standard method (APHA 1992) to determine the 96-h LC50. The zebrafish were divided into four groups: a control group and three experimental groups. 2,4-D stored at +4°C was diluted to give the stock material, and dosing solutions were prepared by dilution to give concentrations of 0.1, 0.5, and 1 ppm. The dosing volume never exceeded 0.2 ml, and the control group received acetone alone. Following the preliminary experiment, all determinations were repeated twice. Groups of experimental animals, each consisting of 10 individuals, were randomly selected and placed into aquaria. Mortality was assessed at 24, 48, 72, and 96-h after the start of the tests. Dead individuals were removed immediately. Behavioral changes were followed closely. Control group was kept in tap water, no solvent was included since 2,4-D is soluble in water; all other conditions were same as experimental groups. After giving different doses of 2,4-diclorophenoxy acetic acid, fishes were dissected in the fifth day of the study.

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). After five days, all fish were taken for postmortem dissection. The ovary of adults were fixed in Bouin's fixative for 18 h and stored in 70% methanol. The fishes were killed instantly by

placing in a jar with a few drops of formalin; then, they were fixed in Bouin's fixative. Dehydration was carried out in an ascending series of ethanol, and the tissue was cleared in xylene. The tissues were then embedded in paraffin wax and cut into 5 µm sections on a microtome. The sections were mounted on glass slides and stained with hematoxylin (H&E) and Periodic Acid Schiff.

Results

Control ovary morphology

The zebrafish ovary consists of a thin epithelium, oogonia, and follicles that contain oocytes surrounded by somatic cells and interstitial tissues (stroma). Zebrafish have asynchronous ovaries, containing follicles of all stages of development, and eggs are spawned throughout the year. Zebrafish follicles possess a large oocyte surrounded by the zona radiata (vitelline envelope) and a follicular layer consisting of an inner layer of granulosa cells separated by a basement membrane from an outer theca cell layer. Follicle development in the adult zebrafish ovary is broadly divided into the growth and maturation stages.

Pioneering work of Selman et al. (1993) described the five stages of follicle growth in zebrafish. Stage I is the primary growth phase in which oocytes begin to enlarge and follicles start to form. In Stage II (cortical alveolus stage), cortical alveoli ("yolk" vesicles containing zona pellucida proteins) accumulate within the oocytes. In Stage III, oocytes undergo vitellogenesis (uptake of vitellogenin), resulting in an increase in the size of follicles. Oocyte maturation takes place in Stage IV. During this period, the germinal vesicle migrates from the center of the oocyte to the periphery, and the nuclear membrane breaks down. In Stage V, mature eggs are ovulated and ready for spawning (Selman et al. 1993; Koç et al. 2009).

Primary oocytes in the control group were oval-shaped cells with oval nucleus and large nucleolus. In the first growth phase, follicle layers were not entirely developed but they were visible, and the diameter of these follicles was small. The nucleuses of oocytes in the previtellogenic phase were central. Under light microscope, nucleoluses were close to the nuclear membrane. Oocytes in the vitellogenic phase were larger in size according to their baseline sizes. The increase in size was attributed to nutrient accumulation in cytoplasm (Figure 1).

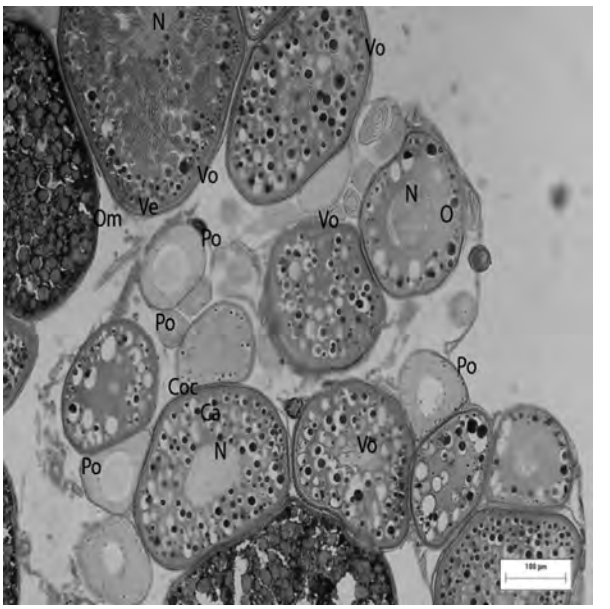

Figure 1. Control group: The ovary of a control fish reveals healthy stages. General appearance of oocytes during the developmental phases [Primary oocytes (Po), Cortical alveolus oocytes (Coc), Vitellogenic oocytes (Vo), Cortical alveoli (Ca), Nucleus (N), Ooplasm (O), and Mature oocytes (Om)]. X10 PAS stain.

Treated ovary morphology

In the ovarium sections of zebrafish, morphological and histological changes were observed due to different doses of 2,4 D. This toxic material as administered in the three groups caused a slow-down in oogenesis, and an increase in the number of atretic follicles and connective tissue in the zebrafish ovaries. Furthermore, histopathological changes were observed, that is, discontinuation in growth of developing follicles and start of follicle breakdown. When compared to controls, degeneration was observed in the integrity of ovarium morphology in the experimental groups.

In the 0.1 ppm 2,4-D group, histopathological changes were observed at minimum levels. But in oocytes administered 0.1 ppm 2,4-D, disintegration in vesicle structures and irregularities in cytoplasm were observed. A decrease in the primary oocyte numbers was observed (Figure 2a). The most striking phenomenon is that 2,4-D is particularly expressed in nuclei of vitellogenic oocytes and additional clumping of karyoplasms in vitellogenic oocyte (Figure 2b). In the periphery of a degenerating large oocyte, former follicle epithelial cells exhibit heavy staining of their nuclei. Figure 3a shows the change of the form of their nuclei to a more round shape. Exposure shows severe damage to follicular epithelium and late vitellogenic stage oocyte with a large number of yolk globules that fill the entire ooplasm (Figure 3b). Figure 3c shows eccentric nucleus and karyoplasmic clumping.

In the 0.5 ppm 2,4-D group, cytoplasmic retraction was more apparent. Atresia and extreme cellular necrosis were visible and an increase in the number of

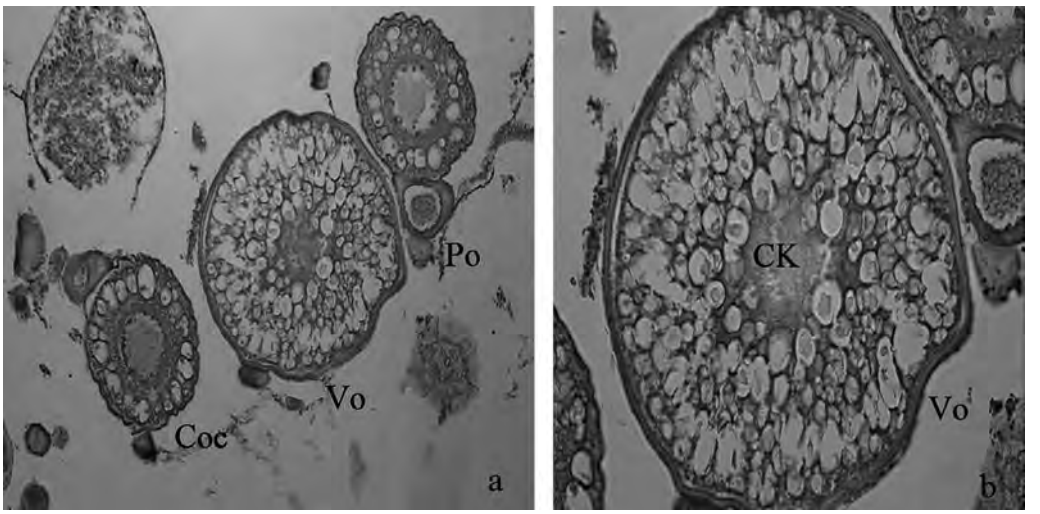

Figure 2. 0.1 ppm 2,4-D group: (a) Decrease in primary oocyte numbers can be observed (X20 HE). (b) The most striking phenomenon is that 2,4-D is particularly expressed in nuclei of vitellogenic oocytes and additional clumping of karyoplasms (CK) in vitellogenic oocyte (Vo).

degenerating oocytes was observed (Figure 4a). The treatment resulted in pathological alterations of ovarian structure, such as irregular folding of oocyte membranes (Figure 4b). Atretic oocytes were characterized by the loss of oocyte shape, a distorted chorion, and a disorganized and fragmented yolk. The breakdown of granulosa layers (which maintain the structural integrity of the oocyte) leads to structural impairment and functional loss. The occurrence of atresia was higher in treatment groups than the control.

When a cell has sustained irreversible damage a succession of morphological changes occur, which are grouped under the term cell necrosis. Cell death, which was perceived to be necrosis, was observed in the early stage oocytes, mainly the early and late primary oocytes. Spaces exist between the karyoplasm and nucleus envelope in the primary oocyte (Figure 4c).

In the 1 ppm 2,4-D group, chromatin material was degenerated. Also, openings were detected between the vitelline membrane and ooplasm. A pronounced decrease was observed in the growth of oocytes. More damage was noted to the oocyte's wall. The oocytes' wall were frayed and broken down. More adhesion, atresia, and extreme cellular necrosis were visible. Ooplasmic retraction and ooplasmic necrosis was more apparent. In addition, the number of primary oocytes and cortical alveolar oocytes were severely reduced and there was an increase in ovarian atretic follicles (Figure 5a, b). Chromatin material in cortical alveolar oocytes disappeared. Vitellogenic oocyte,

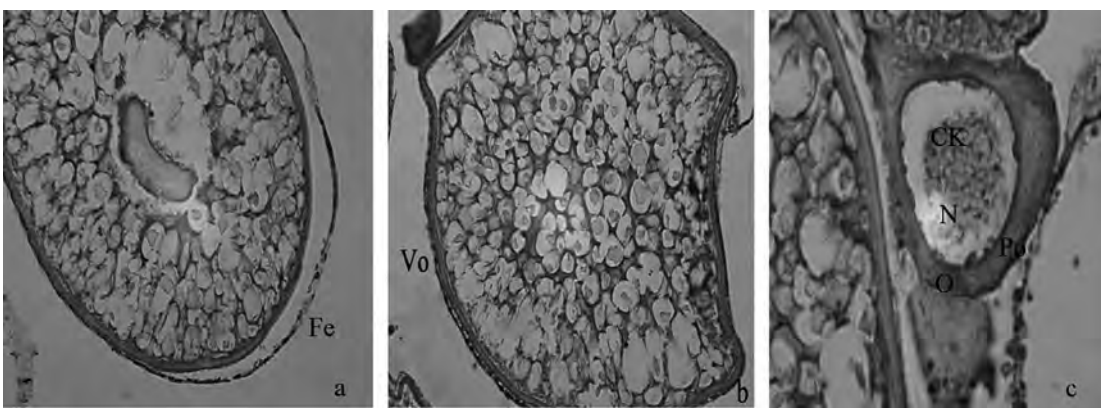

Figure 3. 0.1 ppm 2,4-D group: (a) In this phase, follicle epithelium (FE) showed often groups of stained cells adjacent to the oocyte. In the periphery of a degenerating large oocyte, former follicle epithelial cells exhibit heavy staining of their nuclei. Note the change of the form of their nuclei to a more round shape (×40 HE). (b) Exposure shows severe damage to FE and late vitellogenic stage oocyte with a large number of yolk globules that fill the entire ooplasm (O) (×40 HE). (c) Eccentric nucleus (N) and karyoplasmic clumping. Spaces exist between the karyoplasms (CK) and ooplasm (O) (×100 HE).

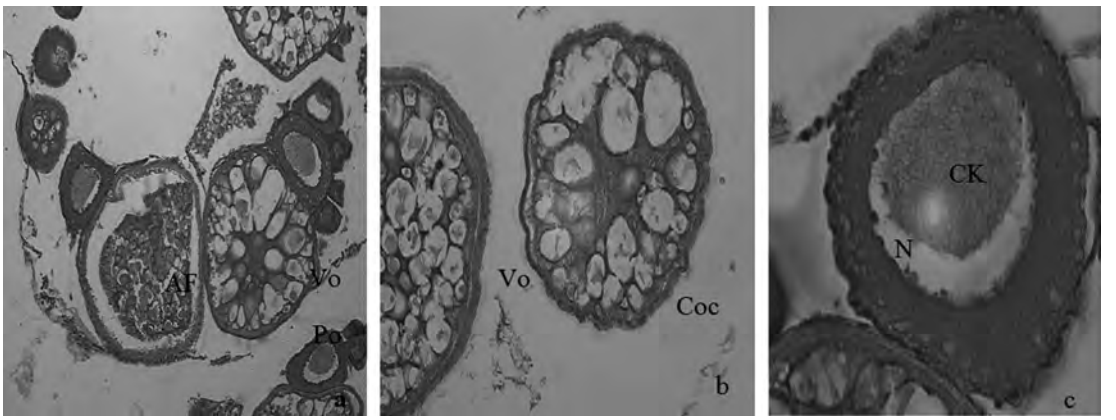

Figure 4. 0.5 ppm 2,4-D group: 2,4-D treatment in the zebrafish ovary. (a) In this overview, oocytes in all developmental stages are present. Cytoplasmic retraction is more apparent. Atresia and extreme cellular necrosis are visible and increased number of degenerating oocytes (× 20 HE). (b) The treatment resulted in pathological alterations of ovarian structure, such as irregular folding of oocyte membranes (× 40 HE). (c) See thinning of follicular lining (FL). Eccentric nucleus (N) and karyoplasmic clumping. Spaces exist between the karyoplasm and nucleus envelope (CK) (× 100 HE).

openings between vitelline membrane and zona radiata anddeformation in the morphology of cortical alveoles were detected (Figure 5c, d).

Discussion

The quality of the natural surroundings of fish has an important role in their development and reproduction. Even slight changes in the concentration of certain chemical compounds can negatively affect the histological properties of fish. Reproductive toxicity of pesticides in zebrafish is similar to that reported in other fish species and consists of reduced fitness as both reproductive capacity and recruitment of offspring are reduced. Most research on pesticides' reproductive toxicity in zebrafish has evaluated the effects on female reproduction and demonstrated that ovarian development and egg release are impaired. Our results are compatible with the results of other researchers.

Green and Abdelghani (2004) investigated the toxicity of a mixture of 2,4-dichlorophenoxyacetic acid and monosodium methanearsonate to the red swamp crayfish, *Procambarus clarkii*. According to the results, the herbicide mixture alone displayed half the toxicity of the individual herbicides. Paul et al. (2006) reported the effects of 2,4-D on several aquatic species: Brook trout (*Salvelinus fontinalis*), walleye (*Sander vitreus*), fathead minnow (*Pimephales promelas*), and the amphipod (*Hyallela azteca*) in static acute toxicity tests in the laboratory. Estrogenic activities of aquatic herbicides using rainbow trout were evaluated by Xie et al. (2005). The other estrogenic compound, bisphenol A, may lead to problems in either mating or sexual behavior due to the difference in growth and disparity of sexual maturation between male and female fish (Na et al. 2002). 2,4-D caused significant induction of vitelogenin, which was reported by Khan et al. (2006). According to Sarıkaya and Yılmaz (2003), the acute toxic effects of 2,4-D on *C. carpio* (L. 1758) estimating LC50 values as well as the behavioral changes in the fish subjected to different concentrations of 2,4-D.

In a recent study, Pandey (1988) observed that when ovaries of the freshwater fish *Colisa fasciatus* was treated with a 1 ppm concentration of endosulfan, ovarian activity was retarded, and the diameter of Stage II and Stage III oocytes was severely reduced. In another study, Park et al. (2002) exposed female mosquitofish (*Gambusia affinis*) with 1 ppb endosulfan, and they demonstrated that endosulfan significantly reduced sexual attractiveness of females. These findings are compatible with our results. When a study using two endocrine inhibitors, carbaryl and endosulfan, was conducted on the freshwater fish *Channa striatus*, the changes in oocyte formation mentioned above were observed and concluded to be correlated with the concentration amount and exposure time. Sublethal doses of each of these endocrine inhibitors showed that endosulfan's doses were more toxic than those of carbaryl (a carbamate) (Kulreshtha and Arora 1984; Dutta and Dalal 2005). It was reported that there was an involution in ovarian size, a delay in ovarium development, and an increase in atretic follicle numbers in female fish exposed to 17-alpha-ethynylestradiol (EE2) administration (Van den Belt et al. 2002). Organophosphate pesticides also caused a drop in the proportion of mature oocytes in fish, and histopathological damage to ovaries as well as disintegration of cortical alveoli and yolk globules in mature oocytes

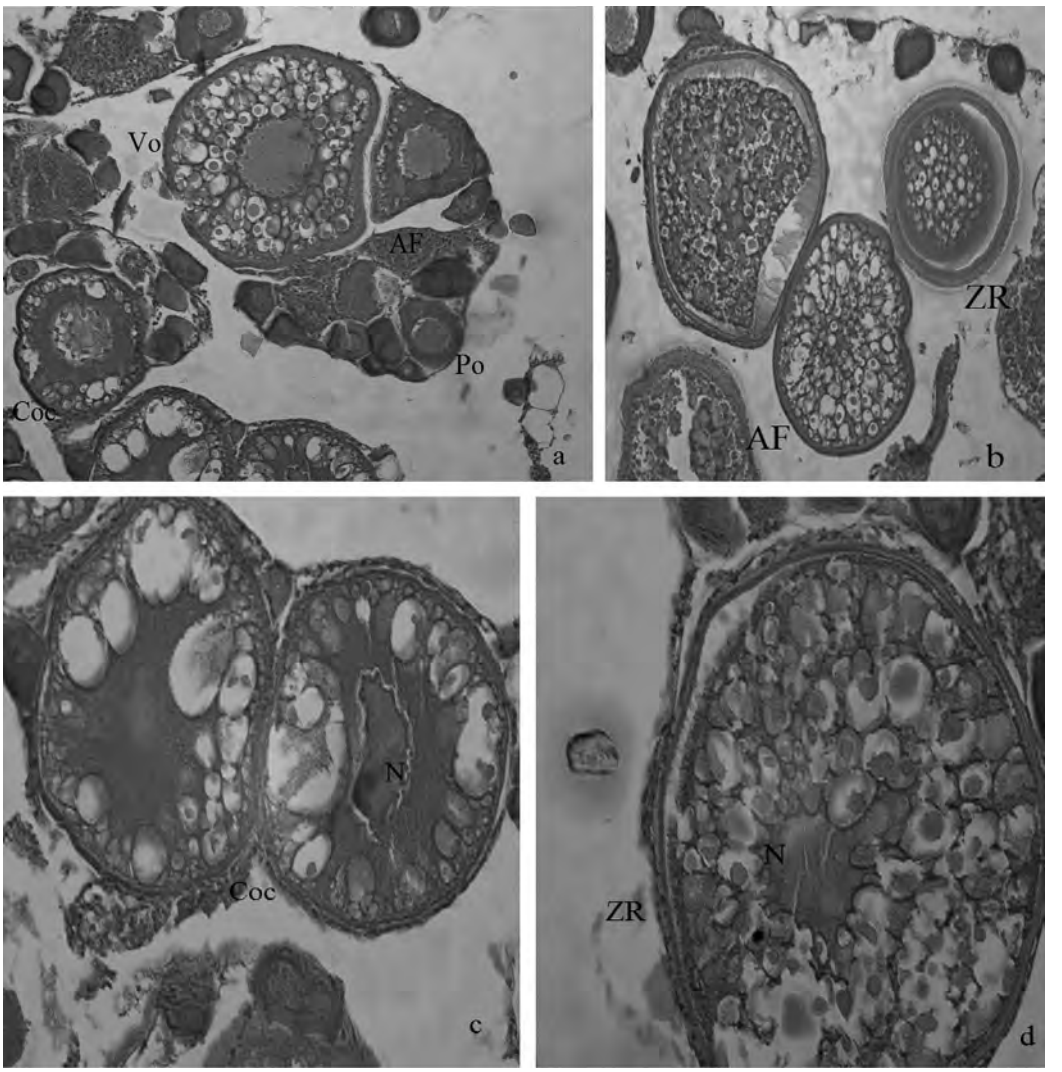

Figure 5. 1 ppm 2,4-D group: (a, b) 2,4-D treatment in the zebrafish ovary. In this picture atretic (AT) oocytes are visible. Considerable damage to oocytes is apparent. More damage is noted to the oocyte's wall. The oocytes wall are thin, frayed, and broken down. More adhesion, atresia, and extreme cellular necrosis are visible. Ooplasmic retraction more apparent and ooplasmic necrosis. Chromatin material in primary oocytes disappeared, vitelline membrane and ooplasm separated (a, $\times$10 HE; b, $\times$20 HE). (c, d) Chromatin material in cortical alveolar oocytes disappeared. Vitellogenic oocyte, openings between vitelline membrane and zona radiata, deformation in the morphology of cortical alveoles [Mature oocyte (Om), Vitelline envelope (Ve), Zona radiata (ZR)] (c, $\times$40 HE; d, $\times$100 HE).

(Rastogi and Kulshrestha 1990; Kogan et al. 2000). These findings are also compatible with our results.

Histopathological analyses of the ovary show that the adverse effects of 2,3,7,8-Tetrachlorodibenzo p-dioxin (TCDD) follicular development and vitellogenesis probably result from a direct effect on the ovary by modulating follicular development and inducing follicular atresia (King-Heiden et al. 2006, 2009). While King-Heiden et al. (2009) did not directly correlate ovary histopathology to reduced reproductive capacity of the TCDD-exposed females, but according to King-Heiden et al. (2011), the results show that ovaries from these TCDD-exposed females contained significantly more atretic follicles, smaller vitellogenic follicles, and approximately half of the TCDD-exposed females had malformed ovaries. According to Korfsmeier (2002), Proliferating cell nuclear antigen (PCNA) is expressed in the nuclei of mitotically active cells in follicle epithelium (FE) of zebrafish oocytes. The number of cells stained for PCNA is a standard measure for the growth of FE in the different stages of oogenesis. In degenerating oocytes, the former follicle cells invade the peripheral ooplasm and are involved in phagocytosis and degradation of remnants of egg yolk (Korfsmeier 1969a, 1969b).

According to Dutta and Maxwell (2003), a sublethal dose of diazinon began to affect the cellular

composition of the ovary after 24 h of exposure, but damage to the oocytes and surrounding tissues were more pronounced after 72 h and kept on increasing until the end of three weeks of exposure. Study also showed that the oocytes at their different stages of maturation get affected differently at various exposure periods of diazinon. The normal ovarian activity was affected after melatonin and 5-methoxytryptophol exposure (Joy and Agha 1991). Seasonal histological changes in the ovary of *Punctius ticto* were caused by heavy metal toxicity (Pundir 1991).

The herbicide 2,4-D used in this study is highly toxic to fish and has very adverse effects on both humans and animals. It also accumulates in tissues and causes acute poisoning (World Health Organization 1984). Despite these grueling facts, the study related to the effects of 2,4-D on animals are highly limited. It is clear that the number of studies directed to the investigation of acute and chronic toxicity of various herbicides in animal tissue is to be increased. The results obtained in this study clearly reveal the fact that it is necessary to control the use of some herbicides, which are commonly employed in agriculture today.

This study demonstrated that 2,4-D in different doses (0,1 ppm, 0,5 ppm and 1 ppm) caused histopathological changes in zebrafish ovaries. It has been suggested that 2,4-D adversely affects ovarian tissue in zebrafish. It was observed that this substance leads to growth inhibition of oocytes, an increase in atretic oocyte numbers, and a decrease in the developing oocyte ratio. In conclusion, our research is the first study demonstrating that 2,4-D gives rise to retardation of oogenesis in zebrafish.

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