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RESEARCH PAPER

Effects of Diazinon on 17β-estradiol, Plasma Vitellogenin and Liver and Gonad Tissues of Common Carp (*Cyprinus carpio*, L., 1758)

Cengiz Korkmaz[1,*], Ahmet Erdem Dönmez[1]

[1]Mersin University, Faculty of Fisheries, Department of Basic Sciences, 33169 Mersin.

* Corresponding Author: Tel.: +90.546 4018705; Fax: +90.324 3413025;
E-mail: cengizkorkmaz@mersin.edu.tr

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Abstract

Both sexes of Common carp (*Cyprinus carpio*) exposed to sublethal concentrations (0.488 mg/L and 0.976 mg/L) of the widely used insecticide diazinon for a period of 7, 15, 30 days and effects on plasma 17β-estradiol (E2), vitellogenin (VTG), gonad and liver tissues were examined. In male carps, 0.488 mg/L and 0.976 mg/L diazinon elevated E2 levels following on the 7, 15 and 30 days. Both concentrations of diazinon elevated VTG levels after 15 days in males. In female carps, 0.976 mg/L diazinon elevated E2 levels after 30 days. However, 0.488 mg/L and 0.976 mg/L diazinon reduced VTG levels in female carps, at the end of experiment. Histopathological examination of liver showed dilatation of the bile ducts and sinusoids, congestion, lymphocytic infiltration, pigment accumulation and necrosis in both sexes and concentrations. Degeneration, congestion and fibrosis were observed in testis. Adhesions between oocytes and necrosis in ovary were also observed. The results showed that diazinon can affect the male and female individuals differently and has an endocrine disrupting potential.

Keywords: *Cyprinus carpio* , diazinon, vitellogenin, 17β-estradiol, histopathology.

Introduction

Diazinon – CAS 333-41-5 (O, O-diethyl O-2-isopropyl–6-methylpyrimidinyl-4-g-1-phosphorothioate) is a widely used insecticide (Fattahi, Parivar, & Jorsaraei, 2009) for control of household and soil insects, pest on fruits, vegetables, field crops, lawns and ornamentals (Kime, 1998). Additionally, diazinon used to dip sheeps to control parasites (Watterson, 1999) and as a biocide to suppress excessive propagation of daphnia zooplankton (Máchová *et al*., 2007). After its application on crops and plants, diazinon can easily be washed into surface waters and enters the ground water and under the conditions of low temperature, low moisture, high alkalinity and lack of suitable microbiological degraders, it may remain biologically active in soils for 6 months or longer (Dutta & Meijer, 2003). It has been reported that diazinon concentrations were found between 0.72 – 315.95 ng/L in four Mediterranean River Basins, Spain, between 2010-2011 (Campo, Masiá, Blasco, & Picó, 2013).

Because of its aquatic distribution, diazinon affects a wide range of non-target organisms, like mammals, birds and fish (Aydın & Köprücü, 2005). Also diazinon is highly toxic for freshwater fish and invertebrates following acute exposures (EPA, 2004). For common carp the LC_{50} of commercial diazinon was reported as 9.76 mg/L (Ahmad, 2011).

Like the other organophosphates (OPs), diazinon inhibits acetycholinesterase (AChE) (Fulton & Key, 2001). This results acetylcholine accumulation in postsynaphtic cells of end organs (Pope, 1999). Accumulation of acetycholine causes symptoms of autonomic dysfunction (e.g., excessive secretions of the airways, excretory systems, salivary glands, and lacrimal glands), involuntary movements (e.g., tremors, convulsions), muscle fasciculations, and ultimately respiratory depression (Nostrandt, Padilla, & Moser, 1997). Moreover, sublethal exposures of some OPs lead to alterations in reproductive performance (Pope, 1999) and may show endocrine disrupting potential (Kitamura, Suzuki, Ohta, & Fujimoto, 2003; Goad, Goad, Atieh, & Gupta, 2004; Hotchkiss *et al*., 2008).

Earlier studies reported that diazinon does not have an endocrine disrupting potential, however recent studies showed that sublethal concentrations of diazinon was able to reduce serum 17 β-Estradiol (E2) (Maxwell & Dutta, 2005; Jamili, Hoseini, & Mashinchian, 2008), progesterone (17,20βP) (Wall, 1999), testosterone (T) and gonadotrophin II levels

(Moore & Waring, 1996), inhibits the cortizol secretion (Bisson & Hontela, 2002), decline in gonadosomatic index (Larkin & Tjeerdema, 2000), hypertrophy, necrosis and pyknosis of hepatocytes in various fish species (Rahman, Hossain, & Mollah, 2002). Therefore, the main objective of this study was to find out whether diazinon has an endocrine disrupting potential in fish such as common carp, *Cyprinus carpio*.

Materials and Methods

One-year old male and female common carps were obtained from hatchery unit of the State Hydraulic Works, Adana, Turkey. The fish were acclimated to the laboratory conditions prior to the experiment for two months Average body weight and length of test materials at the beginning of the experiment were 19.88±6.68 g and 9.42±0.96 cm, respectively. Firstly, fish were divided into two groups by stripping them, as males and females. Male and females groups divided between three groups, one as a control, and each group had eighteen indivudials. One hundred and eight fish were totaly used. Experiments were run in duplicate. During experiments, fiber-glass tanks (120x120x50 cm) supplied with 400 L dechlorinated tap water were used. Some physical and chemical properties of test water were as follows, temperature 24±1°C, pH 7.30±0.20, dissolved oxygen 7.10±0.12 mg/L and total hardness of 252.16±4.03 mg/L $CaCO_3$. The photoperiod was 12:12 h (L:D). The fish were fed daily with commercially available pond sticks (Tetra Pond Koi Sticks®) at a rate of 2% body weight.

Sub-Lethal Toxicity Experiments

The commercial preparation of Basudin 60 EM® (O, O-diethyl O-(6-methyl-2-(1-methylethyl)-4-pyrimidinyl) phosphorothioate, Syngenta, Diazinon, 630 g/L) was used in this study. Stock solutions of the test substance were prepared by dissolving the insecticide in tap water. These solutions were further diluted to obtain the experimental concentrations in tanks. Control group was maintained in tap water without diazinon. Fish were exposed to 5% and 10% of 96 h LC_{50} (0,478 mg/L and 0, 976 mg/L) of diazinon over 7, 15, and 30 days. Test media were refreshed every 24 h to maintain constant insecticide nominal concentrations.

Sampling

Six treated and control fish were randomly removed from each aquarium at the end of 7, 15 and 30 days. They were anesthetized by 0.1 g/L MS-222 (Velisek & Svoboda, 2004) and blood samples were then collected in eppendorf tubes by cutting the caudal peduncle. Samples centrifuged at 3000 g for 10 min at 4°C and the collected plasma was stored at -80°C until analysis (Lomax, Roubal, Moore, & Johnson, 1998).

Hormone Analysis

Plasma levels of 17β-estradiol (E2) were measured by acetylcholinesterase-based competitive enzyme-linked immunosorbent assay (ELISA), using EIA kits, according to manufacturer's instructions (Cayman Chemical Company). Samples run in duplicate. The antiserum to E2 (Cayman Chemical, Ann Arbor, MI, USA) was reported to cross-react with estradiol-3-glucoronide (14%), estrone (12%), estriol (0.30%), T (0.01%) and 5-dihydrotestosterone (0.06%). Results were expressed as absorbance's at 420 nm wavelength with microplate reader.

VTG Analysis

Plasma levels of VTG was measured by sandwich enzyme-linked immunosorbent assay (ELISA), using ELISA kit, according to manufacturer's instructions (Biosense Laboratories). Samples run in duplicate with two different dillutions. Results were expressed as absorbance's at 492 nm wavelength with microplate reader.

Histopathological Examination

Three treated and control fish were randomly removed from each aquaria at the end of 7, 15 and 30 days. Ovary, testis and liver tissues were dissected immediately after taking the blood samples, transferred to 10% neutral buffered formalin for 24 h for fixation. Fixed tissues were prepared for sectioning and embedded in paraffin. Five micrometer thick sections were then taken and stained with hematoxylin and eosin. Tissues were visualized with Nikon Eclipse 80i (Nikon Corporation, Japan) fluorescent microscope and measured with Nikon Digital Sight (DS-L1, Nicon Corporation, Japan).

Statistical Analysis

Statistical analysis were carried out by SPSS 11.5 for Windows. All the data were tested for homogeneity of variance. The results obtained from each experimental group were analyzed by one-way analysis of variance (ANOVA). The significance level was considered to be $P<0.05$. Results are expressed as mean ± SD.

Results

No mortality was recorded during the experimental period for all treatment groups. The behavior of fish were visually monitored during the test periods. Fish exposed to diazinon showed no signs of symptoms like lethargic and erratic swimming, loss of schooling behavior, hyperactivity, convulsions and loss of buoyancy.

Serum E2 Level

Sera E2 levels in control males ranged between 372.80 and 398.40 mg/L over the study period. Sera E2 levels of males increased with increasing concentrations ($P<0.05$) except between the two concentrations of diazinon on day 7 (Figure 1).

Sera E2 levels in control females ranged between 507.60 and 614.85 mg/L over the study period. No significant concentration or time-related effects of diazinon were observed for sera E2 levels in females. However, 0.976 mg/L of diazinon significantly increased sera E2 levels in females after 30 days (Figure. 2).

Serum VTG levels

Sera VTG levels in control males ranged between 217.00 and 315.00 mg/L over the study period. Sera VTG levels of males were significantly higher in 7 days, in response to high concentration of diazion ($P<0.05$). Both low and high concentrations of diazinon on day 15 and only low concentration on day 30 increased sera VTG levels of male *C. carpio* significantly ($P<0.05$) (Figure. 3).

Sera VTG levels in control females ranged between 2880.00 and 7045.00 mg/L over the study period. Sera VTG levels of females were significantly lower in 15 and 30 days, in response to both low and high concentration of diazinon ($P<0.05$). Low concentration sof diazinon decreased sera VTG levels of female *C. carpio*'s significantly after 7 days ($P<0.05$) (Figure 4).

Liver histopathology

Hepatic artery and portal veins, sinusoids, hepatocytes, pancreatic tissue and bile ducts which are tapped into liver were determined in control group. The tissues and hepatocytes showed no abnormalites in control group (Figure 5).

Fish livers exposed to 0.448 mg/L diazinon showed dilatation of sinusoids and veins (Figure 6), lymphocyte infiltration (Figure 7) and pycnotic nucleus (Figure 6). Moreover, in portal veins that surrounded by pancreatic tissue there was an increased amount of proteinaceous fluid (Figure 8).

These proteinaceous fluid were also observed in 0.976 mg/L of diazinon exposure. In addition, accumulation of proteinaceous fluid in liver sinusoids (Figure 9), congestion in hepatoportal veins (Figure 10), pycnotic nucleus in hepatocytes (Figure 9), melanomacrophage centers (Figure 12) and necrosis (Figure 10) were also determined in high concentration of diazinon.

Gonad histopathology

Testis tissues of control group were normal. They were composed of different sized and shaped seminiferous tubules that contain numerous germ cells. Also, intertistial tissues and blood vessels were determined between seminiferous tubules (Figure 13).

Degenerations in seminiferous tubules (Figure 14) and fibrosis (Figure 15 and Figure 17) were observed after low and high concentration of

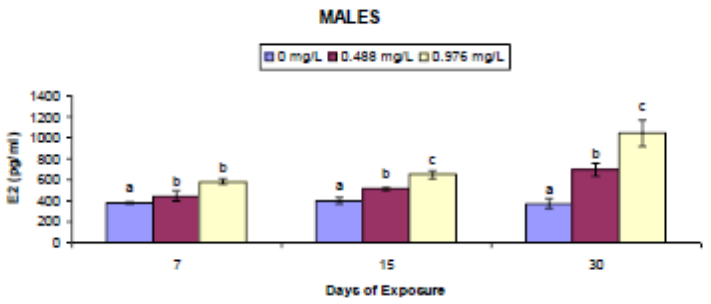

Figure 1. Sera E2 levels of males *C. carpios* exposed to 0.488 and 0.976 mg/L of diazinon after 7, 15 and 30 days (mean±SD).

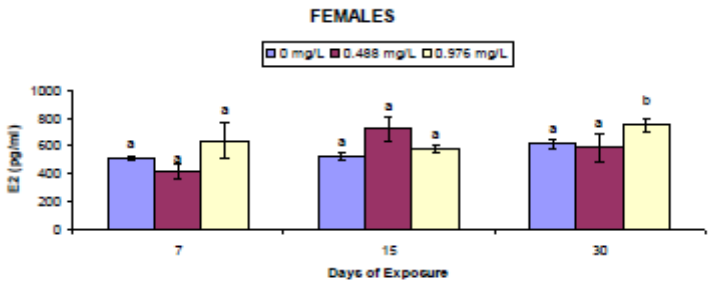

Figure 2. Sera E2 levels of females *C. carpios* exposed to 0.488 and 0.976 mg/L of diazinon after 7, 15 and 30 days (mean±SD).

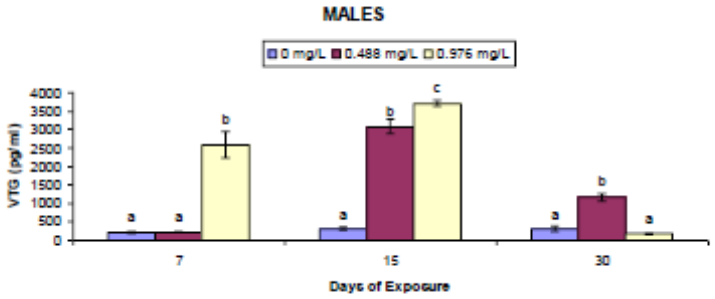

Figure 3. Sera VTG levels of males *C.carpio* exposed to 0.488 and 0.976 mg/L of diazinon over 7, 15 and 30 days (mean±SD).

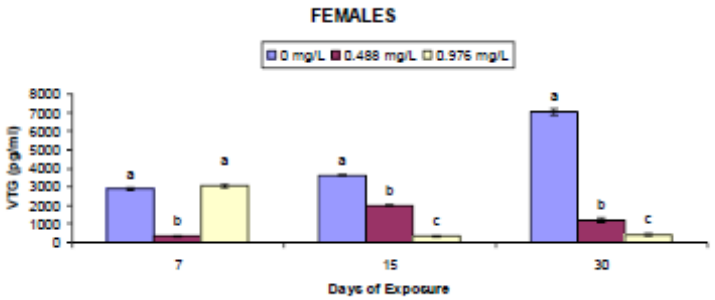

Figure 4. Sera VTG levels of female *C.carpio* exposed to 0.488 and 0.976 mg/L of diazinon after 7, 15 and 30 days (mean±SD).

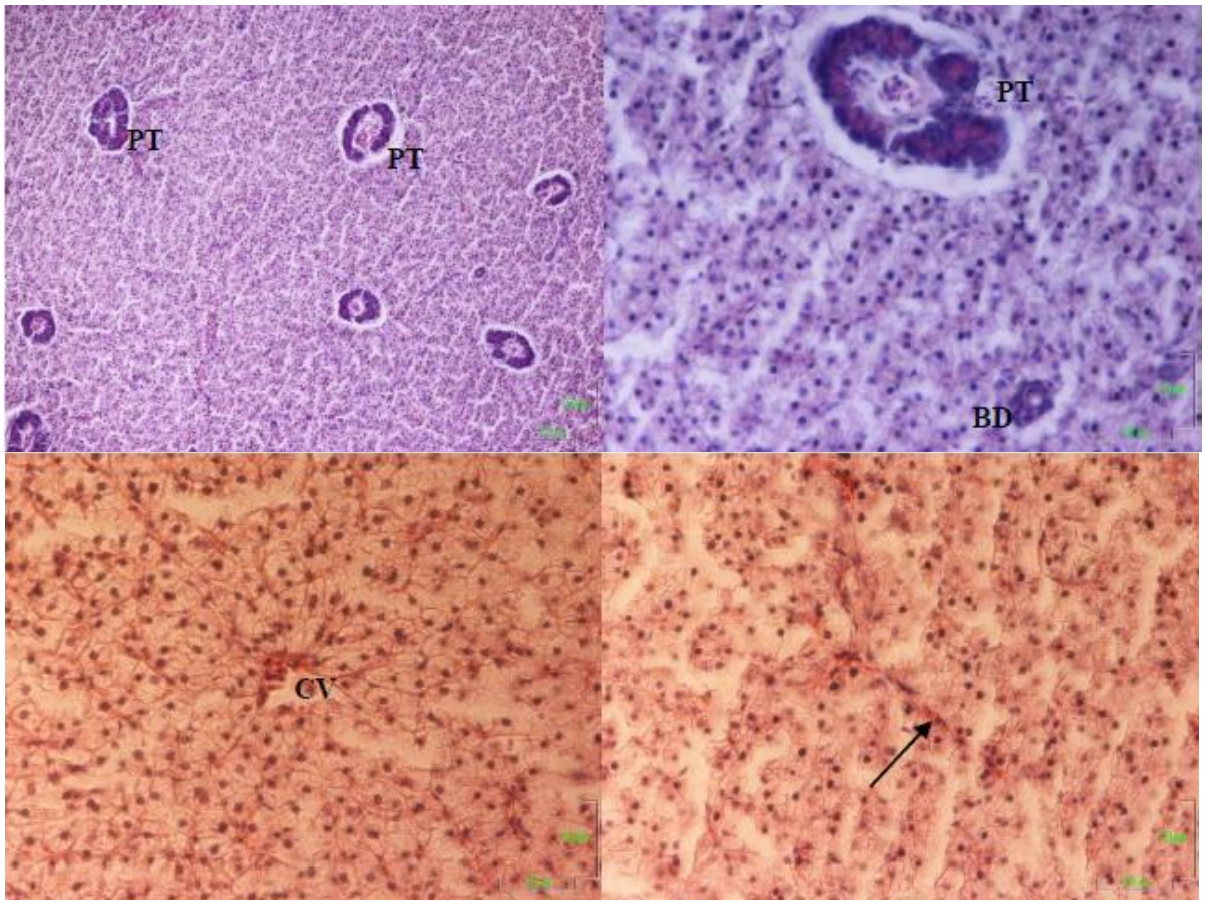

Figure 5. Liver sections of control group. Pancreatic tissue (PT), bile ducts (BD), central veins (CV), sinusoids (arrow). (HE, x100, Scale bar 10 µm).

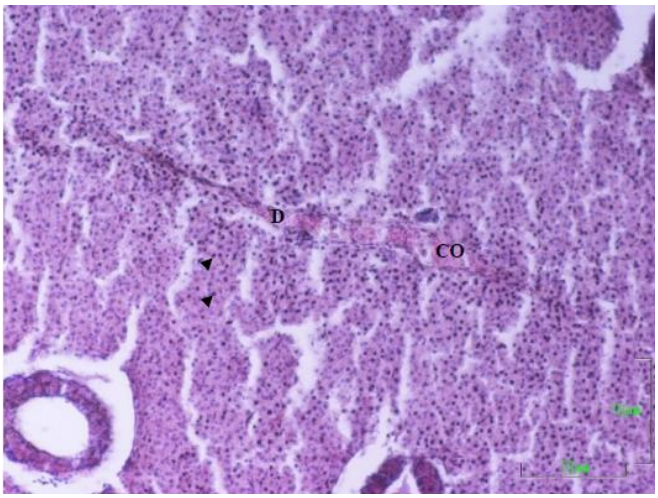

Figure 6. Liver sections of 0.488 mg/L diazinon treated fish after 30 days. Dilatation (D) and congestion (CO) of sinusoids and arrow heads indicate pycnotic hepatocyte nuclei. (HE, x200, Scale bar 10 µm).

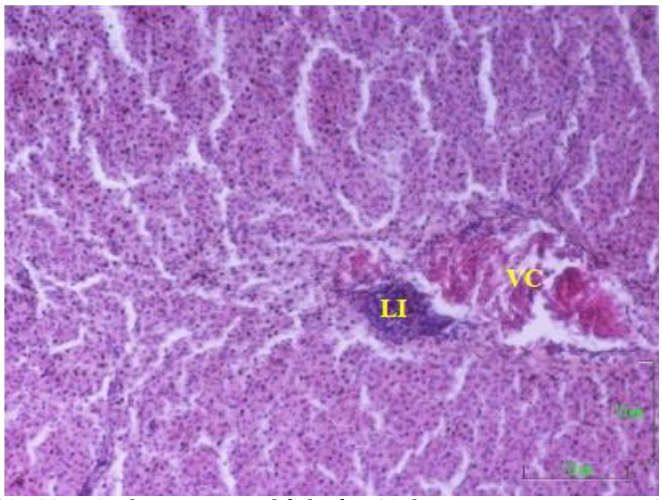

Figure 7. Liver sections of 0.488 mg/L diazinon treated fish after 30 days. Venous congestion (VC), lymphocyte infiltration (LI). (HE, x200, Scale bar 10 µm).

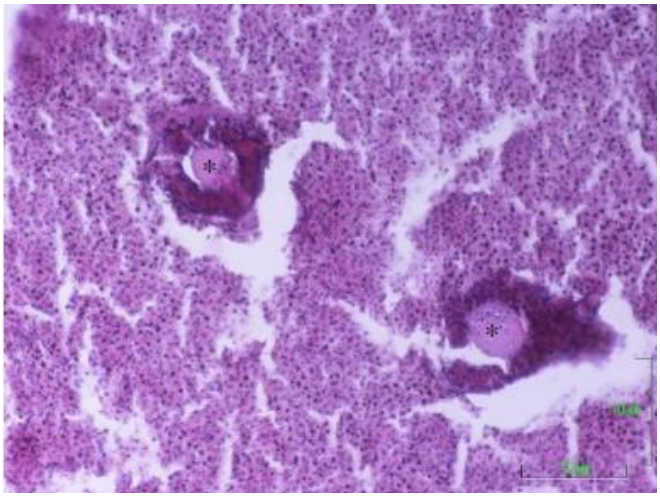

Figure 8. Liver sections of 0.488 mg/L diazinon treated fish after 30 days. Accumulation of proteinaceous fluid in portal veins of liver (*).(HE, x200, Scale bar 10 µm).

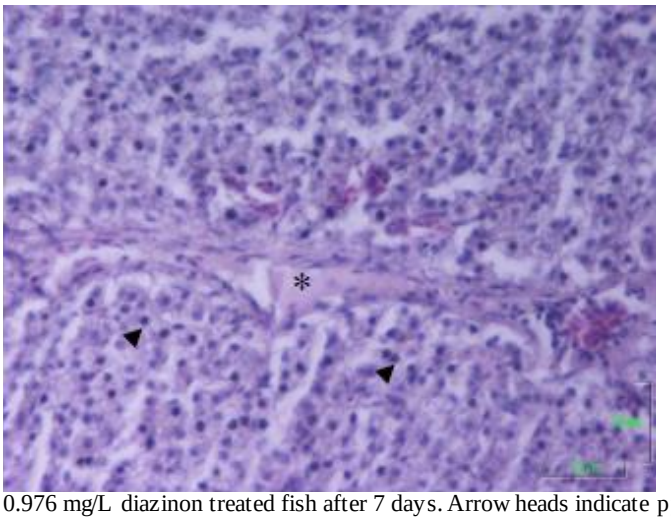

Figure 9. Liver sections of 0.976 mg/L diazinon treated fish after 7 days. Arrow heads indicate pycnotic hepatocyte nuclei. Accumulation of proteinaceous fluid in sinusoids (*).(HE, x200, Scale bar 10 µm).

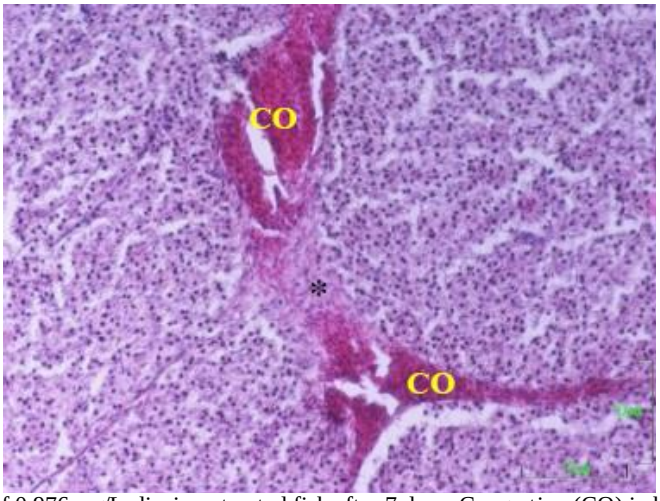

Figure 10. Liver sections of 0.976 mg/L diazinon treated fish after 7 days. Congestion (CO) in hepatoportal veins of liver and necrosis (*).(HE, x200, Scale bar 10 µm).

Figure 11. Liver sections of 0.976 mg/L diazinon treated fish after 7 days. Dilatations of bile ducts (ellipse) and melanomacrophage centers (*).(HE, x200, Scale bar 10 µm).

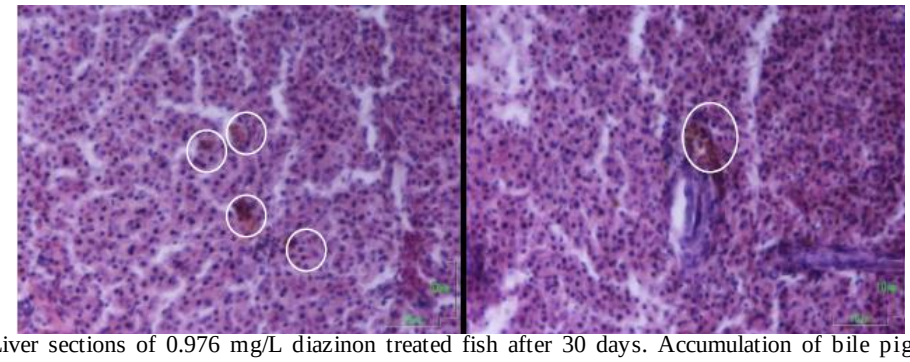

Figure 12. Liver sections of 0.976 mg/L diazinon treated fish after 30 days. Accumulation of bile pigments in liver parenchyma. (HE, x400, Scale bar 10 µm).

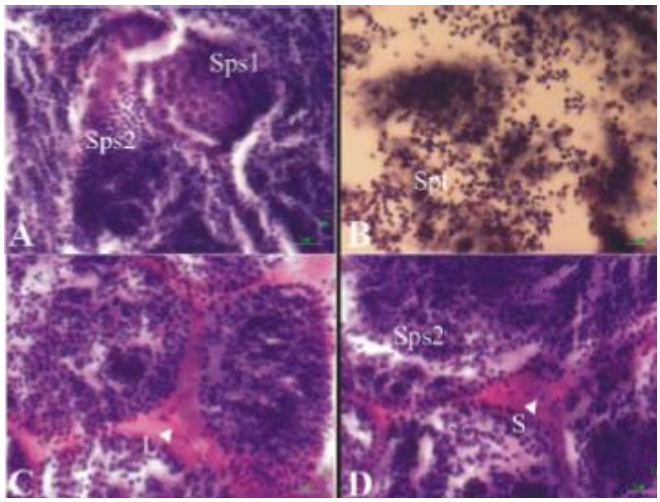

Figure 13. Testis sections of control group (HE, x1000, Scale bar 1 µm). (A: Primer and seconder spermatocytes, B: Spermatids, C: Seconder spermatocytes, interstitial cells, D: Seconder spermatocytes and cells in the seminiferous tubule basement membrane. Sps1: Primer spermatocytes, Sps2: Sekonder spermatocytes, Spt: Spermatid, L: Leyding cell, S: Sertoli cell).

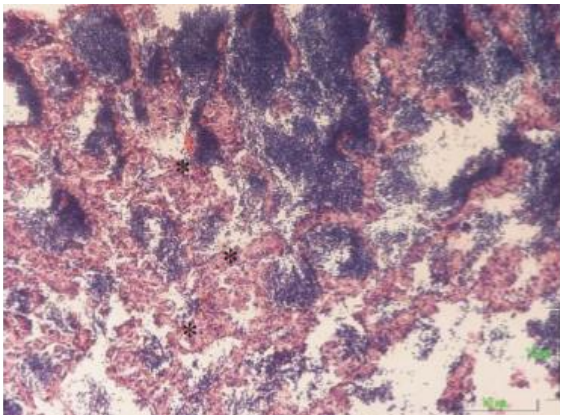

Figure 14. Testis sections of 0.448 mg/L diazinon treated fish. Degenerations of seminiferous tubules (*).(HE, x200, Scale bar 10 µm).

diazinon. The number of cells inside the intertubuler space seemed to have increased. In addition, congestions in vena's of intertubuler spaces (Figure 16) were determined after high concentration of diazion.

The follicles of control group ovaries were normal. They were containing numerous oocytes at different developmental stage. Perinuclear oocytes, cortical alveoli and vitellogenic oocytes were widespread found (Figure 18). Necrosis in ovary was observed after both low and high concentration of diazinon. Additionally, adhesions between the oocytes

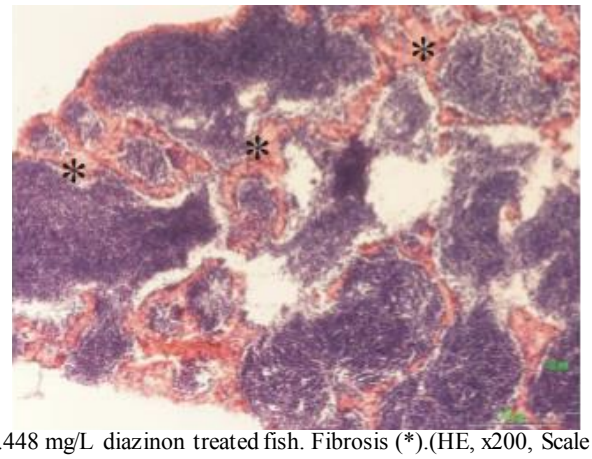

Figure 15. Testis sections of 0.448 mg/L diazinon treated fish. Fibrosis (*).(HE, x200, Scale bar 10 µm).

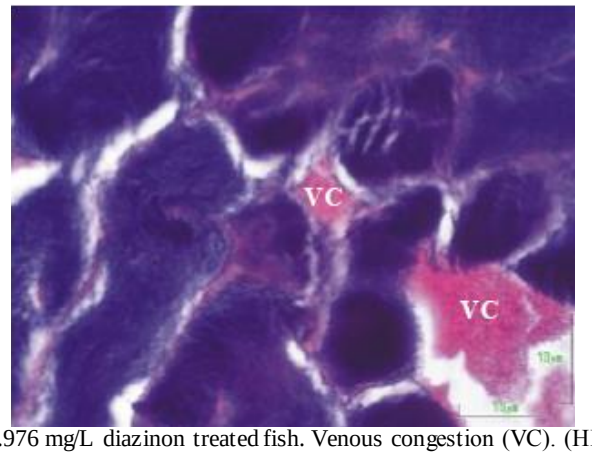

Figure 16. Testis sections of 0.976 mg/L diazinon treated fish. Venous congestion (VC). (HE, x200, Scale bar 10 µm).

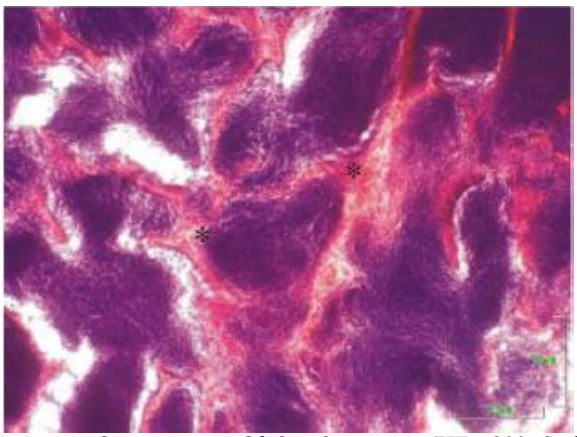

Figure 17. Testis sections of 0.976 mg/L diazinon treated fish. Fibrosis (*). (HE, x200, Scale bar 10 µm).

were determined after 0.976 mg/L concentration of diazinon (Figure 19).

Discussion

In this study the results provide clear evidence that diazinon differently alters E2 and VTG levels in male and female carps and has histopathologic impacts on tissues such as ovary, testis and liver.

In males, both concentrations of diazinon induced E2 levels after 7, 15 and 30 days. Like the other OPs, bio conversation of diazinon occurs in peripheral tissues by monooxygenases (MFO) (Yang, Hodgson, & Dauterman, 1971; Fuji & Asaka, 1982; Demirdöğen, 2010) and MFO activity depends on a family of P-450 enzymes such as aromatase (Kime, 1998). Since aromatase is responsible for conversion of the male hormone (T) to the female hormone (E2)

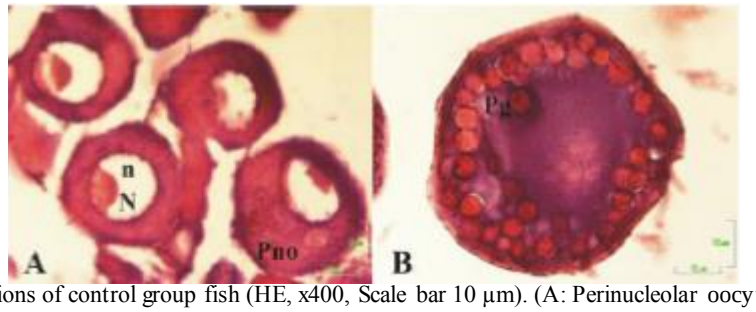

Figure 18. Ovary sections of control group fish (HE, x400, Scale bar 10 µm). (A: Perinucleolar oocyte, B: Vitellogenic oocyte. Pno: Perinucleolar oocytes, N: Nucleus, n: nucleolus, Pg: Protein granules).

Figure 19. Ovary sections of 0.976 mg/L diazinon treated fish. Adhesions in oocytes (AD) and cytoplasmic necrosis (CN). (HE, x400, Scale bar 10 µm).

(Hong *et al*., 2007), increased E2 in males could be a result of an increase in aromatase activity. In past, numerous studies indicated that exposure to OP pesticides induces or enhances the activity of P 450 enzymes (Munkittrick *et al*., 1994; Bucheli & Fent, 1995, Husoy, Myers, & Goksoyr, 1996; Dong *et al*., 2013).

It is known that in teleosts, such as carp, circulating E2 levels direct the synthesis of VTG (Sole, Raldua, & Piferrer, 2003a). In this study, the VTG levels of control group males showed no variations. After 7 days, high concentration of diazinon induced VTG levels significantly. However, no alterations were observed at low concentration. This is probably caused by inadequacy of circulating E2 levels to attain the critical threshold for inducing VTG synthesis in males (Sole *et al*., 2003a). The increase in plasma VTG concentrations was paralleled with E2 fluctuations in males, after 15 days. However, a decrease in plasma VTG concentrations were observed after 30 days. It is known that liver of both male and female fish have reseptors for estrogens and they are capable of producing vitellogenin (Kime, 1998). Generally a decline in vitellogenin concentrations parallels with a decline in E2 levels. But histological disruptions on estrogen receptors in liver can able to cause results like this. It is known that some OPs such as disulfoton can adversely effect estradiol receptors of hepatocytes (Arnold, Pluta & Braunbeck, 1996) and may cause disruption on vitellogenin synthesis (Kime, 1998). Our histological examinations showed more pathologies in the livers of 30 days-treated male fish. Long-time treatment with diazinon could possibly disturb ultra-structure of hepatocytes and could cause a decline in plasma VTG. Another possible explanation for reduction in VTG levels is elemination of plasmatic VTG in some tissues of fish. Normally, in female fish some part of plasma VTG incorporates into developing oocytes, however, in males a reduction in plasma VTG concentrations must be accomplished by alternate pathways. The most direct route for elimination of VTG from blood of male fish is through the kidney (Folmar, George, & Schreibman, 2001). But, we have no data to prove this notion.

In our study, contrast to males, no significant alterations were seen in E2 levels of females. At the end of experiment VTG concentrations decreased significantly. In past numerous studies have demonstrated that pollutants can differently affect endocrine system of male and female fish of same species (Folmar *et al*., 1996, Sole, Barcelo, & Porte, 2002; Sole *et al*., 2003a; Isibashi *et al*., 2004; Spano *et al*., 2004; Lee, Seo, Kim, Yoon, & Lee, 2006; Dong *et al*., 2013). One of the possible reasons for depleted VTG levels in females can be related with negative feedback of gonadotropin secretion. Sole, Raldua, Piferrer, Barceló, and Porte, (2003b) indicated that estrogen mimics could possibly stimulate a negative feedback of gonadotropin secretion and this results reduction in VTG synthesis without a decline in E2 levels. In the same study, they also stated that vitellogenin and hormone variations in female carps appeared to be determined more by biological factors than by xenobiotic exposure.

Cortisol has been shown to enhance the transcription of silent VTG gene in male *Oreochromis aureus* (Ding, Lim, & Lam, 1994) but suppress

plasma VTG concentrations in female catfish (Folmar *et al*., 1996). It also been demonstrated that OP pesticides can reduce (Cericato *et al*., 2008; Oruç, 2010) or induce (Kime, 1998; Nieves-Puigdoller, Björnsson, & McCormick, 2007) cortisol secretion in different fish species. The possible effects of diazinon on cortisol secretion can be another result for VTG depletions in female carps.

To supplement hormone and vitellogenin biomarkers and to show toxic effects of diazinon, gonads and livers were evaluated for abnormalities. Many studies have confirmed that exposure to diazinon or OPs leads to dilatation of bile ducts and sinusoids (Ogueji, Auta, & Balogun, 2007; Kunjamma, Philip, & Bhanu, 2008), cytoplasmic degeneration of hepatocytes (Deka & Mahanta. 2012), congestions of sinusoids (Langiano & Martinez. 2008; Gawish, Issa, & Ali, 2011; Shiogiri *et al*., 2012), necrosis (Rahman, Hossain, & Mollah, 2002; Guimaraes, Silva de Assis, & Boegera, 2007; Velmurugan, Selvanayagam, Cengiz, & Unlu, 2009) and hemorrhages (Rodrigues & Fanta, 1998; Langiano & Martinez. 2008) in livers of many fish species. Our results showed similarities to previous studies. Additionally, in portal veins that surrounded by pancreatic tissue there is an increased amount of proteinaceous fluid was observed. Ruehl-Fehlert, Bomke, and Dorgerloh, (2005) have also observed these proteinaceous fluids in livers of *Pimephales promelas* and stated that they could be vitellogenin molecule.

In this study, degeneration, congestion and fibrosis in testis and adhesions between oocytes and necrosis of oocytes cytoplasm in ovary were investigated. These findings have also been demonstrated by other researchers after diazinon administration (Dutta & Meijer, 2003; Dutta & Maxwell, 2003, Maxwell & Dutta, 2005).

Conclusion

Diazinon is an organophosphate pesticide, it is widely used in agriculture. This study clearly indicates that sublethal and environmentally reliable concentrations of diazinon may cause endocrine disruption and histopathological alterations in gonads of male and female fish that may adversely affect reproduction, while hepatotoxicity indicates its potential adverse effect on metabolic functions. Additionally the presence of diazinon in fresh water reservoirs, even in small concentration, could cause deleterious effects on fish physiology and may potentially disturb their survivability in the natural environment.

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