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Reproductive health indicators of immature common carp exposed to municipal wastewater of Behbahan, Iran

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Original Article

Abstract

Exogenous estrogens or pollutants with estrogen-like activity can induce vitellogenin (VTG) synthesis in male and juvenile fish, making this protein a useful indicator of chemicals that mimic estrogenic activity. The purpose of this study was to investigate the impact of municipal wastewater on blood biochemical parameters of common carp (Cyprinus carpio). Under experimental conditions, biomarkers such as sex steroid levels, alkali-labile phosphate levels, cholesterol and triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were assessed in immature fish exposed to municipal wastewaters collected from a sewage canal in Behbahan, Khuzestan Province, Iran. No significant changes were found in testosterone levels on day 21; however, estradiol, alkali-labile phosphate, triglycerides, cholesterol, and LDL-cholesterol significantly increased in the fish exposed to municipal wastewater compared with the control group. A significant decrease in HDL-cholesterol levels was observed in the fish exposed to municipal wastewater at the end of the experiment. In conclusion, the results of the present study indicated that sewage effluent of Behbahan may contain endocrine disrupters and exposure to sublethal concentrations of municipal wastewater may cause dysfunction in reproductive health indicators of common carp.

KEYWORDS: Alkali-Labile Phosphate, Carp, Endocrine Disrupting Chemicals, Municipal Wastewater

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Introduction

While 300 cities and towns in Iran are thinking of new ways to use wastewater, most cities leave their wastewater untreated to find its way to ground basins. Moreover, 75 cities use traditional systems of wastewater collection and the raw wastewater is used for irrigation or is directly discharged into channels.[1] The total wastewater generated annually in this country is increasing faster than the development of wastewater treatment plants. In 2004–2005, 4% of the wastewater produced in Iran was from households and other municipal sources, while about 96% of it was generated by industrial and commercial sectors. However, there is no exact figure for the amount of wastewater generated in the agricultural section and only 10 to 30% of it is treated in wastewater treatment plants. In fact, a significant portion of municipal and industrial sewage is discharged into underground and surface water or used in farmlands without any treatment.[1]

The millions of cubic meters of untreated sewage discharged into surface water reservoirs can have a significant effect on the

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health of aquatic organisms.[2-4] Literature reviews show that sewage effluents contain a complex mixture of substances including endocrine disrupting chemicals (EDCs) and pharmaceuticals.[5] These chemicals are poorly removed in the sewage system,[5,6] thus increasing their presence in the aquatic environment. This results in substantial effects of EDCs on aquatic organisms; effects which are amplified by almost continual exposure to manufactured compounds designed to persist in the environment.

Changes in the levels of sex hormones and gonad size, increased or decreased vitellogenin (VTG)[7,8] and delayed sexual maturation, decreased fecundity, testicular and ovarian damage[9], and steroidogenesis disruption[10], alterations in reproductive and parental behavior[11,12], impaired olfactory function and reproductive migration disorder[13,14], and courtship behavior deficits of male and female fish, and delay time of spawning[10,15] are the most important physiological changes observed in fish exposed to municipal wastewater.

Many rivers have been contaminated with effluents in recent decades; therefore, their fish populations have experienced a degree of chemical exposure throughout their lifetime. Lack of urban wastewater treatment plants and a considerable volume of surface runoff are among the most important environmental issues in Khuzestan Province, Iran. These issues have adverse effects on the health of the area's wildlife and residents.

The hypothesis for this study is that a high percentage of EDCs and pharmaceuticals are found in sewage canals in Behbahan, Khuzestan Province. It is therefore hypothesized that fish directly exposed to sewage effluents will exhibit signs of changes in blood biochemical parameters. Therefore, the main purpose of this study was to investigate estrogenic and androgenic effects of EDCs in municipal wastewater on blood biochemical parameters of common carp (Cyprinus carpio). Common carp were chosen as the studied species because they are more resistant to temperature fluctuations and laboratory conditions compared to other fish species, and have been shown to respond to sewage effluent exposure in a way similar to fish species native[16,17] to Maroon River (Behbahan).

Materials and Methods

In the present study, 144 immature common carp (mean weight: 42.75 ± 5.45 g) were obtained from a private farm (Behbahan, Iran). The fish were maintained in 80-l tanks filled with 70 l of aerated water at the animal holding facilities of the Department of Fisheries and Aquaculture, Behbahan Khatam Alanbia University, Iran. Water quality was monitored daily for deionized ammonia (< 0.05 mg/l), dissolved oxygen (6.5 ± 0.5 mg/l), temperature (24 ± 2 °C), and pH (7.4 ± 0.2). The experiment was performed after a 2-week acclimation period. During the experiment, the fish were fed a formulated diet obtained from Beyza Feed Mill (Shiraz, Iran).

Sewage samples were collected from 6 different stations of the sewage canal in Behbahan on 26 April 2015. During the 7 days prior to sampling, there was no rain in the sampling area and the minimum and maximum air temperature was 17–32 °C. Duplicates of each sample were collected in brown glass bottles with Teflon stoppers. In order to disinfect and remove pathogens from wastewater, it was filtered and autoclaved at 121 °C for 15 minutes before use.

The fish were placed into anesthetic solution (200 mg/l clove powder) for 3-5 minutes before being weighed individually. The fish were randomly assigned to 4 groups and were placed in 12 80-l plastic tanks which were filled with 70 l of water. Group I fish were maintained in tap water as the control group. Group II was considered as a positive control in this experiment. Anesthetized common carp were intramuscularly injected with an estradiol valerate (50 mg/Kg body weight/week) in 2 stages with an interval of 1 week.[18,19] Injections were carried out using sterile 1 ml syringes and 26-gauge needles. Group III and IV fish were maintained for 21 days[19] in water, respectively, polluted with 0.1 and 0.2 ml per liter of municipal wastewater collected from a sewage canal in Behbahan (equivalent of 7 ml and 14 ml per 70 l). Tanks were cleaned via siphoning and 40% of the water was changed daily to reduce the production of metabolic wastes and municipal wastewater was added to maintain municipal wastewater concentration constant (equivalent of 0.1 and 0.2 ml per l).

At the end of the experiment, each group of fish was harvested using a scoop net and immersed in anesthetic solution (200 mg/l clove powder). Using heparinized syringes, blood samples were collected from the caudal vein, centrifuged immediately at 6000 × g for 10 minutes, and stored at -25 °C.

Plasma samples were extracted twice with diethyl ether, and concentrations of testosterone and 17ß-estradiol were measured using competitive enzyme-linked immunosorbent assay (ELISA) as described by Hecker et al.[20]

Levels of VTG-like proteins in the plasma were immediately determined using an alkali-labile phosphate method and the quantity of alkali-labile phosphate in the plasma was obtained in a way similar to that used by Gange and Blaise.[21] Briefly, 500 µl of plasma was mixed with 500 µl of t-butyl methyl ether and incubated at room temperature for 30 minutes. The emulsion was centrifuged at 10000 × g for 10 minutes at 4 °C. The supernatant was mixed with 100 µl of 2 M NaOH for 60-90 minutes at 37 °C. Levels of free phosphates were determined according to the phosphomolybdenum method, and the optical absorbance was read at 660 nm.

The plasma biochemical parameters were assayed by enzymatic procedures using a UV/VIS spectrophotometer (UNICO 2100, USA). Plasma biochemical parameters including cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and triglycerides were tested using assay kits obtained from Pars Azmoon Co., Iran.

Difference in the biochemical characteristics of fish exposed to different concentrations of municipal wastewater was examined using one-way ANOVA. Data were examined for normality (Kolmogorov-Smirnov test). Significant means were compared using Duncan's test and P-values of less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS software (version 19, IBM Corp., Chicago, IL, USA). Data are presented as mean ± SD.

Results and Discussion

In this study, the effects of exposure to municipal effluent on the health and physiological response of fish were examined. Toxicants and xenobiotics which are found in sewage effluents have multiple effects on the health and biodiversity of aquatic organisms.[2-4] Many xenobiotics show endocrine disrupting properties and are mainly the result of human activities. Three main categories of estrogenic EDCs including steroidal estrogens, phenolic compounds, and phthalate esters are found in sewage effluents in low concentrations.[22-24] The main source of these chemicals in domestic sewage is human waste.[25] No mortality was observed during the experiment.

Figure 1 shows the effects of intramuscular administration of estradiol valerate, and exposure to municipal wastewater on estradiol in immature fish. Plasma levels of 17β-estradiol (E2) significantly increased in experimental fish when compared to the control group ($P < 0.05$). However, E2 levels in the fish treated with estradiol valerate was significantly higher than those in fish exposed to municipal wastewater (Figure 1). The present study indicates that municipal wastewater shows some estrogenic activity, as suggested by the increased E2 levels in exposed immature common carp. E2 seems to be affected by exposure to municipal sewage effluent, as shown by E2 increase both in fish exposed to 0.1 and 0.2 ml of municipal wastewater. Therefore, municipal wastewater may have a specific effect on estradiol levels in the plasma of immature fish. Alterations in sex hormone were reported in crucian carp, Carassius carassius, exposed to treated sewage effluent.[26]

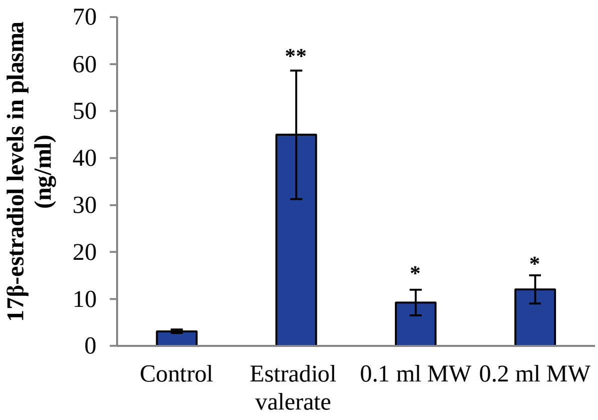

Figure 1. Plasma 17β-estradiol levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at P < 0.05

Xenobiotics with estrogenic properties can bind to nuclear estrogen receptors in fish. This in turn may affect testicular androgen production.[27] Nevertheless, in the present study, no statistical differences were detected in testosterone levels (Figure 2). Loomis and Thomas found that xenoestrogens have a negative effect on androgen production in fish.[27] Decreased plasma levels of 11-ketotestosterone, and VTG induction were observed in wild male chub living in water contaminated with sewage effluent.[28]

Figure 3 shows that the alkali-labile phosphate levels in the plasma of fish treated with estradiol valerate were much higher than those in other groups. Our results show that the alkali-labile phosphorus levels increased in fish exposed to sewage effluents, which may suggest induction of VTG synthesis caused by endocrine disruptor compounds present in sewage effluents (Figure 3). VTG is a blood protein normally synthesized by females during oocyte maturation, but VTG in male fish living downstream of wastewater outfalls can serve as a biomarker of exposure to environmental estrogens.[29]

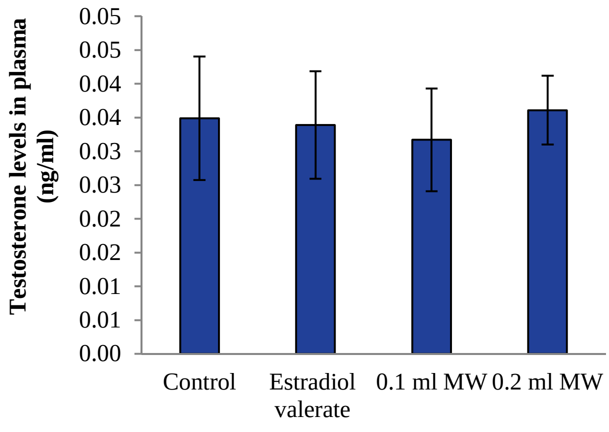

Figure 2. Plasma testosterone levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at P < 0.05

Males and juveniles are also capable of VTG gene expression, but typically do not have sufficient circulating estrogens to stimulate a significant production of the protein.[29] Common carp VTG is a lipophosphoprotein (79% protein, 19% lipid, 0.6-0.8% alkali-labile phosphorus) that contains carbohydrate and binds calcium (0.3%).[30] The 19% lipid contains 13% phospholipids, 4% triglycerides, and 2% cholesterol. The presence of alkali-labile protein phosphorus in fish plasma is specifically associated with the lipophosphoprotein VTG. Increases in VTG induction in adult male fathead minnows, Pimephales promelas,[31] and mirror carp, Cyprinus carpio,[32] exposed to treated municipal sewage effluent confirms the results of the present study. Elevated serum VTG levels were reported in C. carassius exposed to treated sewage effluent in laboratory conditions.[26]

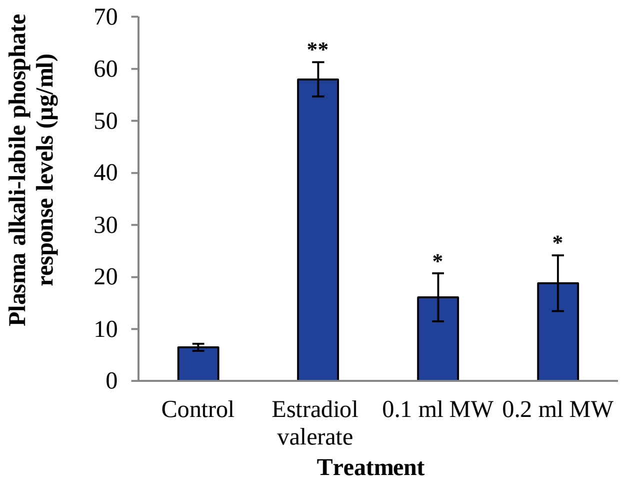

Figure 3. Plasma alkali-labile phosphate levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at P < 0.05

Cholesterol serves as the substrate for all steroid hormones.[33] Results show that exposure to municipal wastewater significantly ($P < 0.05$) increased plasma cholesterol levels in fish (Figure 4). This effect appears to be due to an increase in the detoxification rate of environmental estrogens in liver tissue. Increase in stress hormones such as cortisol in blood of fish exposed to municipal wastewater, which stimulates lipid breakdown in adipose tissue, has been reported by Ikonomou et al.[34] and Quinn et al.[35] Moreover, destruction of cell membranes can also lead to increased levels of cholesterol in plasma. Significant changes in cholesterol, HDL-C, and LDL-C levels in plasma of fish exposed to treated sewage effluents were reported by Samuelsson et al.[36]

Triglyceride levels significantly ($P < 0.05$) increased in both fish treated with estradiol valerate and municipal wastewater compared with the control group (Figure 5). The higher triglyceride levels may be associated with both a reduction in the uptake of triglycerides in adipose tissue and liver dysfunction.

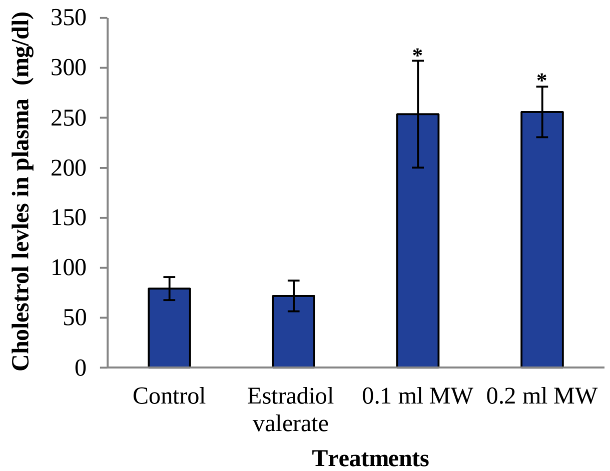

Figure 4. Plasma cholesterol levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at P < 0.05

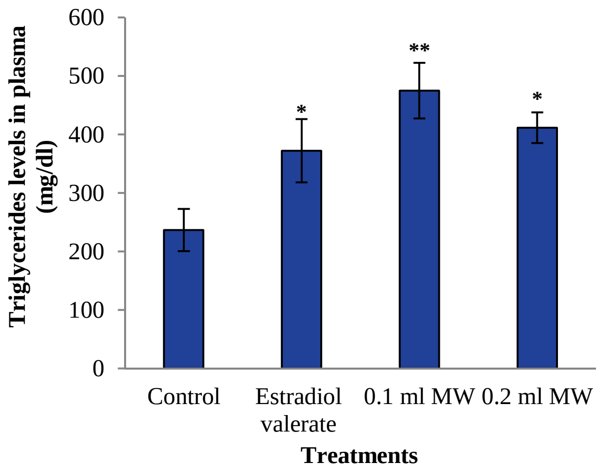

Figure 5. Plasma triglyceride levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at P < 0.05

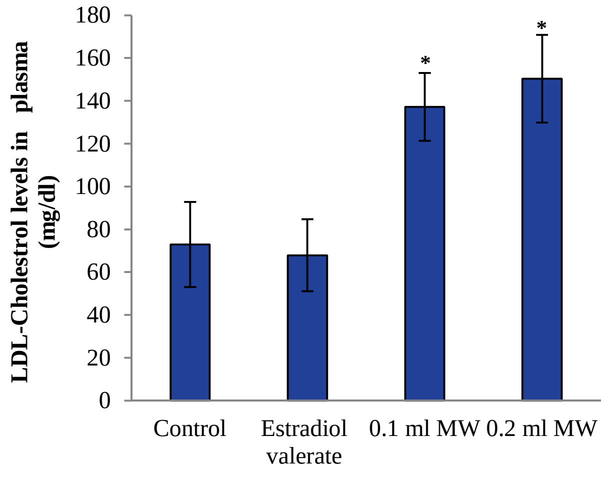

Figure 6. Plasma low-density lipoprotein cholesterol levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at $P < 0.05$

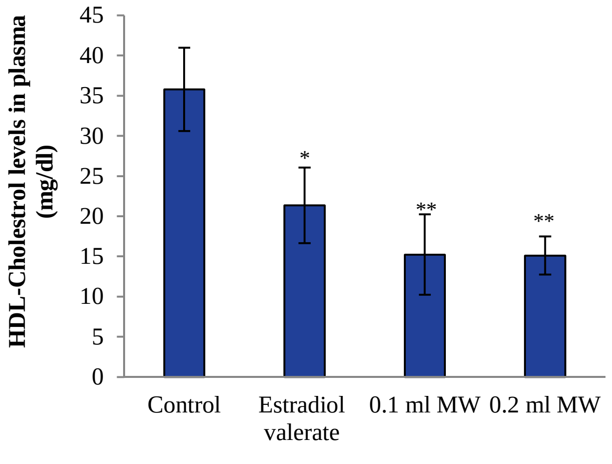

Figure 7. Plasma high-density lipoprotein cholesterol levels in immature common carp treated with estradiol valerate and different concentrations of municipal wastewater for 21 days (mean ± SD) (n = 9)
Asterisk (*) indicates that the difference between the experimental and control groups was significant at $P < 0.05$

Although a significant increase was observed in plasma LDL-C levels of fish exposed to municipal wastewater (Figure 6), no significant difference was observed in LDL-C levels between fish treated with estradiol valerate and the control group. The significant increase in plasma LDL-C levels in fish exposed to municipal sewage effluent may be correlated with the role of LDL in delivering cholesterol to cells for the synthesis of steroid and corticosteroid hormones in the adrenal glands.

Plasma HDL-C levels were significantly ($P < 0.05$) lower in immature common carp exposed to municipal wastewater than in the control group (Figure 7). Available evidence indicates that estrogens can have significant effects on the rate of syntheses and secretion of HDL in the liver and intestines.[37] The decreased HDL in the fish exposed to sewage effluent might affect the excretion of excess cholesterol from the body via the liver, which secretes cholesterol in bile or converts it to bile salts. Studies have shown that administration of estrogen can decrease HDL-C.[37]

Conclusion

This study shows that municipal wastewater effluents collected from a sewage canal in Behbahan possess estrogenic activity. This is clearly shown by increased alkali-labile phosphate and estradiol levels, which provide evidence of the presence chemical compounds capable of affecting the endocrine system of fish in municipal sewage effluents in Behbahan.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgements

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