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Effects of sodium chloride and methylthioninium chloride on Persian sturgeon, *Acipenser persicus* (Borodin, 1897): A histopathological and bacteriological study

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ABSTRACT

360 pieces of Persian sturgeon fingerlings (1.94 ± 0.75 g) were exposed to various sublethal doses of disinfecting chemicals commonly used in aquaculture such as sodium chloride and methylthioninium chloride in order to investigate their impacts on bacterial loads of skin, gill and surrounding water and to define the histopathological status of gill and liver tissues. The sublethal concentrations were determined after a pre-test, then the experiment was performed in four treatments with three replicates inside the glass aquariums with stocking density of 10 fish (1-3 g) per aquarium. The treatments included 5, 6.3, 8.06 and 10.23 mg L^{-1} sodium chloride and also 1, 1.56, 2.45 and 3.83 mg L^{-1} methylthioninium chloride. A control group (with no chemical added) was set up for each experiment. After exposure to treatments during 96 h, the microbial and histopathological examinations were carried out. Hemorrhage, elongation of secondary lamellae, adhesion of secondary lamellae, hypertrophy of supporter cartilage, mucus coagulation and secretion, hyperplasia, lamellar necrosis and clubbing of gill lamellae were observed on the fish gills. Biliary depression, hemorrhage, cell necrosis and degeneration of lipid were also found in liver. The severity of these effects was dose-dependent. Only the sublethal concentration of methylthioninium chloride significantly influenced bacterial load (CFU g^{-1}) on skin, while other treatments of sodium chloride and methylthioninium chloride did not exhibit any significant effects. In conclusion, the sublethal doses of methylthioninium chloride and sodium chloride showed no obvious disinfecting effect on gill, skin and surrounding water of Persian sturgeon fingerling. Nevertheless, histopathological alterations were observed on fish gill, skin and also liver of all treatments.

Keywords: Persian sturgeon, Bacterial, Sodium chloride, Methylthioninium chloride, Histopathology.

INTRODUCTION

The rapid development of intensive aquaculture over the last few decades involves the use of various disinfecting chemicals and drugs against numerous infectious diseases. Aquaculture needs the availability of disinfecting products to avoid severe economic losses, and disinfection will remain one of the main means of controlling diseases in the future (Lafont 1992). At present, various disinfecting chemicals such as malachite green, formalin, copper sulfate, potassium permanganate, chloramines, chlorine dioxide and quaternary ammonium compounds are commonly used for the control of bacterial and fungal pollution in cultured fish and surrounding water (Torgersen & Hastein 1995). Irrespective of their disinfecting use, these chemicals are subsequently released in the aquatic environment and affect aquatic ecosystems (Boyd & Massut 1999; Nash 2003; Erondu & Anyanwu 2005).

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Therefore, to limit the undesired impacts of disinfecting chemicals on aquatic ecosystems, the protocols of the use of these chemicals should be optimized. In fact, a disinfecting chemical in addition to its disinfecting role on fish and water, should be safe for fish and natural environment. The Persian sturgeon, *Acipenser persicus* is a critically endangered anadromous species that has been considered for restocking programme in the southern part of the Caspian Sea (Kiabi *et al.* 1999). Due to the use of artificial propagation for the rebuilding of reserves, today it is used for breeding in freshwater. So that, a large number of fingerlings are produced annually via artificial reproduction and then released to the environment. At present, some disinfecting chemicals such as malachite green, formalin, copper sulfate and potassium permanganate, sodium chloride and methylthioninium chloride are used routinely for disinfection of fish and water in Iranian sturgeon hatcheries. Methylene blue (methylthionine chloride) is a heterocyclic aromatic chemical compound with molecular formula ($C16H18ClN3S, 3H_2O$) with the chemical name [3, 7-bis (Dimethylamino)-phenazathionium chloride tetramethylthionine chloride] (Faber *et al.* 2005; Wiklund *et al*. 2007). Methylene blue (MB) is a cationic thiazine dye that is deep blue in the oxidized state while it is colorless in its reduced form (leucomethylene blue). Chambel *et al.* (2014) evaluated the effect of hydrogen peroxide, iodine solution (PVP) and methylene blue on eggs disinfection of three ornamental fish species, *Danio rerio*, *Pterophyllum scalare* and *Gymnocorymbus ternetzi*. Bolivar *et al.* (2001) evaluated the effect of two chemicals (sodium chloride and methyleneblue) on bacterial load in the transport water containing *Oreochromis niloticus* fingerlings under farm practice with those reported by the literature.

Nevertheless, there is very little information on the toxicity and histopathological impacts of these chemicals on sturgeons. The general objective of this study was to determine the effect of methylthioninium chloride and sodium chloride 1) on the bacterial load of skin, gill and surrounding water and 2) on the histopathological status of gill and liver tissues of the cultured Persian sturgeon fingerlings.

MATERIALS AND METHODS

The Persian sturgeon fingerlings (1-3 g) were obtained from the International Sturgeon Research Institute, Rasht, Iran. Before the experiment, a pre-test was performed to determine the sublethal dose range of sodium chloride and methylthioninium chloride to be tested. So that, LC_{10}, LC_{50} and LC_{90} were determined after 24, 48, 72 and 96 h exposure of fingerlings to various concentrations of sodium chloride and methylthioninium chloride (Tables 1 and 2) finding 5-10.2 mg L^{-1} and 1-3.8 mg L^{-1} as their sublethal range respectively. The experiment were conducted in 30 glass aquariums (15 aquariums per each disinfectant and 10 fish per each aquarium) containing 20 L of dechlorinated and gentle aerated water. Water temperature of each aquarium was maintained at 20 °C by an aquarium heater during experiment. The water exchange was also performed entirely every 24 h. Four treatments and one control group with three replicates were employed for each disinfectant including 6.3, 8 and 10.2 mg L^{-1} for sodium chloride and 1, 1.5, 2.4 and 3.8 mg L^{-1} for methylthioninium chloride. During experiment, fish fed with special commercial feed for sturgeon i.e. Ex-AS (diet form: Granular and 1-1.5 mm in diameter; manufacturing Company: Beysa, Iran) three times a day (Table 3). After 96 h exposure, the histopathological status of gill and liver and also the bacterial load on gill, skin and surrounding water were examined.

Table 1. Lethal concentrations of sodium chloride during four days on Persian sturgeon (ppt)

LC	24 h	48 h	72 h	96 h
LC_{10}	16.13	6.61	6.03	5.77
LC_{50}	22.29	10.94	7.93	7.67
LC_{90}	30.81	21.15	10.38	10.15

Table 2. Lethal concentrations (per ppt) of methylthioninium chloride during four days on Persian sturgeon

LC	24 h	48 h	72 h	96 h
LC_{10}	1.25	1.12	0.86	0.89
LC_{50}	5.60	4.48	2.23	1.69
LC_{90}	25.13	17.98	6.22	3.19

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Table 3. Composition of the experimental diet (dry weight).

Ingredients	g kg^{-1} dry weight
Fish meal	620
Meat powder	60
Wheat flour	100
Soybean cake	50
Fish oil	40
Soybean oil	40
Lecithin	30
Vitamin mixture (Vitamin E free)[a]	15
Vitamin mix (riboflavin-free)b	25
Mineral mixture [c]	10
Salt	2
Proximate composition	%
Crude protein	49.18
Crude lipid	14.12
Moisture	14.25
Ash	20.70
Crude Energy (kcal kg^{-1})	3012

a: Vitamin mixture was manually provided according to feed requirements of the fish and ingredients were obtained from Science Laboratories (Ghazvin, Iran); which each 1000 g vitamin mixture provides vitamin A, 1,600,000 I.U; vitamin D3, 400 000 I.U; thiamin, 6 g; riboflavin, 8 g; niacin, 12 g; pantothenic acid, 40 g; pyridoxine, 4 g; folic acid, 2 g; cyanocobalamin, 8 mg; vitamin C, 60 g; vitamin K3, 2 g; biotin, 240 mg and inositol, 20 g.

b: Riboflavin-free vitamin mix (mg/kg diet): Thiamin hydrochloride, 10; pyridoxine hydrochloride, 10; calcium pantothenate, 40; niacin, 50; folk acid, 5; biotin, 2; vitamin BIZ, 0.02; vitamin A, 4,000 IU; vitamin D3, 2,000 IU; vitamin E, 50; menadione-Na-bisulfite, 10; inositol, 400; ascorbic acid, 300; choline chloride, 3.000.

c: Aquatic mineral premix, for cold and warm water fish, is manufactured by Science Laboratories (Ghazvin, Iran); which each 1000 g contains mineral trace elements such as ferrous, 6000 mg; zinc, 10 000 mg; selenium, 20 mg; cobalt, 100 mg; copper, 600 mg; magnesium, 5000 mg; iodine, 600 mg. In addition, choline chloride (6000 mg), which is vital for fish and can not be combined with other vitamins, is also included in mineral premix.

Sample preparation for bacterial examinations

After 96 h exposure, three fish from each experimental aquarium were used for bacterial examinations of the gill and skin tissues. Three small pieces (each pieces = 1 cm^2) of skin and gill tissues were collected. Each sample was sepeately analysed by ensuring homogeneity of the samples using a sterile pipette. The 1 mL of each sample was suspended into 9 mL sterile water ascepticallyaseptically in a macCartney bottle which was then shaken together. After shaking and mixing in glass tube for 5 min, the tissue samples were separated and was used for microbial culture. For this purpose, 1 ml of each solution was added separately into a sterile Petri dish containing Tryptic Soy Agar (TSA) and then incubated at 25 ^oC for 24-48 h. To measure the bacterial load, the water samples were plated into TSA in three replicates and incubated for 24-48 h at 25 ^oC. After incubation, the bacterial load (CFU g^{-1}) in plates and Petri dishes were assessed with counting of formed bacterial total (Ampofo & Clerk 2010).

Gill and liver histopathology

Fixation was done in Bouin's fixative solution and then tissue processing (it contains three stages of dehydration, clearing and paraffin embedding) was performed: dehydration was done in ethanol 50, 70, 80 and 96 percent and 1-butanol, clearing by chloroform and paraffin embedded was conducted in a mixture of chloroform and purified paraffin at 56 ° C, then molding, assembling sample template on wooden base, sectioned at 7 µm, mounted on slides coated with gelatin tissue (tissue to bind on the slide), stained with hematoxylin and eosin (H&E), and after that slides were studied by light microscopy (model Nikon E600, Japan) connected to the computer and using Biocom program to study and photograph the tissues (Hallajian 2009; Sharifpour *et al.* 2013).

Statistical analysis

The SPSS software was used for data analysis. All data had normal distribution according to Kolmogorov Smirnov test. One-way analysis of variance (ANOVA) was employed to compare the means. When significant F-ratios were calculated by ANOVA, the Tukey test was applied to identify which means were different. The significant level was considered as $p < 0.05$

RESULTS

Preliminary tests were performed to determine the tolerable concentration of methylene blue and sodium chloride for fingerlings of Persian sturgeon, with a mean weight of 1.74 ± 045 g. Then 6 treatments (with 3 replicates

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apiece) were established to determine the 96-h LC_{50} of these two substances to Persian sturgeon. The results showed that the 96-h LC_{50} of methylene blue and sodium chloride to juveniles of Persian sturgeon was 1.69 mg L^{-1} and 7.67 mg L^{-1} , respectively. There was no alive subject in treatments 6 mg L^{-1} of methylene blue and 13 mg L^{-1} of sodium chloride after 96 h to be sampled for the bacterial count of the skin, gills, and water. Total bacterial count in exposure to sodium chloride after 96 hours was lower in the control group (5.09 ± 0.10 log CFU mL^{-1}) than other treatments. Moreover, the lowest number of bacteria in water in exposure to methylene blue was observed in the 3.83 mg L^{-1} treatment (4.43 ± 0.51 log CFU mL^{-1}). Total skin bacterial count was significantly lower in treatments 6.3 mg L^{-1} of sodium chloride (5.46 ± 0.20 log CFU mL^{-1}) and the 1.56 mg L^{-1} of methylene blue (2.77 ± 0.26 log CFU mL^{-1}) than other treatments.The results also indicated that total bacterial count in the skin, gills, and water was lower in exposure to 8.06 mg L^{-1} of sodium chloride (3.46 ± 0.32 log CFU mL^{-1}) and 1 mg L^{-1} of methylene blue (3.52 ± 0.48 log CFU mL^{-1}) compared to other treatments. However, there was a significant difference only between the subjects exposed to 1.56 mg L^{-1} of methylene blue and those in other treatments in terms of changes in their skin. No significant change was observed in the skin, gills, and water in any of the sodium chloride treatments (Figs. 1 and 2). The results also demonstrated that the increased concentration of the two studied substances led to behavioral changes such as imbalance, abnormal movements, and mucus secretion on the body. The amount of mucus secreted on the body of subjects exposed to methylene blue was higher than that of those exposed to sodium chloride.

After 96 h exposure of Persian sturgeon fingerlings to various doses of sodium chloride and methylthioninium chloride, a range of histopathological alterations were observed in gill and liver tissues as follows:

Gill: haemorrhage, elongation of secondary lamellae, adhesion of secondary lamellae, hypertrophy of supporter cartilage, mucus coagulation and secretion, hyperplasia, lamellar necrosis and clubbing of gill lamellae (Figs. 1 and 2).

liver: biliary depression, haemorrhage, cell necrosis and lipid degeneration (Figs. 4 and 5). The severity of these alternations increased with increasing of the doses of sodium chloride and methylthioninium chloride so that the highest histopathological alternations were observed in doses of 10.23 and 3.83 mg L^{-1} for sodium chloride and methylthioninium chloride, respectively. According to bacterial study, only the sublethal doses of methylthioninium chloride had significant effects on bacterial load (CFU g^{-1}) of skin ($P<0.05$) while other treatments with sodium chloride ($P>0.05$) and methylthioninium chloride ($P>0.05$) did not show significant impacts on gill, skin and surrounding water.

Table 4. Bacterial total (CFU g^{-1}) in gill, skin and surrounding water of Persian sturgeon fingerlings after exposure to various concentrations of sodium chloride (mg L^{-1}). Mean ± SD letters indicate significantly different ($p<0.05$).

Treatments	Gill	Skin	Water
Control	3.6 ± 0.60 [a]	4.1 ± 0.95 [a]	5.09 ± 0.10 [a]
5	4.03 ± 0.5 [a]	4.2 ±1.76 [a]	5.3 ± 0.00 [a]
6.3	4.2 ±1.17 [a]	3.43 ± 0.80 [a]	5.46 ± 0.20 [a]
8.06	3.46 ± 0.23 [a]	3.96 ± 0.05 [a]	5.16 ± 0.66 [a]
10.23	4.35 ±1.06 [a]	3.85 ± 0.21[a]	5.45 ± 0.21[a]

Table 5. Bacterial load (CFU g^{-1}) in gill, skin and surrounding water of Persian sturgeon fingerlings after exposure to various concentrations of methylthioninium chloride (mg L^{1}). Mean ± SD letters indicate significantly different ($p<0.05$).

Treatments	Gill	Skin	Water
Control	3.93 ± 0.79 [a]	2.67 ± 0.58 [ab]	5.05 ± 0.05 [a]
1	3.52 ± 0.48 [a]	3.66 ± 0.57 [a]	5.36 ± 0.58 [a]
1.56	3.96 ± 0.35 [a]	2.77 ± 0.26 [b]	4.9 ± 0.1[a]
2.45	3.96 ± 0.65 [a]	3.37 ± 0.96 [ab]	4.78 ± 0.61[a]
3.83	4.3 ± 0.15 [a]	5.22 ± 0.53 [c]	4.43 ± 0.51[a]

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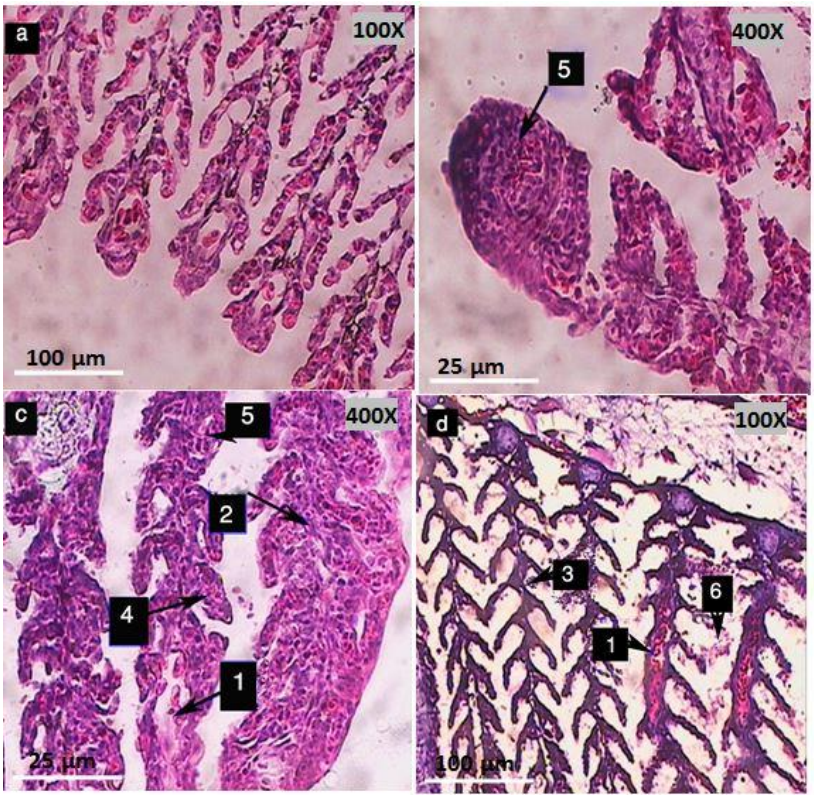

Fig. 1. Cross section of gill of Persian sturgeon fingerlings (*Acipenser persicus*) exposed to various concentrations of sodium chloride (a: 5 mg L^{-1}; b: 6.3 mg L^{-1}, c: 8.06 mg L^{-1}; d: 10.23 mg L^{-1}). (1: haemorrhage, 2: hyperplasia, 3: sloughing of primary lamellae, 4: hypertrophy of supporting cartilage, 5: clubbing of gill lamellae; 6: mucus secretion).

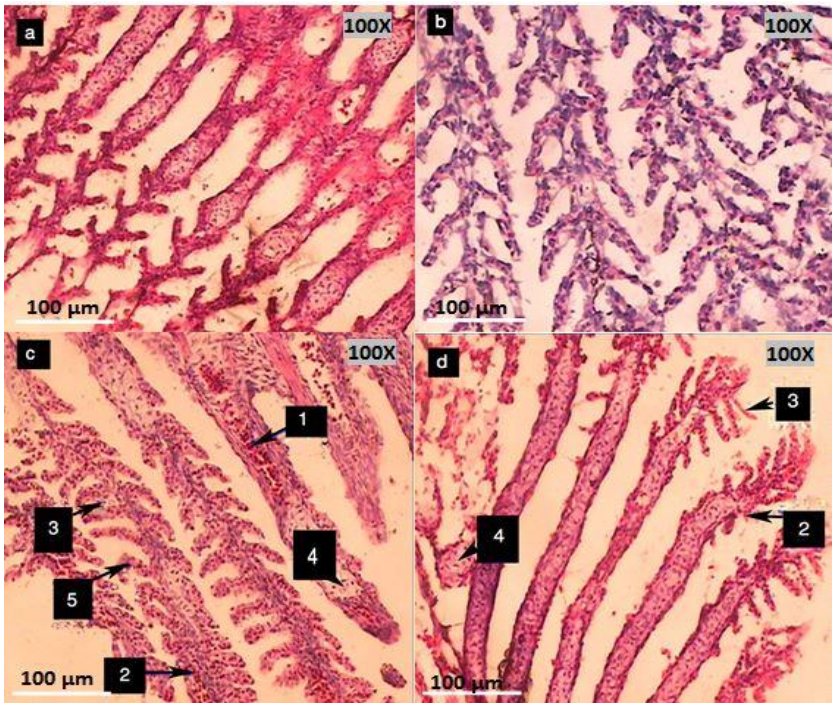

Fig. 2. Cross section of gill of Persian sturgeon fingerlings (*Acipenser persicus*) exposed to various concentrations of methylthioninium chloride (a: 1 mg L^{-1}; b: 1.56 mg L^{-1}, c: 2.45 mg L^{-1}; d: 3.83 mg L^{-1})
(1: haemorrhage, 2: hyperplasia, 3: narrowing of primary lamellae, 4: hypertrophy of supporting cartilage, 5: clubbing of gill lamellae).

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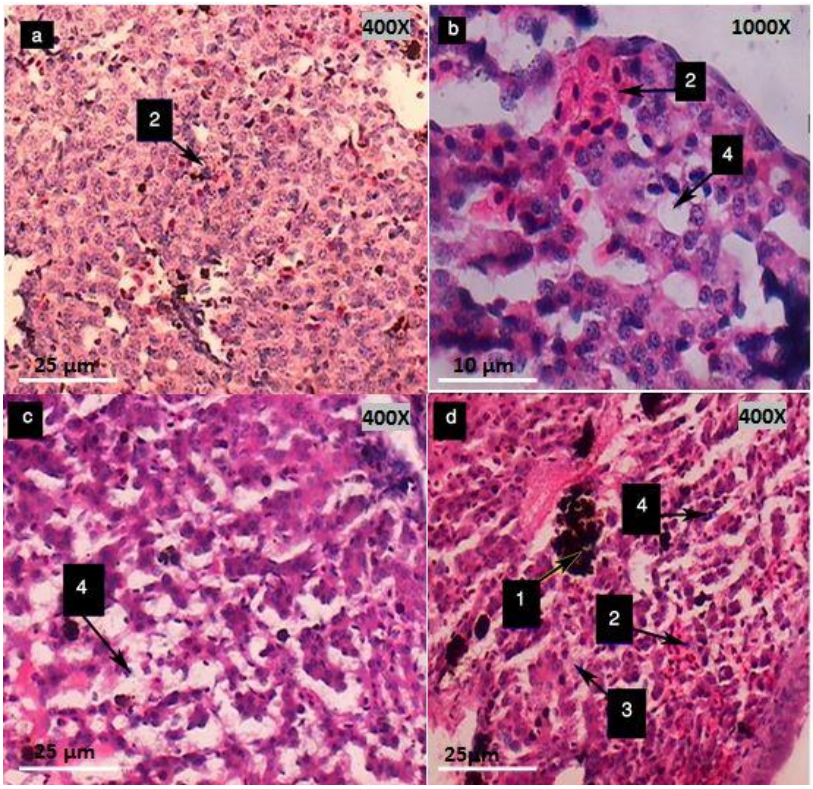

Fig. 3. Cross section of liver of Persian sturgeon fingerlings (*Acipenser persicus*) exposed to various concentrations of sodium chloride (a: 5 mg L^{-1}; b: 6.3 mg L^{-1}, c: 8.06 mg L^{-1}; d: 10.23 mg L^{-1}). (1: biliary deposition, 2: haemorrhage, 3: cell necrosis, 4: hydropic degeneration).

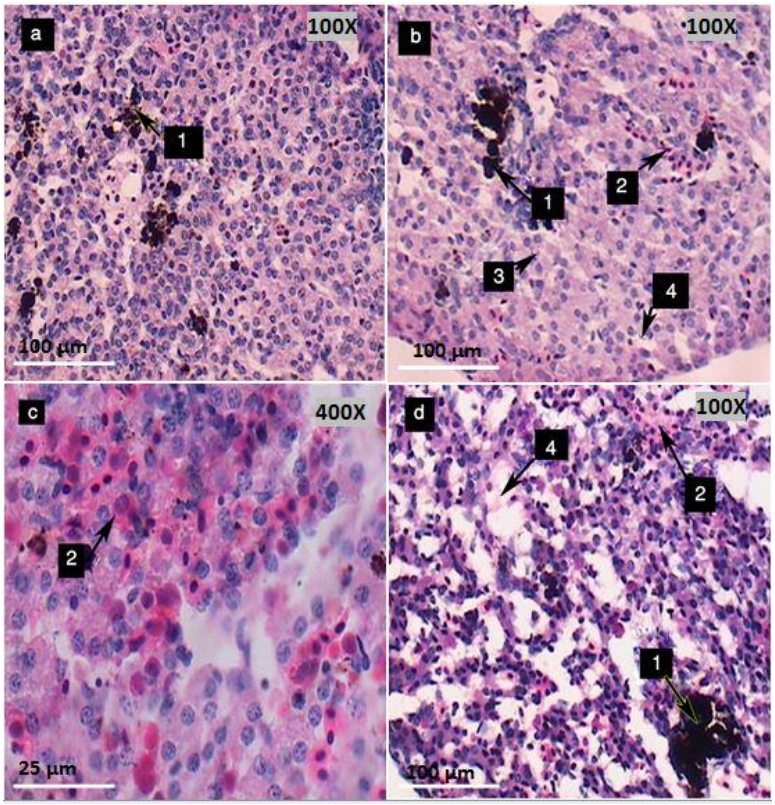

Fig. 4. Cross section of liver of Persian sturgeon fingerlings (*Acipenser persicus*) exposed to various concentrations of methylthioninium chloride (a: 1 mg L^{-1}; b: 1.56 mg L^{-1}, c: 2.45 mg L^{-1}; d: 3.83 mg L^{-1}). (1: biliary deposition, 2: haemorrhage, 3: cell necrosis, 4: hydropic degeneration).

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DISCUSSION

To protect against pathogen agents, disinfecting product such as methylthioninium chloride and sodium chloride are used in aquaculture industry of Iran for several years. Nevertheless, governmental control on allowed using concentrations is limit and almost absent. In the present study, the histopathological effects of two disinfecting product i.e. methylthioninium chloride and sodium chloride, the most commonly used disinfecting products in aquaculture (Swann & Fitzgerald 1993), were investigated in order to determine their safe concentrations for Persian sturgeon fingerlings.

Salt is inexpensive, readily available, and when properly administered is safe for use in aquaculture (Van Duijn 1973; Schaperclaus 1992; Anderson 1992; Plumb 1992; Baticados & Paclibare 1992; Swann & Fitzgerald 1993). The disinfecting action of methylthioninium chloride on bacteria and other parasites is probably due to its binding effect with cytoplasmic structures within the cell and also its interference with oxidation-reduction processes. In the present study, elevating sodium chloride concentration from 5 mg L^{-1} to 10.2 mg L^{-1} did not exhibit obvious effect on the bacterial load of gill, skin and surrounding water of fish compared to control. It seems that the bacterial flora develop efficient resistance to sublethal doses of sodium chloride. Disinfecting effects of sodium chloride is likely appeared on concentrations higher than 10.2 mg L^{-1}. Likewise, the increase of methylthioninium chloride concentration from 1 mg L^{-1} to 3.83 mg L^{-1} did not exhibit significant impact on the bacterial load. Nevertheless, the sublethal concentration of methylthioninium chloride decreased the bacterial load of water, which may be due to their different chemical structure and influencing potential of methylthioninium chloride. In the present study, although disinfecting agents had no considerable effects on bacterial flora of Persian sturgeon fingerling and water, but a wide range of histopathological were observed in gill and liver tissues. The severity of these lesions had a dose-dependent pattern once exposure to sodium chloride and methylthioninium chloride. Similar histopathological alterations were reported for various fish species exposed to disinfecting chemicals (Peyghan *et al.* 2007). These alterations may play a defensive role against disinfecting chemicals. However, these alterations can induce adverse effects on fish health, and may increase their susceptibility to secondary infectious diseases and even death (Hawkins *et al.* 1984).

In conclusion, in the present study, we observed no disinfecting effects on bacterial flora of Persian sturgeon which may be due to the use of very low concentrations of sodium chloride and methylthioninium chloride. Nevertheless, some low concentrations had adverse impacts on gill and liver tissues.

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اثرات کلرید سدیم و متیلن بلو روی تاسماهی ایرانی (*Acipenser persicus*): یک مطالعه آسیب‌های بافتی و باکتری شناسی

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چکیده

۳۶۰ قطعه بچه ماهی انگشت قد تاسماهی ایرانی (۰/۷۵±۱/۹۴ گرم) در معرض مقادیر تحت حاد مواد ضد عفونی کننده شیمیایی رایج در آبزی‌پروری شامل کلرید سدیم و متیلن بلو منظور بررسی تأثیر آنها بر بارهای باکتریایی پوست، آبشش و آب محیط پرورش و وضعیت اثرات بلفتی آبشش و کبد را قرارگرفتند. غلظت‌های تحت حاد پس از آزمایش اولیه تعیین شدند، سپس این آزمایش در چهار تیمار با سه تکرار در داخل آکواریوم‌های شیشه‌ای با تراکم ذخیره‌سازی ۱۰ ماهی (۱-۳ گرم) در هر آکواریوم انجام شد. تیمارها شامل ۵ ، ۶/۳، ۸/۰۶ و ۱۰/۲۳ میلی‌گرم در لیتر کلرید سدیم و همچنین کلرید ۱ ، ۱/۵۶ ، ۲/۴۵ و ۳/۸۳ میلی‌گرم درلیتر متیلن بلو بودند. یک گروه کنترل (بدون افزودن مواد شیمیایی) درنظر گرفته شد. پس از قرار گرفتن در معرض تیمارها در طی ۹۶ ساعت، بررسی‌های میکروبی و آسیب‌های بافت انجام شد. خونریزی، کشیدگی لاملای ثانویه، چسبندگی لایه‌های ثانویه، هیپرتروفی غضروف حامی ، انعقاد مخاط و ترشح مخاط ، هایپرپلازی ، نکروز لاملار و لخته‌شدن لاملار در آبششهای ماهی مشاهده شد. رکود صفراوی، خونریزی، نکروز سلول و دژنراسیون چربی نیز در کبد مشاهده شد. شدت این اثرات وابسته به دوز بود. تنها مقادیر تحت حاد متیلن بلو اختلاف معنی‌داری روی با باکتریایی (CFU/g) پوست داشت، درحالی که سایر تیمارهای کلرید سدیم و متیلن بلو تفاوت معنی‌داری را نشان نداد. در نتیجه، مقادیر تحت حاد متیلن بلو و کلرید سدیم هیچ اثر ضد عفونی کننده آشکاری بر روی آبشش، پوست و آب محیط پرورش بچه ماهیان انگشت قد تاس ماهی ایرانی نشان ندادند. با این وجود، تغییرات هیستوپاتولوژیکی روی آبشش ماهی، پوست و همچنین کبد در همه تیمارها مشاهده شد.

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