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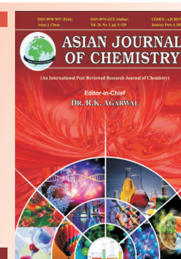

Influence of Fe^{3+} on Motility Parameters and Antioxidant Responses in Chinese Loach Sperm†

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Effects of Fe^{3+} on the motility parameters and oxidative stress of Chinese loach (*Misgurnus anguillicaudatus*) sperm were investigated *in vitro*. Loach sperm were exposed for 1, 2, 4 h to Fe^{3+} at concentrations of 10, 25, 50, 100 and 500 μM. Results revealed that 10 μM of Fe^{3+} had significant influence on motility parameter and antioxidant responses indices in loach sperm, except for sperm viability and SOD activity, which suggest Fe^{3+} have a potential impairment for reproductive of male loach and that a reduction of sperm motility is not necessarily the result of sperm death. Results of *in vitro* experiments suggest that using of sperm *in vitro* assays may provide a novel and efficiently means for evaluating the effects of residual heavy metals in aquatic environment on loach.

Keywords: Spermatozoa, Fe^{3+}, Motility, Viability, Antioxidant enzymes, Lipid peroxidation, Chinese loach.

INTRODUCTION

Pollution of the aquatic environment with heavy metals has become a serious health concern in recent years. Although environmental concentrations of heavy metal are rarely directly dangerous for fish survival, heavy metals are known to accumulate in fish tissues, leaching concentrations of up to thousands of times higher than in the surrounding water environment and becoming extremely harmful[1]. A significant amount of literature has demonstrated that iron is an essential element for human, while heavy metal is directly correlated to a human carcinogen and acute toxicity of aquatic organisms, some trace metal, like iron and copper, are essential element for animals and human[2]. The ability of iron to catalyze the formation of reactive oxygen species (ROS), including the hydroxyl radical ($^{\cdot}OH$), has been extensively reviewed[3]. These oxygen-derived radicals may produce oxidative stress and damage such cellular constituents as proteins and nucleic acids and also damage the membranes of cellular organelles by initiating lipid peroxidation[4].

Although it has been well established that iron toxicity can produce significant reproductive damage, the relationships between the cellular events surrounding iron-induced reproductive toxicity remain uncertain. Sperm motility is considered one of the most important parameters in evaluating the fertilizing ability of sperm. Because modulation of sperm motility might give information regarding quantitative and qualitative evaluation of toxic effects of exposed contaminants, monitoring of sperm motility parameters has been used as an important approach in toxicity experiments[5]. Using fish sperm in toxicity test *in vitro* has been developed for past decade. Several studies have shown a good relationship between sperm motility and sperm quality, as measured by subsequent fertilization success and hatching success in different species[6]. The large number of cells isolated from fish facilitates designing experiments to assess both the toxic effects of exposed compounds and the reproductive risk[7].

Fish endogenous protection to oxidative stress occurs through a cellular antioxidant system, which includes enzymes, such as superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase is an antioxidant enzyme and crucial for maintaining cellular homeostasis in fish[8], which plays important roles in the line of defenses against oxidative stress SOD catalyzes the conversion of reactive superoxide anions (O_2^-) into yield hydrogen peroxide (H_2O_2), which is an important ROS. Hydrogen peroxide is subsequently detoxied by two types of enzymes, namely, CAT and glutathione peroxidase (GPx). Catalase is one of the most efficient enzymes known and has been frequently reported in toxicity studies. The function of this enzyme is to convert hydrogen peroxide into

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oxygen and water[9]. Oxidative stress frequently induces lipid peroxidation (LPO). Lipid peroxidation in fish could be estimated by measuring malonaldialdehyde (MDA) and has been used as a biomarker in a large number of studies[10]. Lipid peroxi-dation results in the production of lipid radicals and in the subsequent formation of a complex mixture of lipid degradation products[11].

According to available data, most studies of the adverse effects of heavy metals on fish have focused on lethal or sub-lethal effects of Cd, Hg and Pb. The aims of the present study were to investigate how motility parameters and antioxidant responses were affected by short-time *in vitro* exposure (1,2 and 4 h) of Chinese loach (*Misgurnus anguillicaudatus*) sperm to Fe^{3+}, by analyzing oxidative stress indices (MDA) and antioxidant enzymes activities (SOD and CAT) of Chinese loach sperm, as well as sperm quality parameters (viability and motility). Toxicity screening using animal sperm has been taken as a simple and valid model during the past decades since its use does not require expensive sterile cell culture conditions[12]. Therefore, the need for sensitive, short-term tests has led to the increasing use of loach sperm in toxicity test. In this study an attempt was made, for the first time, to determine *in vitro* the effects of Fe^{3+} on the motility parameters and antioxidant responses in loach sperm.

EXPERIMENTAL

Iron(III) chloride hexahydrate ($FeCl_3·6H_2O$, 99.0 % purity) was purchased from sinopharm chemical reagent Co. Ltd. The stock solutions of ferric iron was prepared by dissolving suitable amounts of chemicals into water, sealed and stored at 4 ºC. The working solutions were made by diluting the stock solutions with ultrapure water.

Fish: The fish used in this study were Chinese loaches. Healthy Chinese loaches were obtained from the market of Beijing, China, and were acclimated in stainless glass tanks with dechlorinated tap water for at least 2 weeks prior to the exposure experiment. The mean weight and length of fish were (9.76 ± 0.92) g and (11.94 ± 0.58) cm, respectively. The day/ night cycle was set as 12 h/12 h. Through the study, water quality was monitored by detecting pH (7.0-8.0), oxygen concentration (5.2-7.1 mg L^{-1}) and temperature (21.6-24.8 ºC). At the beginning of the experiment, 18 fish were randomly allocated to 30 L glass tanks with 20 L dechlorinated tap water. Water was changed every other day during the exposure. The fish were fed with *Oligochaetes limnodirlus* twice a week.

Experimental design: For the experiment, the semen obtained from five untreated males was pooled. The pooled sample was then diluted with an immobilization medium (135 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 6.5, osmolality = 300 mosmol kg^{-1}) to obtain a sperm density of 1×10^8 cells mL^{-1}. The sperm motility was assayed after sub-samples (n = 5) were exposed for 1, 2 and 4 h to final concentrations of 0, 10, 25, 50, 100 and 500 μM of Fe^{3+}. To study the correlation between sperm motility and sperm viability, the sperm viability were assayed after sub-samples (n = 5) were exposed for 2 h to final concentrations of 0, 10, 25, 50, 100 and 500 μM of Fe^{3+}. Finally, the antioxidant enzymes and MDA were assayed after sub-samples (n = 5) were exposed for 2 h to final concentrations of 0, 10, 100 and 500 μM of Fe^{3+}.

Sperm motility and viability parameters: The Chinese loach sperm is typically non-motile in the semen since the semen has high sperm density and high viscosity. After the sperm was *in vitro* exposed to the toxicant in the immobilization medium, for estimation of sperm motility, 5 μL of sperm suspension were placed on a glass slide. Then 45 μL of low osmolarity (60 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 6.5, 150 mosmol kg^{-1}) solution was added to activate the sperm, and the motility was observed at 200 × magnification using dark-phase microscopy (Olympus IX81, Japan). The motility was expressed as the percentage and duration of sperm and sperm vibrating in place were not considered to be motile.

Sperm viability was analyzed using eosin-nigrosin stain[13]. 10 μL of each semen sample were placed on a slide and stained with 10 μL of eosin-nigrosin (1 g eosin + 5 g nigrosin/100 mL deionized water). The live (nonstained) and dead (purple-stained head) sperm were analyzed at 400 ×.

Antioxidant enzymes: Activity of SOD (E.C. No. 1.15.1.1) was estimated by the standard colorimetric method of Marklund and Marklund[14]. Activity of CAT (EC 1.15.1.6) was assessed by the colorimetric method of Sinha[15]. SOD activity is expressed as units/10^8 sperm and CAT activity is expressed as amount of H_2O_2 consumed/min/10^8 sperm.

Lipid peroxidation: MDA, as a marker for LPO, was determined by the double heating method of Draper and Hadley[16]. The principle of the method was spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 mL of 100 g L^{-1} trichloroacetic acid (TCA) solution was added to 0.5 mL sperm in a centrifuge tube and placed in a boiling water-bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 g for 10 min and 2 mL of the supernatant was added to 1 mL of 6.7 g L^{-1} TBA solution in a test-tube and placed in a boiling water-bath for 15 min. The solution was measured using UV762 spectrophotometer (China) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex.

Statistical analysis: Statistical analysis was performed using the statistical package SPSS 16.0. Data are presented as the Mean ± SD. The data were tested for homogeneity and normality. If these assumptions were met, one-way analysis of variance (ANOVA) with Tukey's test was used to determine whether results of treatments were significantly different from the control group ($p < 0.05$ and $p < 0.01$).

RESULTS AND DISCUSSION

Sperm motility and sperm viability: As an approach to evaluate and assess the potential for reproductive toxicity of the Fe^{3+}, the present study has examined Chinese loach sperm viability percentage, motility percentage and duration. The effects of Fe^{3+} on sperm motility are shown in Table-1. Results of the present study show that loach sperm motility duration had been decreased by Fe^{3+} at concentration of 10 μM, whereas no significant reduction of sperm motility percentage could be measured at the same concentration. For fish, fertilization

TABLE-1
EFFECTS OF Fe^{3+} TO MOTILITY OF LOACH SPERM AFTER 1, 2 AND 4 h EXPOSURE. DATA ARE MEANS ± S.D, n = 5

Concentration (µM)	Percentage (%)			Duration (s)		
	1 h	2 h	4 h	1 h	2 h	4 h
Control	94 ± 4.08	94 ± 5.48	90 ± 5.48	315 ± 31.46	305 ± 39.87	285 ± 31.46#
10	93 ± 4.18	89 ± 4.92	90 ± 3.76	295 ± 35.07#	290 ± 30.98#	280 ± 30.98#
25	83 ± 9.35*,#	80 ± 11.40*,#	71 ± 7.48*,#	245 ± 39.87*,#	240 ± 30.98*,#	223 ± 29.28*,#
50	73 ± 6.32*,#	75 ± 10.00*,#	71 ± 12.42*,#	230 ± 24.50*,#	222 ± 26.84*,#	215 ± 29.50*,#
100	50 ± 4.47*,#	48 ± 4.47*,#	48 ± 5.24*,#	160 ± 30.99*,#	150 ± 18.98*,#	155 ± 29.50*,#
500	0 ± 0*,#	0 ± 0*,#	0 ± 0*,#	0 ± 0*,#	0 ± 0*,#	0 ± 0*,#

*:Significant differences compared with control value $p < 0.05$; **: Significant differences compared with control value $p < 0.01$.

ability may depend on the capacity of sperm to move fast enough to find the egg in water after their releases[17]. As such, Fe^{3+} affecting sperm motility may significantly reduce fertilization success.

There was a high sperm viability of loach in all concentration. However, sperm viability with Fe^{3+} was significantly affected at concentrations of 100 and 500 µM and had significant differences compared with control group (Fig. 1A).

The Fe^{3+} effects on viability and motility were compared, considering the control group as 100 % (Table-2). At 25, 50, 100, 500 µM, Fe^{3+} decreased viability by 3, 1, 13 and 17 %, respectively, whereas Fe^{3+} decreased motility by 15, 20, 49 and 100 %, respectively. These data show that the reduction in motility is not necessarily the result of cell death. Rather, the sperm are viable but lose the capacity to move.

TABLE-2
Fe^{3+} EFFECTS ON THE VIABILITY AND MOTILITY AFTER 2 h EXPOSURE, IN RESPECT TO THE CONTROL

	Concentration (µM)					
	Control	10	25	50	100	500
Viability percentage	100	99	97	99	87	83
Motility percentage	100	95	85	80	51	0

Data were normalized considering the control group as 100 %.

In the present study, the decrease in sperm viability was most pronounced with a significant with 100 and 500 µM of Fe^{3+}. Regarding sperm motility duration, all concentrations of Fe^{3+} produced a significant reduction. Our findings at higher concentrations (100 and 500 µM) suggest that the reduction in motility is not necessarily caused by cell death. The results are consistent with the effects of insecticides malathion and diazion, and herbicides atrazine and fenoxaprop-eheyl on porcine sperm viability and motility[18]. Since the quality of fish sperm is a major factor contributing to successful production, measurement of its motility could provide a sensitive and accurate bio-indicator of aquatic pollution[19].

Antioxidant enzymes: The SOD-CAT system provides the first defense against oxygen toxicity. Superoxide dismutase catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which detoxified by the CAT activity. Various responses of SOD and CAT activities have been observed in animals exposed to organic or metallic contaminants in both field and laboratory experiments. These enzymes have been shown to be either induced or inhibited by metals depending on the dose, the species or the route of exposure[9]. It has been reported that pollutants such as phoshomidor, trichlorfon and dichlorvos caused inhibition of SOD activity[20]. However, Maiti and Kar[21] showed that dimethoate increased hepatic SOD activity. It was also shown that *in vitro* SOD activity was increased in human erythrocyte whereas it was decreased in plasma by phosphomidon[22].

In this study, SOD activity was significantly increased by high concentrations of Fe^{3+} in Chinese loach sperm (Fig. 1B). Usually, a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants[23]. However, our results showed that the exposure to Fe^{3+} for 2 h resulted in increase in SOD but reduce in CAT at same concentration (Fig. 1C). Thus, the results suggested that CAT was more sensitive than SOD as an indicator of evaluating oxidative stress of loach spermatozoa. It has been seen that CAT activity was inhibited by free radicals such as singlet oxygen and superoxide and peroxyl radicals[24]. Therefore, CAT may be inhibited by both Fe^{3+} itself and increased ROS induced by iron.

Lipid peroxidation: Lipid peroxidation has been used as an indicator of oxidative damage in fish tissues[25]. Thomas and Wofford[26] showed that lipid oxidation, as measured by MDA, significantly increased in liver and ovarian homogenates after Atlantic croaker (*Micropogonias undulatus*) were exposed to a PCB mixture (Aroclor 1254). In this study, LPO level increased significantly when sperm were exposed to 10 and 500 µM of Fe^{3+} for 2 h (Fig. 1 D). Similar to mammalian sperm, fish sperm also contain a high level of polyunsaturated fatty acids (PUFA), which are particularly susceptible to ROS induced lipid peroxidation[26]. Increased MDA may indicate an increased oxygen free radical generation and has been associated with mid-piece abnormalities and decreased spermatozoa quality[27]. This may be due to not only largely lack of protective cytoplasmic enzymes, but also the high content of PUFA in the spermatozoa plasma membrane which make them highly susceptible to free radical attack[5].

Conclusions

The present study shows that Fe^{3+} can have adverse influence on Chinese loach sperm motility parameters, which enhancing along with concentrations increasing or durations prolonging. However, the reduction in motility is not necessarily the result of cell death as the sperm are viable but lose the capacity to move. In addition, Fe^{3+} can cause oxidative stress, leading to the elevation of SOD activities and LPO level, the reduction of CAT activities. It may be concluded that Fe^{3+}-induced ROS production and corresponding oxidative damage may be one of the major causes damaging fish reproduction processes. From a more practical point view, the use of loach sperm for *in vitro* assays may provide a quick, cost effective

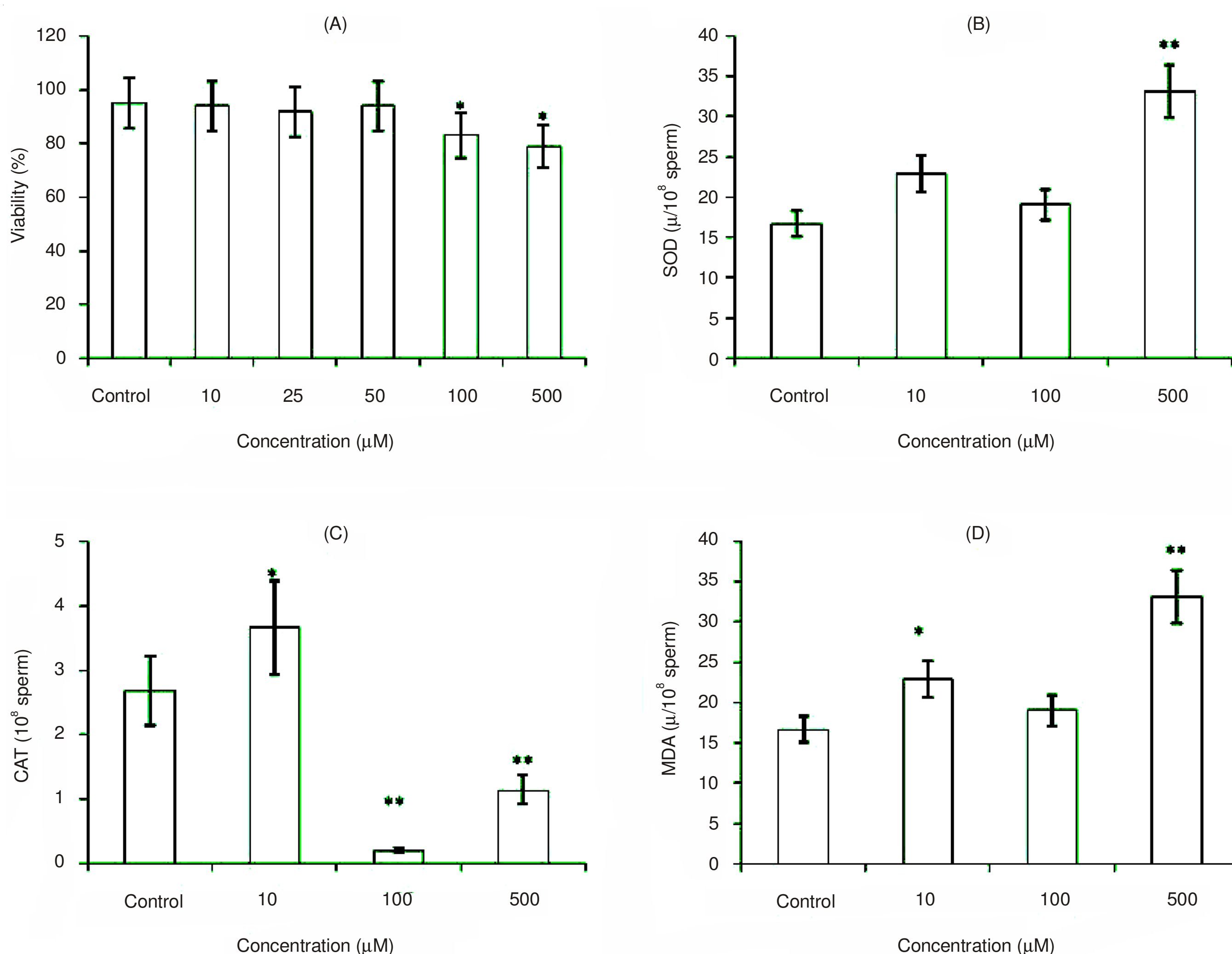

Fig. 1. Effect of Fe^{3+} on loach sperm after 2 h exposure. (A) viability. (B) SOD activity, (C) CAT activity. (D) MDA

means for routine monitoring and toxicological assessment of chemicals.

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