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Influence of Polluted Sea Water in the Red Sea on the Osteoclasts and Osteoblasts of Goldfish, *Carassius auratus*

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To analyze the influences of environmental pollutants and bioactive substances on fish bone metabolism, we have developed an original *in vitro* bioassay using goldfish scales that have osteoclasts, osteoblasts, and bone matrix. We used tartrate–resistant acid phosphatase (TRAP) for osteoclasts and alkaline phosphatase (ALP) for osteoblasts as respective markers. Using this assay system, we have reported the influences of heavy metals such as cadmium (Cd) and mercury (Hg) on fish bone metabolism. In the case of Cd, we found that its concentration (even at 10^{-13} M) influenced osteoclastic activity in goldfish scales only at 6 hrs of incubation. Thus, in the present study, we examined the effects of polluted seawater on osteoclastic and osteoblastic activities with this scale *in vitro* bioassay. Polluted seawater was collected from the Suez Gulf site on the Red Sea. Polluted seawater was added into culture medium at dilution rates of 50, 100, and 500 times and incubated with the goldfish scales for 6 hrs. Subsequently, the influences of polluted seawater on TRAP and ALP activities were compared with that of artificial seawater as a non–polluted seawater. As a result, TRAP activity was significantly suppressed by the polluted seawater samples diluted at least 500 times; in contrast, ALP activity did not show any change. This response was similar to the response of Cd and Hg. As heavy metal contamination of the Suez Gulf site has been reported, there is a high possibility that heavy metals are contained in the seawater. Considering our data together with environmental pollution in Egypt that has been reported until now, we should conduct a heavy metal risk assessment to protect the ecosystem in the Red Sea.

Key words: heavy metal pollution, fish scales, osteoclasts, osteoblasts, risk assessment

INTRODUCTION

Heavy metals are one of the main groups of pollutants due to rapid urbanization and industrial development (Liu *et al*., 2016). Some discharged heavy metals are put into aquatic systems and can be accumulated in fish *via* water, sediment, and the aquatic food chain (Omar *et al*., 2014; Saleh and Marie, 2015). The Egyptian Red Sea has also been reported to be contaminated with heavy metals (Lasheen *et al*., 2012; Attia and Ghrefat, 2013). The muddy and sandy sediments of the Suez Gulf in the Red Sea contained high concentrations of heavy metals such as cadmium (Cd), Nickel, and lead (Pb) (El Nemr *et al*., 2006). These metals seem to influence living fish and marine ecosystems in the Red Sea.

On the other hand, it has been known that fish scales are useful for the assessment of contaminated water because the chemical composition of fish scales have been reported to reflect the composition of the environmental water (Lake *et al*., 2006; Cobelo–García *et al*., 2017; Sultana *et al*., 2017). In addition, fish scales have been used as a suitable tool for reconstructing past contamination in aquatic systems (Cobelo–García *et al*., 2017). Therefore, fish scales have been used to assess polluted water. In our previous study, we noted the bone–like character, having both osteoclasts (bone–resorptive cells) (Azuma *et al*., 2007) and osteoblasts (bone–formative cells) (Yoshikubo *et al*., 2005), in fish scales and we have developed an original *in vitro* bioassay (Suzuki *et al*., 2000; Suzuki and Hattori, 2002; 2003; Suzuki *et al*., 2008). Using this assay system, we have examined toxicological studies regarding heavy metals. Namely, inorganic mercury (10^{-5} to 10^{-3}M) (Suzuki *et al*., 2011) and methylmercury (10^{-8} to 10^{-6}M) (Suzuki *et al*., 2004) significantly suppressed the osteoclastic activity of goldfish scales. Furthermore, in the case of Cd, its concentration (even at 10^{-13}M) influenced osteoclastic activity in scales (Suzuki *et al*., 2004). Actually, heavy

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metals induced spinal deformities in natural populations of grass goby, *Zosterisessor ophiocephalus* (Messaoudi *et al.*, 2009).

Thus, we should pay attention to fish bone metabolism caused by heavy metal contamination. To evaluate the toxicity of polluted seawater in fish bone metabolism, in the present study, we collected polluted seawater from the Suez Gulf site on Red Sea (El Nemr *et al.*, 2006). The polluted seawater was added into culture medium and incubated with the goldfish scales. Subsequently, the influences of polluted seawater on osteoclasts and osteoblasts in the goldfish scales were compared with that of artificial seawater as a non–polluted seawater.

MATERIALS AND METHODS

Animals

In our previous study (Suzuki *et al.*, 2000), we found that sensitivity for calcemic hormones, such as estrogen and calcitonin, was higher in mature female than mature male goldfish (*Carassius auratus*). Therefore, female goldfish (n = 12, 37.50 ± 1.50 g) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the scale *in vitro* assay (n = 6 for osteoclastic activity; n = 6 for osteoblastic activity). Moreover, all experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Effects of polluted seawater samples on scale osteoclastic and osteoblastic activities using cultured scales of goldfish

Surface water was collected from the Suez Gulf site on the Red Sea (Fig. 1), and the influence of the polluted water on the osteoclasts and osteoblasts of goldfish scales was examined.

Goldfish were anesthetized with ethyl 3–aminobenzoate, methanesulfonic acid salt (Sigma–Aldrich, Inc., St. Louis, MO, USA), and the normally developed scales on both sides of the body were removed to allow the regeneration of scales. Hence, on day 14, goldfish were anesthetized again, and the regenerating scales were extracted. Then, using the extracted regenerating scales, we examined the influences of seawater samples on the osteoclasts and osteoblasts using tartrate–resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) markers, respectively, because, in mammals, the effects of hormones and some bioactive substances on osteoclasts and osteoblasts have been investigated using TRAP and ALP, respectively (Vaes, 1988; Dimai *et al.*, 1998; Suda *et al.*, 1999). Therefore, the scales were incubated for 6 hrs in Leibovitz's L–15 medium (Phenol Red–free, Invitrogen, Grand Island, NY, USA) containing a 1% penicillin–streptomycin mixture (ICN Biomedicals, Inc., Aurora, OH, USA) supplemented with polluted seawater (diluted 50, 100, and 500 times with L–15 medium). Finally, the influences of polluted seawater on osteoclasts and osteoblasts were compared with those of artificial seawater (Allen seawater: NaCl 3%, $MgSO_4 \cdot 7H_2O$ 0.358%, $MgCl_2 \cdot 6H_2O$ 0.272%, $CaCl_2 \cdot 2H_2O$ 0.06%, KCl 0.039%, $NaHCO_3$ 0.01%) (Suzuki *et al.*, 1992) as a non–polluted seawater.

From one goldfish, 24 scales from the left or right side were used for the present study. The 24 scales were utilized as follows: (1) 8 scales for TRAP analysis with 500–time dilution, (2) 8 scales for TRAP analysis with 100–time dilution, (3) 8 scales for TRAP analysis with 50–time dilution. The respective mean for TRAP (obtained from 8 individual scales of one goldfish) was compared with that of the right side (control group: diluted artificial seawater samples). After incubation at 15°C for 6 hrs, TRAP activities were measured using the same methods previously described (Suzuki *et al.*, 2009). The results are shown as the means ± SEM (n = 6). In the case of ALP, the same experiment was done repeatedly using 6 individual goldfish.

The methods for measuring TRAP and ALP activities were as follows. An aliquot of 100 μl of an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) or an alkaline buffer (100 mM Tris–HCl, pH 9.5; 1 mM $MgCl_2$) was added to each well. Then, each scale was put into its own well in a 96–well microplate. Moreover, this microplate was immediately frozen at –80°C and then kept at –20°C until analysis. Also, an aliquot of 100 μl of 20 mM para–nitrophenyl phosphate in an acid or alkaline buffer was then added to each well of melted solution in the microplate. Furthermore, this plate was incubated at 23°C for 20 min, while being shaken. After incubation, the reaction was stopped by adding 50 μl of 3 N NaOH. One hundred fifty μl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of para–nitrophenol (pNP) produced using a standard curve for pNP.

After measuring both TRAP and ALP activities, the scales were measured with Image J. Thereafter, TRAP and ALP activities were normalized to the surface area (mm^2) of each scale (Suzuki *et al.*, 2009).

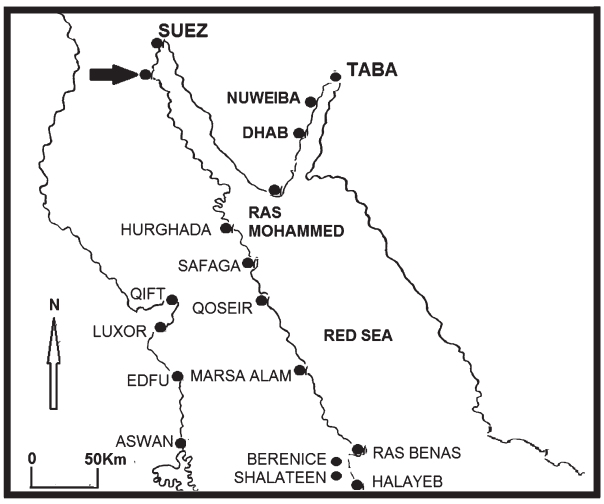

Fig. 1. Location of the sampling site (Suez Gulf site on the Red Sea). Arrow indicates sampling site.

Statistical analyses

All results are expressed as the means ± SEM (n = 6). The data were assessed using the paired t–test, and the significance level chosen was $p < 0.05$.

RESULTS

Effect of polluted seawater on TRAP activity in the cultured scales of goldfish

The polluted seawater sample was added to culture medium diluted 50, 100, or 500 times and incubated with the goldfish scales for 6 hrs. Then, TRAP activity was measured. Results showed that all of the diluted seawater significantly suppressed TRAP activity (500–time dilution: $P < 0.05$; 100–time dilution: $P < 0.05$; 50–time dilution: $P < 0.01$) as compared with diluted artificial seawater (Fig. 2). Furthermore, the activity responses to the polluted seawater were dose dependent (Fig. 2).

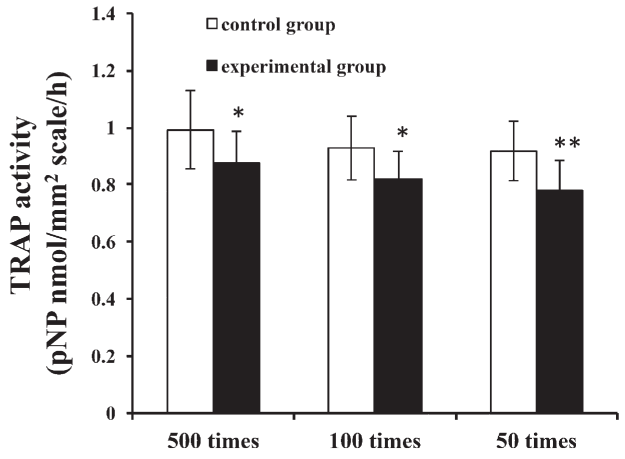

Fig. 2. Effect of polluted seawater on TRAP activity in the cultured scales at 6 hrs. * and ** indicate statistically significant differences at $P<0.05$ and $P<0.01$, respectively, from the value in the control scales.

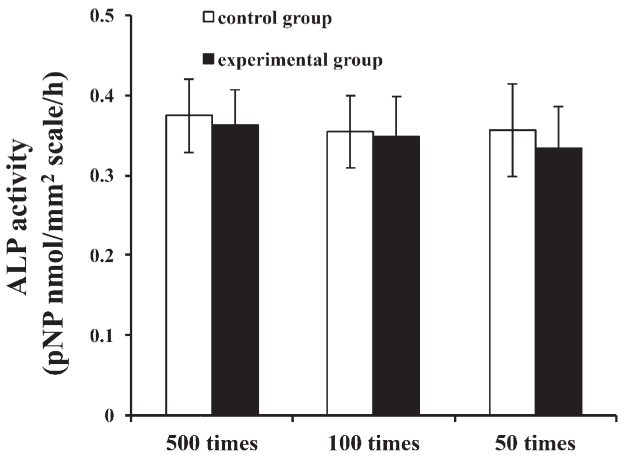

Fig. 3. Effect of polluted seawater on ALP activity in the cultured scales at 6 hrs. There was no significant difference between experimental and control scales.

Effect of polluted seawater on ALP activity in the cultured scales of goldfish

In ALP activity, as well as TRAP activity, the polluted seawater sample was added to culture medium diluted 50, 100, or 500 times and incubated with the goldfish scales for 6 hrs. Thereafter, the ALP activity was measured. We found that the ALP activity did not change significantly during 6 hrs of incubation when compared with the values in the control (Fig. 3).

DISCUSSION

In the present study, we detected the toxicity for osteoclasts (Fig. 2) in seawater collected from the Suez Gulf site (Fig. 1). Osteoclastic activity was significantly suppressed by polluted seawater samples diluted at least 500 times. Our results are in agreement with those of Suzuki *et al*., 2004; Suzuki *et al*., 2011; and Yachiguchi *et al*., 2014a; who reported very similar responses to Cd and mercury (Hg). Also, as heavy metal contamination of the Suez Gulf site has been reported (El Nemr *et al*., 2006), there is a high possibility that heavy metals are contained in the seawater.

In the case of osteoblasts, the ALP enzyme activity did not change during 6 hrs of incubation when compared with the values of the control (Fig. 3). In our previous study regarding Cd and Hg, the ALP activity in the Cd– or Hg–treated scales did not change significantly (Suzuki *et al*., 2004; Yachiguchi *et al*., 2014a) with short incubation times of less than 18 hrs. Conversely, the mRNA expression of metallothionein (MT), a metal–binding protein that protects the organism from heavy metals (Hamer, 1986; Klaassen *et al*., 1999), significantly increased with either Cd or Hg treatment (Suzuki *et al*., 2004; Yachiguchi *et al*., 2014a). As it has been reported that osteoblasts expressed MT, which protected against heavy metals (Angle *et al*., 1990; Nagata and Lönnerdal, 2011), the activation of MT in osteoblasts may be involved in resistance to heavy metals.

Our original *in vitro* bioassay system can detect the activities of both scale osteoclasts and osteoblasts with TRAP and ALP, respectively, as specific markers (Persson *et al*., 1994; Suzuki *et al*., 2007; de Vrieze *et al*., 2010). In addition, we can analyze the enzyme activity in each scale by transferring each scale into a 96–well microplate and incubating directly the scale with the substrate in each well. Using this *in vitro* system, we have analyzed the effects of endocrine disrupters such as bisphenol–A (Suzuki and Hattori, 2003), tributyltin (Suzuki *et al*., 2006), and polychlorinated biphenyl (Yachiguchi *et al*., 2014b) on osteoblasts and osteoclasts. Even 10^{-10}M tributyltin significantly suppressed osteoblastic activity. Furthermore, polychlorinated biphenyl 118 (around 10^{-8} to 10^{-7}M) influenced osteoclastic activity (Yachiguchi *et al*., 2014b). In the present study, we can sensitively evaluate the effect of polluted seawater on osteoclasts for short incubation times. Thus, our bioassay system is very useful for evaluating the effect of environmental pollutants on fish bone metabolism.

In African catfish (*Clarias garipinus*) and Nile tilapia (*Oreochromis niloticus*), Pb, zinc, copper, and Cd were present in the liver and muscle, although the concentration of heavy metals in the water was within the permissible limits for discharge drain water into the Nile River (Lasheen *et al*., 2012). In the Red Sea, the bioaccumulation of heavy metals in fish has been reported (Idris *et al*., 2015). Namely, heavy metal concentrations in studied fish species were recorded: vanadium (0.004–0.561), chromium (0.013–0.477), manganese (0.073–0.128), arsenic (0.002–0.935), selenium (0.083–3.058), tin (2.835–5.540), and Pb (0.150–0.386) (μg/g, wet weight) in muscle tissues of commercially obtained fish from the Southern Red Sea (Idris *et al*., 2015). Therefore, we should conduct a risk assessment of heavy metals to protect the ecosystem in the Red Sea.

AUTHOR CONTRIBUTIONS

N. Suzuki, H. F. Nassar, F. K. Abdel–Gawada, K. Hayakawa, N. Shimizu, Y. Oshima, and H. M. Mahmoud designed the study. M. I. Zanaty, M. Sato, N. Suzuki, A. Hattori, K. Yachiguchi, K. Mukai, Y. Shimasaki, and Y. Oshima analyzed the data and wrote the paper. Y. Oshima and Y. Shimasaki supervised the work. All authors assisted in editing the manuscript and approved the final version.

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