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Effects of a Styrene Trimer (2,4,6-triphenyl-1-hexene) on Plasma Mineral Concentrations of Nibbler Fish, *Girella punctata*

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Abstract: To investigate the toxic effect of plastic-derived styrene oligomers, we examined the influence of a styrene trimer (ST) (2,4,6-triphenyl-1-hexene) which is the main styrene oligomer in the ocean, on the plasma mineral concentrations of nibbler fish (marine teleosts), *Girella punctata*. We intraperitoneally administered the ST (100 µg/L) into nibbler fish and measured the plasma calcium (Ca), magnesium (Mg), inorganic phosphorus (Pi), sodium (Na), chlorine (Cl), and potassium (K) at 12 and 24 h after injection. Plasma Ca levels in the ST-treated fish did not change significantly at 12 and 24 h as compared with those in untreated fish. However, plasma Mg and Pi concentrations were influenced by the ST administration. At 12 h after injection, plasma Mg and Pi concentrations in the experimental group increased significantly as compared with those in the control group. At 24 h after injection, however, there were no significant changes in the plasma Mg and Pi levels between the control and experimental groups. In the case of Cl levels, the values of plasma Cl in experimental group decreased significantly as compared with those in the control group at 24 h after administration, although there was no significant difference between the control and experimental groups at 12 h after injection. On the other hand, the plasma Na and K levels did not change under the present conditions at either 12 or 24 h after injection. It has been reported that seawater along the coast of Japan contained a ST between 0.35 to 6.97 µg/l (mean: 3.26 µg/l). Similar concentrations of the ST have been detected in other parts of the world's oceans. This study is the first to demonstrate that the ST influenced plasma Mg, Pi, and Cl levels in nibbler fish, suggesting that STs have harmful effects on marine fish. We should pay more attention to STs existing as polluted substances in the ocean.

Keywords: Styrene trimer, Microplastics, Plasma magnesium, Plasma inorganic phosphorus, Plasma chlorine, Marine fish, *Girella punctata*

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Introduction

The problem of large amounts of plastic waste floating in the sea is becoming very serious (Schmid *et al.*, 2021). Plastics released in the ocean environment undergo various natural processes and are fragmented into particles of different sizes (Andrady, 1990; Song and Andrady, 1993). Degraded and decomposed plastic particles with diameters of less than 5 mm are called micro/nano plastics (Cózar *et al.*, 2014; Law and Thompson, 2014).

The top three polymer types reported are polyethylene, polypropylene, and polystyrene (PS) (Hidalgo-Ruz *et al.*, 2012). In the present study, we focus on PS because plastic-derived styrene oligomers (SOs) were actually present in the ocean (Saido *et al.*, 2014; Kwon *et al.*, 2017; Amamiya *et al.*, 2020). Along the coast of Japan, concentrations of SOs ranged from 0.65 to 8.65 µg/l (mean: 4.03 µg/l) (Amamiya *et al.*, 2020). Particularly, the styrene trimer (ST) (2,4,6-triphenyl-1-hexene) (Fig. 1) between 0.35 to 6.97 µg/l (mean: 3.26 µg/l) has been detected in seawater along the coast of Japan (Amamiya *et al.*, 2020). Similar concentrations of the ST have been detected in other parts of the world's oceans (Kwon *et al.*, 2017, 2018). Recently, the reasons for the high concentrations of STs in the ocean have been found. It has been reported that purified polystyrene (SO-free PS) decomposed at 30 to 150 °C, and that the composition ratio of styrene monomer (SM) : styrene dimer (SD) : ST in decomposed PS was 1:1:5 (Kimukai *et al.*, 2020). These facts indicate that STs originated from PS have an influence that is possibly harmful to marine teleosts in natural marine environments.

We previously reported that environmental pollutants such as bisphenol-A (Suzuki *et al.*,

2003), tributyltin (Suzuki *et al.*, 2006), polychlorinated biphenyl 118 (Yachiguchi *et al.*, 2014), and benz[a]anthracene (Suzuki *et al.*, 2017; Zanaty *et al.*, 2020) influenced plasma calcium (Ca) levels and disrupted bone metabolism. In the present study, we examined the effects of the ST on plasma mineral concentrations in marine fish, *Girella punctata*. This study is the first to demonstrate that a ST influence plasma mineral levels in fish.

Fig. 1: Chemical structure of 2,4,6-triphenyl-1-hexene used in the present study as a styrene trimer.

Materials and Methods

Animals:

Nibbler fish (*Girella punctata*) (both sexes, n = 19, bw 26.25 ± 4.72 g) (Fig. 2) were collected by fishing in Tsukumo Bay of the Noto Peninsula (Ishikawa Prefecture). After acclimation for 2 weeks, these nibbler fish were used in the present experiments.

Fig. 2: Nibbler fish (*Girella punctata*).

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

The intraperitoneal administration of a ST (100 µg/l) into nibbler fish:

Nibbler fish (marine teleosts) were divided into two groups: the control group and the experimental group. The ST were first dissolved in ethanol, and then 0.9% NaCl solution was added to the ethanol solution so that the ethanol concentration became 0.1%, as described by Suzuki *et al.* (2017).

The experimental group (n=10) was anesthetized with 0.04% 2-phenoxyethanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and a ST (100 µg/l) were administered intraperitoneally with a syringe. In the control group (n=9), a vehicle solution containing 0.1% ethanol was also administered into the abdominal cavity using a syringe under anesthesia with 0.04% 2-phenoxyethanol.

Measurement of plasma Ca, magnesium (Mg), inorganic phosphorus (Pi), sodium (Na), chlorine (Cl), and potassium (K) concentrations at 12 and 24 hours after the administration of a ST into nibbler fish:

The fish (control and experimental) were anesthetized with 0.04% 2-phenoxyethanol at 12 and 24 h after injection. Blood samples were taken from the caudal vessels using heparinized syringes. The collected blood was put into a 1.5 ml tube and centrifuged at 15,000 rpm for 3 min. Then, the separated plasma was immediately frozen and kept at -80 C until use. The plasma total Ca, magnesium (Mg), and inorganic phosphorus (Pi) levels (mg/dL) were determined using assay kits (Ca: Aqua-auto Kainos Calcium Reagent Kit, KAINOS Laboratories, Inc., Tokyo, Japan; Mg: L-type Mg N, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; Pi: Phospha C-test, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with a microplate reader (CORONA ELECTRIC Co., Ltd., Hitachinaka, Ibaraki, Japan) as

described by Sato *et al.* (2017). To measure sodium (Na), chlorine (Cl), and potassium (K) in the plasma of control or ST-treated nibbler fish, plasma samples were sent to a commercial vendor (Oriental Yeast Co., Ltd., Tokyo, Japan) and measured by an ion electrode method with a Hitachi 7180 automatic analyzer (Hitachi High Technologies Corporation, Tokyo, Japan).

Statistical analysis:

All results are expressed as the means $\pm$ SE. The statistical significance between the control and experimental groups was assessed by student t-test. In all cases, the selected significance level was $P < 0.05$.

Results

Changes in plasma Ca, Mg, and Pi levels of nibbler fish at 12 and 24 h after the administration of a ST:

The plasma Ca, Mg, and Pi levels are illustrated in Figures 3 and 4. In nibbler fish, plasma Ca levels in the experimental group did not change significantly as compared with those in the control group at 12 and 24 h after the injection of a ST.

On the other hand, plasma Mg concentrations in the ST-treated fish increased significantly as compared with those in untreated fish at 12 h after the injection of a ST. Similarly, there were significant differences in plasma Pi levels between control and experimental groups at 12 h after administration. However, 24 h after injecting a ST into fish, plasma Mg and Pi levels were not significantly different between control and experimental groups.

Changes in plasma Na, Cl, and K levels of nibbler fish at 12 and 24 h after the administration of a ST:

The plasma Na, Cl, and K levels at 12 and 24 h after injection are shown in Figures 5 and 6, respectively. Plasma Na and K levels did not change under the present conditions at either 12 or 24 h after injection.

The values of plasma Cl in the experimental group decreased significantly as compared with those in the control group at 24 h after

Fig. 3: Changes in the plasma calcium (Ca) (A), magnesium (Mg) (B), and inorganic phosphorus (Pi) (C) levels of nibbler fish at 12 h after the administration of a styrene trimer (100 µg/l). * indicates $P < 0.05$.

Fig. 4: Changes in the plasma calcium (Ca) (A), magnesium (Mg) (B), and inorganic phosphorus (Pi) (C) levels of nibbler fish at 24 h after the administration of a styrene trimer (100 µg/l).

Fig. 5: Changes in the plasma sodium (Na) (A), chlorine (Cl) (B), and potassium (K) (C) levels of nibbler fish at 12 h after the administration of a styrene trimer (100 µg/l).

Fig. 6: Changes in the plasma sodium (Na) (A), chlorine (Cl) (B), and potassium (K) (C) levels of nibbler fish at 24 h after the administration of a styrene trimer (100 µg/l). * indicates $P < 0.05$.

administration, although there was no significant difference at 12 h after injection.

Discussion

The present study is first to demonstrate that the ST (2,4,6-triphenyl-1-hexene) significantly increased plasma Mg and Pi levels. In mammals, it has been reported that a high-Mg diet resulted in decreased renal Pi excretion associated with modulation of the sodium-dependent Pi cotransporter (Thumfart *et al.*, 2008). In teleost fish as well as mammals, the sodium-dependent Pi cotransporter is present in the kidney and contributes to Pi homeostasis (Verri and Werner, 2019). Therefore, we imply that a ST acts on fish kidneys and increase plasma Pi levels, resulting from the suppression of renal Pi excretion. The elevation of plasma Mg levels seems to be associated with excretory organs such as the kidneys and gills. The detailed mechanism will be investigated in the near future.

In the present study, on the other hand, plasma Ca levels did not change significantly with ST treatments. We previously reported that tributyltin acetate (TBTA) treatments induced the secretion of calcitonin that functions as a hypocalcemic hormone (Suzuki *et al.*, 2006). It is possible that the ST has probably no effect on the ultimobranchial gland which secretes calcitonin in fish (Suzuki *et al.*, 2004; Sekiguchi *et al.*, 2021) and thus plasma Ca levels did not change in spite of ST treatments. The other possible reason for not detecting any effect on plasma Ca in treated fish may be due to the short duration of the present experiment. Hence, long-term experiment is needed to explore the effects of a ST on plasma Ca.

In the case of monovalent ions, a ST induced toxic influences in nibbler fish. Plasma Cl levels in the experimental group decreased significantly as compared to those in the control group at 24 h after administration, although there was no significant difference at 12 h after injection. It has been reported that environmental pollutants induced a decline of Cl ions by the toxic influence

of excretory organs such as the kidneys and gills (Rogers *et al.*, 2003; Pane *et al.*, 2003). Therefore, we imply that a ST functions on excretory organs and decreases plasma Cl ions.

Microplastics were found in the stomachs of several fishes (Chan *et al.*, 2019; Nie *et al.*, 2019; Chenet *et al.*, 2021). Therefore, most researchers have focused on fragmented particles of microplastics and investigated the effects of these microplastics on fish toxicology (Greven *et al.*, 2016; Fossi *et al.*, 2017; Pitt *et al.*, 2018; Lei *et al.*, 2018). However, the influence of STs on fish physiology has not been investigated until now. Actually, a ST was detected off the coast of Japan (mean: 3.26 µg/l) (Amamiya *et al.*, 2020). Similar concentrations of a ST, have been detected in seawater of other parts of the ocean (Kwon *et al.*, 2017, 2018). Therefore, the toxicological influence of a ST in marine fish should be investigated. In the present study, we found that a ST administration influenced plasma Mg, Pi, and Cl levels, at least in the present conditions. Harmful effects of a ST were observed in fish, although our experiment period was short. Marine fish are always exposed to environmental STs and SOs in natural ocean conditions. Therefore, we believe that experiments regarding long-term ST exposure are necessary for understanding the exact effect of STs on marine teleosts. We plan to conduct experiments exploring the effects of long-term ST exposure in nibbler fish and elucidating details of its harmful influences on marine teleosts.

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