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FULL PAPER

Toxicology

Absence of histopathological changes in the retina of zebrafish treated with sodium iodate

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ABSTRACT. In ophthalmological research, the use of zebrafish to investigate visual behaviors has been increasing, but can produce misleading, false-positive results if compounds adversely affect their motor functions or central nervous system. Therefore, histological analysis to identify a target organ is important in zebrafish toxicity assay. We investigated the retinal degeneration in zebrafish, using typical retinal toxicants, mainly sodium iodate and N-methyl-N-nitrosourea (MNU). No histopathological changes were found after sodium iodate exposure at 1.0 mM for 5 or 7 days in the retina of larval, juvenile, and adult zebrafish. There were also no obvious histopathological changes in the retina of adult zebrafish at 0.1 mM, even after 30 days treatment with sodium iodate. In addition, many proliferating cell nuclear antigen-positive cells were found not only in the ciliary marginal zone, but also in the outer nuclear layer, especially in larval and juvenile zebrafish with or without sodium iodate exposure. However, the concentrations of iodine in the blood and the eyeballs of adult zebrafish increased remarkably after the treatment. General retinal damage emerged after MNU exposure at 150 mg/l for 60 min in adult zebrafish, but first pyknotic cells appeared in the inner nuclear layer and the ganglion cell layer. Our findings indicate that zebrafish retina have a different reactivity pattern from mammalian animals against some retinal toxicants, and in them it is difficult to detect histopathological changes.

KEY WORDS: eye, histopathology, sodium iodate, toxicity, zebrafish

It is now generally accepted that zebrafish provide an excellent model system for biological research, including drug discovery [7]. The use of zebrafish, especially larval zebrafish, for early safety assessment has been increasing recently because of its relatively good throughput property related with its small body size and rapid growth [4, 9, 11].

In ophthalmological research, the zebrafish is expected to be a useful organism because the visual system of the zebrafish is basically similar to that of humans [1, 8]. For example, a number of methods have been established for investigating zebrafish visual function, such as optomotor response (OMR) assay, optokinetic response assay, startle response, escape response, and visual motor response assay [1, 5]. Among these, the OMR assay and the visual motor response assay have good throughput properties, and have been used to assess effect of compounds that are well known as ocular toxicants on visual function [3, 16]. These studies indicated that the OMR assay using larval zebrafish has a high predictability for drug toxicity.

However, these visual assays based on behavioral response might produce misleading results if the investigated compounds adversely affect in organs, motor functions, or central nervous system, which critically effect behavior. For this reason, histological analysis is thought to be essential to identifying a target organ in zebrafish.

Sodium iodate is a typical retinal toxicant, and is thought to primarily induce retinal pigmented epithelial cell necrosis, resulting in a patchy loss of the retinal pigmented epithelial cells, followed by apoptosis of photoreceptor cells [10]. Sodium iodate-induced retinal toxicity has been reported in many mammals, such as mice [10], rats [21], rabbits [2], and monkeys [12]. Sodium iodate, however, is known not to affect zebrafish OMR activity [16], while N-methyl-N-nitrosourea (MNU), a well-known inducer of photoreceptor degeneration via DNA alkylation of photoreceptors in mammals, induced massive rod photoreceptor degeneration after only 60 min exposure in adult zebrafish [17, 18]. As the other toxicants, gentamicin or chloroquine are known to cause retinal disorder [16, 22].

In this study, we therefore examined whether sodium iodate induces retinal histopathological changes in three different developmental stages of zebrafish. The retinal toxicity of MNU was also tested in adult zebrafish. Such a study is of importance

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to provide information on zebrafish as a model for ocular research. In addition, this study is of significance from a viewpoint of the veterinary field; e.g., accumulating knowledge of comparative toxicology between mammalians and fish, and characteristics of poisoning in aquatic animals. With this background, we employed three different maturation stages of zebrafish, larva, juvenile, and adult, to probe their different sensitivities against the toxicants.

MATERIALS AND METHODS

Animals

Fertilized eggs of the AB strain of zebrafish (*Danio rerio*) were provided by the National Cerebral and Cardiovascular Center Research Institute Department of Cell Biology (Osaka, Japan). Juvenile zebrafish (body length approx. 1.5–2 cm) were purchased from the National Institute for Environmental Studies (Tsukuba, Japan). Adult zebrafish (body length approx. 4 cm) were purchased from the National Institute for Environmental Studies or another supplier (Meito Suien Co., Ltd., Nagoya, Japan). Zebrafish were bred and maintained in accordance with standard procedures [20]. Zebrafish and fertilized eggs were maintained in rooms on a 14-hr light/10-hr dark cycle at approximately 28.5°C, and were bred in 0.3% artificial seawater, composed of distilled water with Sealife® (Marine Tech, Tokyo, Japan) artificial sea salt. The juvenile and adult fish were fed a commercial feed (Otohime B1, SAN-U Fish Farm, Osaka, Japan) twice daily.

Male 7-week-old rats (strain: Sprague-Dawley) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and total of twelve rats were used in this experiment when they were 8 weeks old. These were housed in air-conditioned rooms maintained at 20 to 26°C, 40 to 70% relative humidity, and a 12-hr light/dark cycle. Rats were fed a commercial pellet feed (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan), and supplied with tap water *ad libitum*.

All experimental procedures involving animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Senju Pharmaceutical Co., Ltd.

Exposure of zebrafish to sodium iodate

Larval zebrafish hatched from eggs were exposed by immersion in 0.3% artificial seawater containing the final concentrations of 0.1, 0.3 and 1.0 mM sodium iodate (Sigma-Aldrich, St. Louis, MO, U.S.A.) from 3 days post-fertilization (dpf) to 8 dpf in 12-well plates with 10 larvae per well (N=10). Replacement of 0.3% artificial seawater containing sodium iodate was performed at 6 dpf.

Juvenile zebrafish were treated in 0.3% artificial seawater containing the final concentrations of 0.1, 0.3 and 1.0 mM of sodium iodate for 7 days at a rearing density of 5 or less fish/*l* in 5-*l* tank (N=5).

Adult zebrafish were treated in 0.3% artificial seawater containing the final concentration of 1.0 mM of sodium iodate for 7 days at a rearing density of 3 fish/200 m*l* in 500-m*l* glass beakers (N=3). Replacement of the compound in 0.3% artificial seawater was done every day. The maximum tolerated concentration was estimated in larval and adult zebrafish, and was judged to be 1.0 mM, based on observation for lethality and abnormal behavior. In order to examine long-term toxicity, adult zebrafish were exposed to 0.1 mM of sodium iodate for 30 days (N=3). For determination of iodine concentration, 9 adult zebrafish were used per group. Fish were randomly divided into three subgroups of 3 fish each, and exposed to sodium iodate as described above. The fish were decapitated, and the blood of each was collected into one heparinized hematocrit capillary tube, and then the blood samples were pooled together into a single tube (N=3). The eyes were enucleated, and one eye of each of 3 zebrafish was put into one tube (N=3). In all experiments using zebrafish, 0.3% artificial seawater was used as the vehicle control.

Exposure of zebrafish to MNU

Adult zebrafish were treated in 10 mM phosphate buffer (pH 6.3), containing the final concentration of 150 mg/*l* of MNU (Sigma-Aldrich) for 60 min (N=2), in accordance with the previous description [17, 18].

Exposure of rats to sodium iodate

Sodium iodate was dissolved in sterile saline (Otsuka Pharmaceutical factory, Inc., Tokyo, Japan) as a 2% w/v stock solution. The 8-week-old rats were anesthetized by a 2.5 m*l*/kg intraperitoneal injection of a 4:1 mixture of 5% ketamine hydrochloride (Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and 2% xylazine hydrochloride (Bayer, Leverkusen, Germany). A single dose of sodium iodate (40 mg/kg) was intravenously injected via the tail vein (N=3). The control animals were injected with the same volume of the saline. For investigation of the time-specific effect of sodium iodate, the rats were euthanized under the anesthesia described as above at 5 min, 2 hr, and 7 days after sodium iodate injection. The blood was collected from abdominal veins to determine the exposure levels of sodium iodate. The eyes were enucleated to examine histopathological changes and to determine the exposure levels of sodium iodate.

Histopathological examination

Whole larvae were fixed overnight in a solution of 4% paraformaldehyde (PFA) and 5% sucrose in 0.1 M phosphate buffer at 4°C, and embedded in paraffin. Adult and juvenile zebrafish were euthanized by immersion in ice-cold water or 1:1,000 dilution of 2-phenoxyethanol. After the euthanasia, adult and juvenile zebrafish were decapitated, and the heads were fixed overnight in Bouin's solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 4°C, and thereafter embedded in paraffin. The paraffin blocks were cut in 5 μm thick sections, and stained with hematoxylin and eosin (H&E).

The eyes of rats were fixed overnight in a solution of 10% formalin (Wako Pure Chemical Industries, Ltd.): 25% glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan) (9:1) at 4°C, and thereafter embedded in paraffin. H&E stained specimens were prepared under routine method.

Proliferating cell nuclear antigen (PCNA) staining

The procedure of fixation for the eyeballs was the same as described above, but slightly modified: Whole larvae were fixed at room temperature for 60 min. The eyes of adult zebrafish were enucleated and fixed in a solution of 4% PFA at 4°C overnight.

Paraffin sections were pretreated with heating at approximately 98°C for 45 min in 10 mM citrate buffer (pH 6.0). Immunostaining was performed in accordance with the standard protocols using 5% normal goat serum (Thermo Fisher Scientific, Waltham, MA, U.S.A.) in 0.2% Triton X-100 as a blocking reagent, and primary antibody was diluted with 1% normal goat serum/0.2% Triton X-100. The secondary antibody was diluted with DAKO Real Antibody Diluent (Agilent Technologies, Santa Clara, CA, U.S.A.), as follows: The primary antibody used was anti-PCNA (1:1,000, clone PC10, Sigma-Aldrich), and the secondary antibody used was Alexa 488-conjugated goat anti-mouse IgG (1:1,000, Thermo Fisher Scientific). Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

Determination of blood/tissue iodine concentrations

The iodine from the blood and eyeballs were extracted by adding 0.1 N hydrogen chloride and 1% Triton X-100. The concentrations of iodine were analyzed on an inductively coupled plasma mass spectrometer (Agilent 7700x ICP-MS, Agilent Technologies, Tokyo, Japan) at Shin Nippon Biomedical Laboratories, Ltd., Tokyo, Japan.

RESULTS

Histopathology of sodium iodate-induced lesions and expression of PCNA in the larval, juvenile and adult zebrafish retina

In larval zebrafish, there were no histopathological changes in the retina after sodium iodate exposure from 3 to 8 dpf at any dose (Fig. 1). Many PCNA-positive cells were found in the ciliary marginal zone, where the stem cell niche is located in the retina, and the outer nuclear layer in larval zebrafish with and without treatment (Fig. 1).

Similarly, no histopathological changes were found in the retinas of the juvenile zebrafish after sodium iodate exposure for 7 days at any dose (Fig. 2), and many PCNA-positive cells were also noted in the ciliary marginal zone and the outer nuclear layer in juvenile zebrafish with and without treatment.

In adult zebrafish, there were no histopathological changes after sodium iodate exposure for 7 days at 1.0 mM, in spite of finding much fewer PCNA-positive cells in the ciliary marginal zone and the outer nuclear layer than in larval and juvenile zebrafish (Fig. 3). In the examination for long-term toxicity, no obvious histopathological changes were found at 0.1 mM, even after 30 days treatment with sodium iodate (Fig. 4).

Histopathology of MNU-induced lesions in the adult zebrafish retina

Whereas sodium iodate did not induce retinal changes in any stage of zebrafish, MNU induced retinal damage in adult zebrafish. Compared with the untreated control retina, several pyknotic cells in the inner nuclear layer and a few of ones in the ganglion cell layer appeared at 6 hr after MNU exposure (Fig. 5B, black arrowheads). The number of these cells increased at 24 hr (Fig. 5C, black arrowheads). At day 3, obscuration of retinal structure between the inner nuclear layer and the photoreceptor layer was advanced, and the number of cells likely to decrease in the inner nuclear layer and the outer nuclear layer (Fig. 5D). At day 5, the obscuration of the retinal structure sustained over time, and some parts of the inner nuclear layer were fused with the outer nuclear layer (Fig. 5E, white arrows). In addition, cells appeared in the inner plexiform layer (Fig. 5E, white arrowheads). At day 8, amelioration of retinal structure obscuration was observed, accompanied by an increase in the number of cells in the inner plexiform layer and the revelation of cell nests which had hyperchromatic nuclei (Fig. 5F, black arrow).

Measurement of iodine in the blood and the eyeballs of adult zebrafish and rats

Table 1 shows the concentrations of iodine in the blood from adult zebrafish and rats. After 7 days exposure of adult zebrafish to sodium iodate, the concentration of iodine was approximately 450 times higher as compared with the control group. The concentration of iodine in the blood in rats rapidly increased 5 min after intravenous injection, and was sustained thereafter up to 2 hr (Table 1). Seven days after injection, the concentration of iodine in the blood from rats returned to the base line.

Table 2 shows the concentration of iodine in the eyeballs of adult zebrafish and rats. The concentration of iodine in adult zebrafish was significantly higher in the treatment group at 7 days than that of the control group. The concentration of iodine in the eyeballs of rats also increased rapidly 5 min after intravenous injection, and was sustained 2 hr after injection. Seven days after injection, the concentration of iodine in eyeballs of rats had decreased to the base line.

As shown in Fig. 6, there were no histopathological changes at 5 min or at 2 hr after injection in rat retina. The outer nuclear layer, the IS/OS junction, and the retinal pigment epithelium layer were disrupted 7 days after injection. Simultaneously, retinal folding was evident and the inner nuclear layer showed abnormal shape resulting from compression with the outer nuclear layer. There were no histopathological changes in the ganglion cell layer.

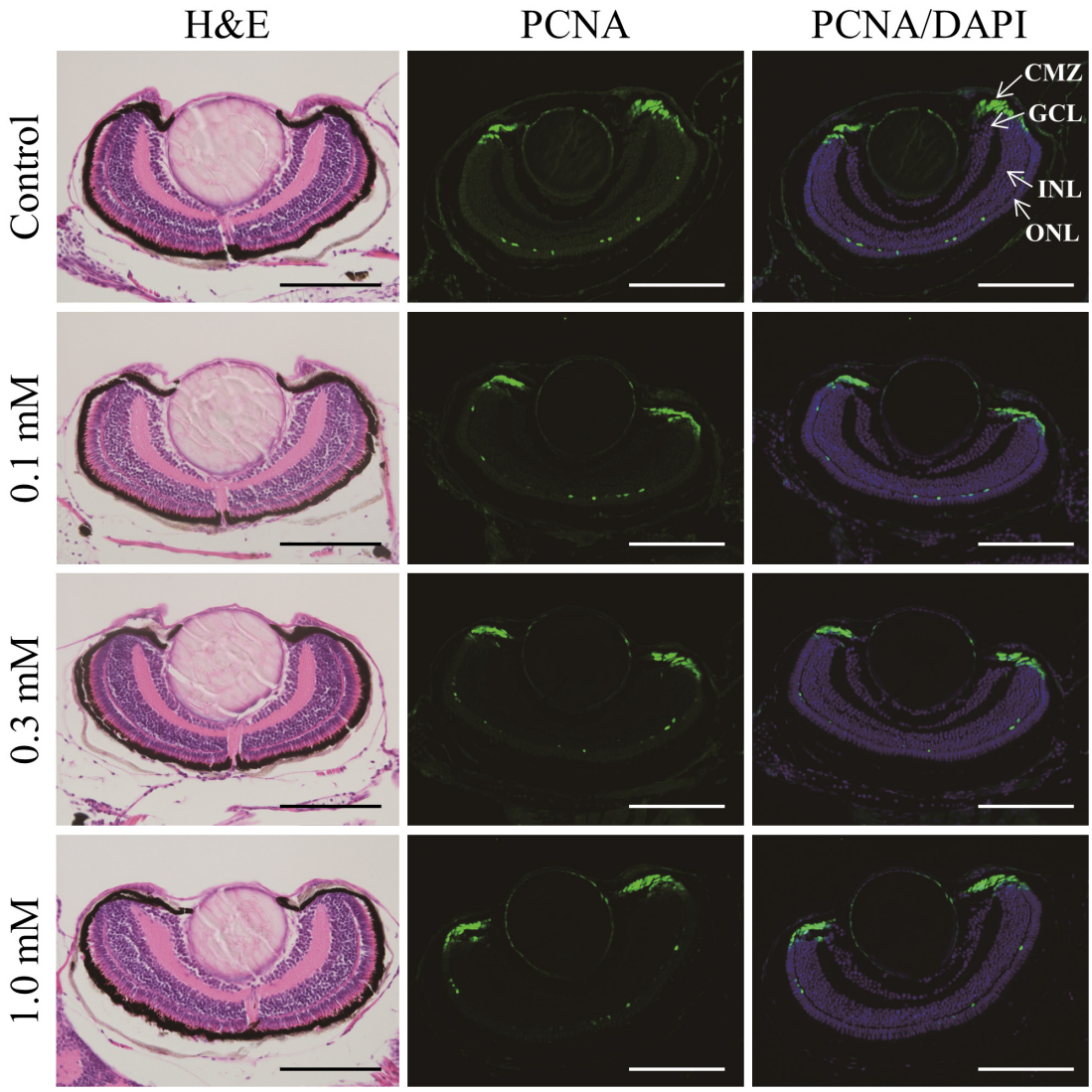

Fig. 1. Representative images of H&E and PCNA staining of larval zebrafish eyes after sodium iodate exposure at 0.1, 0.3 and 1.0 mM, respectively, from 3 to 8 dpf. No significant differences were observed in H&E and PCNA staining between the control group and the treatment groups. PCNA-positive cells were observed in the CMZ and the ONL. Green: PCNA; Blue: DAPI; CMZ: ciliary marginal zone; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars indicate 100 μm.

DISCUSSION

In this study, we investigated sodium iodate-induced histopathological changes in zebrafish retina, but did not observe retinal lesions as histopathological changes at any of three different developmental stages of zebrafish in short-term (larvae for 5 days, juvenile and adult for 7 days) studies and a long-term (adult for 30 days) study. In order to compare the retinal lesions between zebrafish and rats, sodium iodate was injected intravenously in rats at a dose of 40 mg/kg, which generates toxicity consistently to the retina but not to other organs [21]. In contrast to zebrafish, the outer nuclear layer, the IS/OS junction, and the retinal pigment epithelium layer in rats were disrupted 7 days after injection. Furthermore, adult zebrafish were exposed to gentamicin or chloroquine, which are known as retinal toxicants, for 7 days at their maximum tolerated doses in our preliminary study, but these chemicals did not induce histopathological changes in the retina (data not shown). These findings lead us to consider that zebrafish have a different feature of reactivity against sodium iodate from rats.

We examined the effect of MNU on retina in adult zebrafish morphologically. As the result, MNU induced retinal damage in zebrafish. This also verified our experimental procedure for testing retinal damage in zebrafish under chemical exposure in an aquarium. MNU is well known to be an alkylating agent, and induces severe retinal degeneration in mammals such as mice [15] and rats [14]. The lesions are caused by DNA alkylation of the outer nuclear layer [14]. It is noteworthy that the retinal lesion induced by MNU is somewhat different between zebrafish and mammalian species, i.e., we observed the pyknotic cells in the inner nuclear layer and the ganglion cell layer first, and then the number of cells in the inner nuclear layer and the outer nuclear layer

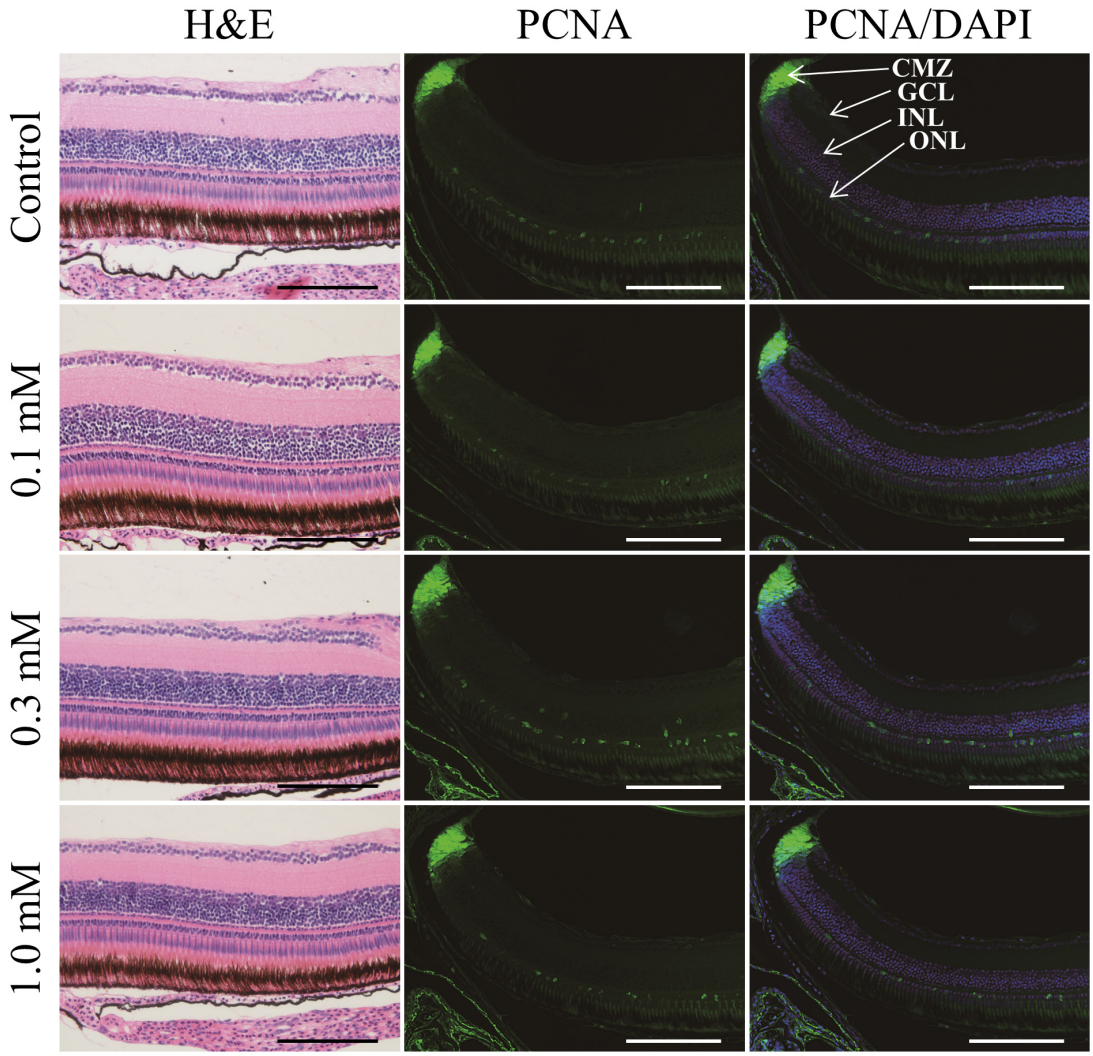

Fig. 2. Representative images of H&E and PCNA staining of juvenile zebrafish retinas after sodium iodate exposure at 0.1, 0.3 and 1.0 mM, respectively, for 7 days. No significant differences were observed in H&E and PCNA staining between the control group and the treatment groups. PCNA-positive cells were observed in the CMZ and the ONL. Green: PCNA; Blue: DAPI; CMZ: ciliary marginal zone; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars indicate 100 μm.

gradually decreased, which is different from the findings in previous reports [17, 18]. These results indicate the difference in MNU-induced retinal morphological changes between zebrafish and mammals.

It is well known that zebrafish regenerate their retinal tissue after physical damage [23], chemical toxicity [6, 17], and light stimulation [19]. As is evident from distribution of PCNA-positive cells in our experiments, many PCNA-positive cells were found in the ciliary marginal zone and the outer nuclear layer in larval and juvenile zebrafish. In adult zebrafish retina, PCNA-positive cells were found in the same sites, but the number of cells in adult zebrafish was much less than that of cells in larvae and juveniles. The number of PCNA-positive cells was comparable between the control and the sodium iodate-treated groups in all three developmental stages of zebrafish. Therefore, it is apparent that zebrafish possess the regenerative activities during maturation and aging, even though some difference exists in the parts of retina. Muto *et al*. [13] presented that zebrafish mutants generated with ethylnitrosourea are morphologically indistinguishable from wild type, implying biological stability in retinal morphology. It appears to be difficult to observe histopathological changes, especialy if they are slight, in zebrafish retina because of their regeneration activity during maturation or morphological stability. Future research is required to clarify these different reactivities of zebrafish from those of mammalian animals against some retinal toxicants.

The concentration of iodine in the blood and the eyeballs of adult zebrafish and rats increased remarkably. The concentration of iodine in zebrafish was higher than that in rats, indicating that zebrafish should obtain sufficient exposure to sodium iodate in aquariums. This result rules out the possibility of low exposure level as a cause of apparent insensitivity of retinal changes to sodium iodate in zebrafish.

We used different strains of zebrafish in this study. However, when adult zebrafish of AB strain were exposed to sodium iodate

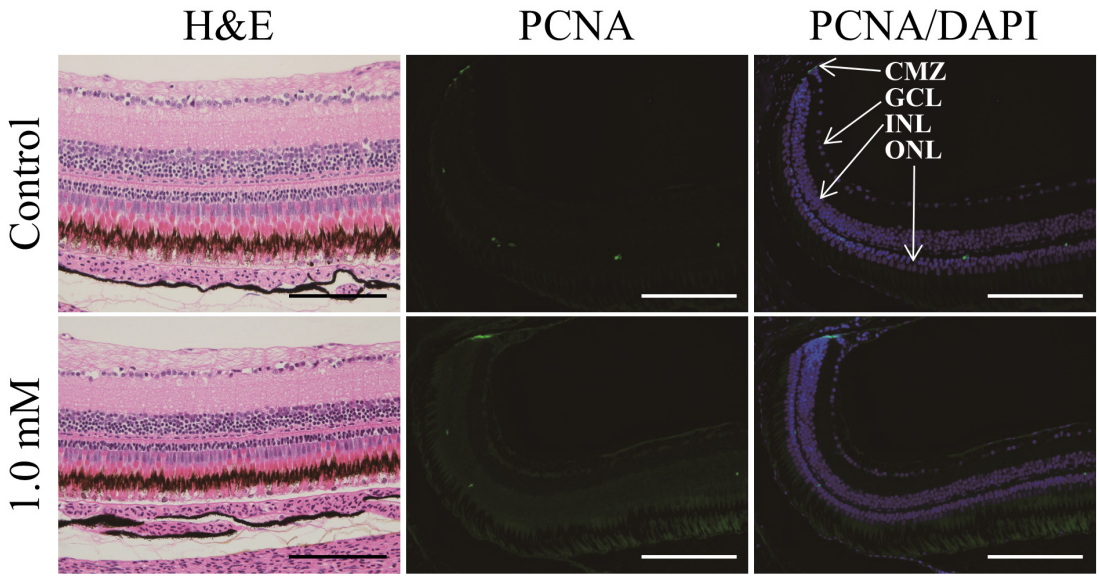

Fig. 3. Representative images of H&E and PCNA staining of adult zebrafish retinas after sodium iodate exposure at 1.0 mM for 7 days. No significant differences were observed in H&E and PCNA staining between the control group and the treatment group. PCNA-positive cells were observed in the CMZ and the ONL. Green: PCNA; Blue: DAPI; CMZ: ciliary marginal zone; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars indicate 100 μm.

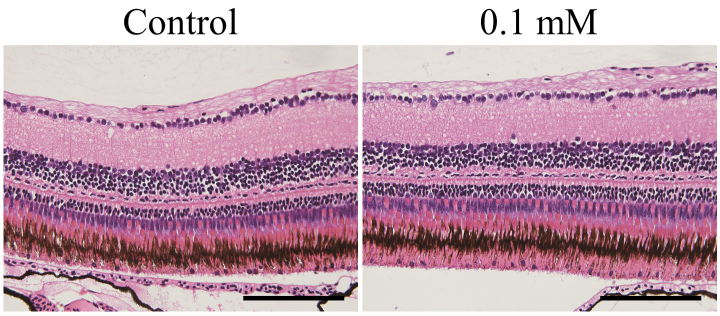

Fig. 4. Representative images of H&E staining of adult zebrafish retinas after sodium iodate exposure at 0.1 mM for 30 days. No significant differences were observed between the control group and the treatment group. Scale bars indicate 100 μm.

Table 1. Concentration of iodine in the whole blood of adult zebrafish and rats after sodium iodate treatment at different time points

Sample No.	Concentration in blood (*ng*/m*l*)					
	Adult zebrafish (1.0 mM)		Rat (40 mg/kg)			
	Control	Day 7	Control	5 min	2 hr	Day 7
1	57.0	24,299.1	253.4	3,681.2	15,341.2	144.8
2	94.9	49,584.8	182.7	11,845.1	10,709.8	100.4
3	70.1	28,923.8	71.5	17,046.8	8,085.6	91.9
Average	74.0	34,269.2	169.2	10,857.7	11,378.9	112.4

for 7 days at 1.0 mM, no histopathological changes were found (data not shown) as well. Therefore, we think that strain differences did not affect the results of this study.

The present results give us cause for caution in using zebrafish as a model of mammals including humans. Taken together, our findings indicate that it is difficult to detect retinal histopathological changes due to tolerance of organism. Moreover, zebrafish have a different reactivity pattern from mammals against retinal toxicants.

In summary, we present that zebrafish have different features of reactivity from mammals against retinal toxicants, which cause

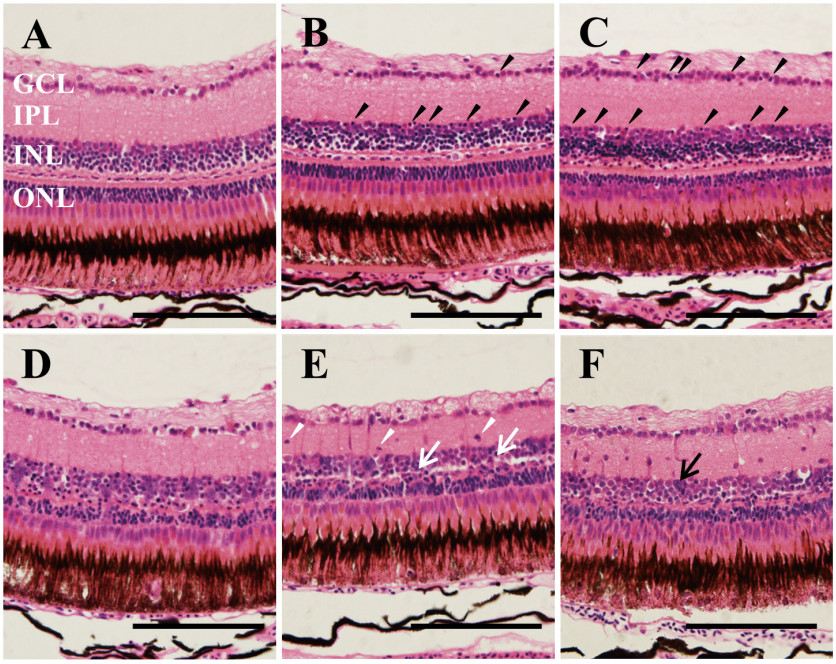

Fig. 5. Representative images of H&E staining of adult zebrafish retinas after MNU exposure. Each group of zebrafish was exposed to MNU for 60 min and thereafter maintained under standard conditions for 6 hr, 24 hr, 3 days, 5 days and 8 days, respectively. (A) Vehicle control; (B) Six hr of maintenance after exposure, the pyknotic cells (black arrowheads) started to appear in the INL and the GCL; (C) Twenty four hr of maintenance after exposure, the number of pyknotic cells increased (black arrowheads); (D) Three days of maintenance after exposure, obscuration of retinal structure between the INL and the ONL was observed; (E) Five days of maintenance after exposure, some parts of the INL were fused with the ONL (white arrow); (F) Eight days of maintenance after exposure, accumulation of cell clusters was observed in the INL (black arrow) and cells appeared in the INL (white arrowheads). GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars indicate 100 μm.

Table 2. Concentration of iodine in the eyeballs of adult zebrafish and rats after sodium iodate treatment at different time points

Animal No.	Concentration in ocular tissue (*n*g/g)					
	Adult zebrafish (1.0 mM)		Rat (40 mg/kg)			
	Control	Day 7	Control	5 min	2 hr	Day 7
1	26.0	4,863.6	97.2	444.5	4,953.5	50.3
2	26.6	4,285.9	117.6	1,541.8	3,603.2	BLQ (33.0)
3	21.6	6,663.9	BLQ (12.9)	4,562.4	2,729.3	BLQ (16.6)
Average	24.7	5,271.1	107.4	2,182.9	3,762.0	50.3

BLQ: Below the lower limit of quantification (50 *n*g/g). (): Extrapolation value.

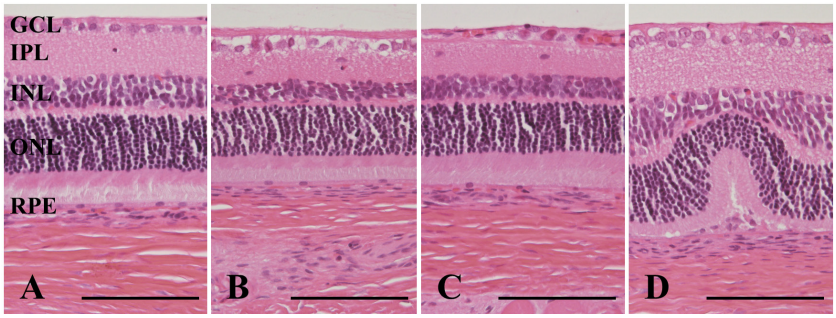

Fig. 6. Representative images of H&E staining of rat retinas after sodium iodate treatment at 40 mg/kg. (A) Vehicle control; (B) At 5 min after the treatment; (C) At 2 hr after the treatment; (D) Seven days after the treatment, the ONL, IS/OS junction and the RPE layer were disrupted and retinal folding was observed. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; RPE: retinal pigmented epithelium. Scale bars indicate 100 μm.

difficulties in detecting histopathological changes from chemical insults, even under sufficient chemical exposure. The present results provide information in regards to the use of zebrafish as a model for mammals for retinal toxicity and basic knowledge concerning characteristics of poisoning in fish.

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