Short Communication

Effects of cadmium exposure on medaka (Oryzias latipes) testes

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Abstract: Adult male medaka (Oryzias latipes) were exposed to 10 ppm of cadmium for 96 h, and the testes were examined histopathologically. Numerous apoptotic cells were found in the spermatogonia and spermatocytes at 72 and 96 h after initiation of cadmium exposure, and the pyknotic index, TUNEL-positive rate, and cleaved caspase-3-positive rate in the spermatogonia and spermatocytes of the cadmium-treated group were higher compared with the control group. No significant difference between the control and cadmium-treated groups was found in the phospho-histone H3-positive rate in the spermatogonia and spermatocytes. No edematous, hemorrhagic, or necrotic changes were observed within the testes in the cadmium-treated group. These results suggest that spermatogonia and spermatocytes in medaka testes are highly sensitive to cadmium. Exposure to 10 ppm of cadmium induced histopathologic changes in the testes that were similar to those described in rodents exposed to low doses of cadmium. (DOI: 10.1293/tox.2017-0015; J Toxicol Pathol 2017; 30: 255–260)

Key words: apoptosis, cadmium, medaka, spermatocyte, spermatogonia, testis

Cadmium is a heavy metal and a major environmental toxicant associated with many industrial processes1, 2. Recent increases in the production and use of cadmium-containing industrial products has resulted in higher exposure of workers in manufacturing industries as well as a greater level of public exposure to cadmium, principally via absorption through cadmium-contaminated water or soil3. In rodents, acute or chronic exposure to cadmium results in cadmium accumulation in many organs, which can lead to a variety of disorders4. Studies of cadmium intoxication in rodents have revealed that the testis is an important target organ1, 3, 4. In rodents, cadmium exposure induces cell death via apoptosis and necrosis of germ cells in the testicular tissue5, 6. In rodent testes, necrosis of germ cells arises secondarily as a result of cadmium-induced disruption of the vascular system7. By contrast, cadmium-induced apoptosis of testicular germ cells in rodents is provoked via upregulation of Bax and caspase-3 expression and downregulation of Bcl-XL and Bcl-2 expression5, 7.

As a result of runoff, aquatic ecosystems are the final receptacle of land-based chemicals; therefore, fish are suitable models for evaluating the toxic effects of cadmium8. Only a few histopathologic studies of cadmium-induced testicular damage in fish have been reported. These have been confined to the following three species: Gymnotus carapo (banded knife fish), Pseudosciaena crocea (large yellow croaker), and Oreochromis niloticus (Nile tilapia)9, 10. With respect to the Organization for Economic Cooperation and Development (OECD) guidelines, medaka (Oryzias latipes) is recognized as a suitable model fish for chemical toxicity testing. Nevertheless, there are no reports examining the histopathologic effects of cadmium on medaka testes, and the pathogenesis of cadmium-induced testicular damage in medaka remains unclear. Therefore, in the present study, we examined the temporal changes in histopathologic characteristics in the testes of adult male medaka exposed to cadmium.

Adult male NIES-R strain medaka at 4–6 months posthatching were used in the study. The fish were maintained at 25–26°C in a recirculating aquaculture system equipped with carbon filtration and biofiltration. The photoperiod was adjusted to a 14 h:10 h (light:dark) cycle. The medaka were obtained from the National Institute for Environmental Studies (Ibaraki, Japan). The present experiments were performed according to the provisions approved by the Animal Research Committee of Tottori University.

A total of 80 fish (body length: 28.34 ± 0.23 mm) were divided into two groups as follows: (1) control group (n = 40) and (2) cadmium-treated group (n = 40). Cadmium chloride (Wako, Osaka, Japan) was dissolved in dechlorinated tap water a concentration of 10 ppm. For the exposure ex-
Experiments, fish were kept in the cadmium-containing water and sampled and examined histopathologically at 24, 48, 72, and 96 h (10 fish per group per time point). The decision to use this concentration was based on the results of a preliminary study in which exposure at 0.1 and 1 ppm for 96 h induced few histopathologic changes in the testes, whereas exposure to 10 ppm caused stable pyknotic changes in the testes. No individual differences in the pyknotic rate were observed in the 10 ppm cadmium-treated group. The water was changed each day, and fresh cadmium was added to it. The fish were euthanized by prolonged immersion in 100 mg/l tricaine methanesulfonate (Tokyo Chemical Industry, Tokyo, Japan) and fixed in Bouin’s fluid overnight before being refixed in neutral buffered formalin, embedded in paraffin, cut into sagittal sections, and routinely stained with hematoxylin-eosin. Histopathologic examinations of whole organs were also carried out.

DNA-fragmented cells in the testicular tissue were detected by terminal deoxynucleotidyl-transferase-mediated deoxuridine triphosphate-digoxigenin nick-end labeling (TUNEL), which was performed using TACS2 TdT-DAB In Situ Apoptosis Detection Kit (Trengen, Inc., Gaithersburg, MD, USA). The TUNEL-positive rate was calculated as the percentage of TUNEL-positive cells among the total number of component cells within the spermatogonia and spermatocytes.

Immunohistochemical staining was carried out according to the labeled-polymer method using Histofine Simple Stain MAX-PO (R) (Nichirei, Tokyo, Japan). To retrieve the antigen, tissue sections for the detection of cleaved caspase-3 were immersed in citrate buffer at pH 6.0 (Dako, Glostrup, Denmark) and autoclaved for 15 min at 121°C. Tissue sections for the detection of phospho-histone H3 were immersed in citrate buffer at pH 6.0 (Dako) and microwaved for 15 min. Histone H3, a protein involved in chromatin structure, is phosphorylated at serine 10 during chromatin condensation in mitosis; therefore, phospho-histone H3 is recognized as a mitosis-specific marker. Endogenous peroxidase activity was quenched by immersing the sections in 3% hydrogen peroxide in methanol for 15 min. The sections were incubated with cleaved caspase-3 rabbit polyclonal antibody (1:50 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at room temperature. The sections were also incubated with phospho-histone H3 rabbit monoclonal antibody (1:1,500 dilution; Abcam, Tokyo, Japan) for 30 min at room temperature. The sections were then treated with Histofine Simple Stain MAX-PO (R) (Nichirei, Tokyo, Japan) for 30 min at room temperature. After incubation in 3,3’-diaminobenzidine solution containing hydrogen peroxide (Nichirei, Tokyo, Japan) to facilitate a peroxidase color reaction, the sections were counterstained with Mayer’s hematoxylin. The cleaved caspase-3-positive rate and phospho-histone H3-positive rate were calculated as the percentage of cleaved caspase-3-positive cells and phospho-histone H3-positive cells among the total number of spermatogonia and spermatocytes.

All values are expressed as the mean ± standard error (SE). Comparisons between the control group and the cadmium-treated group were carried out using Excel Toukei 2015 statistical software (SSRI Co., Ltd., Tokyo, Japan). Data from two groups were analyzed with an F-test. Welch’s t-test was also performed because variances were not homogeneous (P<0.05).

In the control group, all fish survived throughout the experimental period. In the cadmium-treated group, all fish survived for at least 48 h (Table 1). Of a total of 40 cadmium-treated fish, 8 survived for 72 h (mortality rate: 20%), and 6 fish survived for 96 h (mortality rate: 40%) (Table 1). Neither behavioral disorders nor abnormal appearance was observed in fish in the cadmium-treated group until 72 h, although the fish appeared lethargic beginning at 72 h.

Microscopic examination of whole medaka organs revealed histopathologic changes only in the testes. Throughout the experimental period, few pyknotic cells were observed in the spermatogonia and the spermatocytes within the testes in the control group (Fig. 1 and 2). In the cadmium-treated group, there were few pyknotic cells in the spermatogonia and spermatocytes at 24 and 48 h after initiation of cadmium exposure (Fig. 1). At 72 and 96 h, there were more pyknotic cells in the spermatogonia and spermatocytes of the cadmium-treated group than in the control group (Fig. 2). The pyknotic cells in the spermatogonia and spermatocytes of the cadmium-treated group were positive for TUNEL staining and cleaved caspase-3 (Fig. 3 and 4). At 72 and 96 h, the pyknotic index and the TUNEL-positive and the cleaved caspase-3-positive rates in the spermatogonia and spermatocytes in the cadmium-treated group were higher compared with the control group (Fig. 1). Throughout the experimental period, almost equal numbers of phospho-histone H3-positive cells were observed in the spermatogonia and spermatocytes in the cadmium-treated and control groups (Fig. 5). In addition, no statistically significant differences in the phospho-histone H3-positive rate in the spermatogonia and spermatocytes were observed between the control and cadmium-treated groups throughout the experimental period (Fig. 1).

In the present study, exposure to 10 ppm of cadmium for 96 h induced an increase in the number of pyknotic cells in the spermatogonia and spermatocytes in medaka testes. The pyknotic cells in the spermatogonia and spermatocytes were positive for TUNEL staining and cleaved caspase-3.

### Table 1. Effects of Cadmium on Medaka

| Treatment | Total no. of medaka | No. of live medaka | Mortality rate (%) |
|-----------|---------------------|--------------------|-------------------|
| 24 h      | Control             | 10                 | 10                | 0                 |
|           | Cadmium             | 10                 | 10                | 0                 |
| 48 h      | Control             | 10                 | 10                | 0                 |
|           | Cadmium             | 10                 | 10                | 0                 |
| 72 h      | Control             | 10                 | 10                | 0                 |
|           | Cadmium             | 10                 | 8                 | 20                |
| 96 h      | Control             | 10                 | 10                | 0                 |
|           | Cadmium             | 10                 | 6                 | 40                |
Cleavage of caspase-3 is associated with apoptosis and thus serves as an apoptosis marker. These results indicate that the observed pyknotic changes in the spermatogonia and spermatocytes of medaka testes induced by exposure to 10 ppm of cadmium were due to apoptosis. Cadmium exposure is known to induce both apoptotic and necrotic cell death in the germ cells of rodent testes. A previous study involving rodents demonstrated that low-dose (15 and 20 µM/kg) exposure to cadmium is sufficient to induce apoptosis, whereas exposure to high doses (25 or 30 µM/kg) of cadmium induces necrosis in the testes. In the present study, a 96 h exposure to 10 ppm of cadmium induced apoptosis of spermatogonia and spermatocytes in medaka testes. The observed histopathologic findings in the testes were similar to those reported for testes of rodents exposed to a low dose of cadmium. Other studies have shown that cadmium-
induced necrosis in the testes occurs secondarily to primary disruption of the vascular system (e.g., increased vascular permeability in testicular tissue induced by cadmium). Another previous study demonstrated that edema and hemorrhage precede necrotic changes in rodent testicular tissue exposed to cadmium. In the present study, no edematous, hemorrhagic, or necrotic changes were observed in testes of medaka exposed to 10 ppm of cadmium for 96 h.

A previous study in Gambusia holbrooki (mosquito fish) demonstrated that the testes tend to accumulate cadmium to a greater degree than other organs. In the present study, there were no microscopic findings in organs other than testes in medaka exposed to 10 ppm of cadmium; it is thus possible that cadmium tends to accumulate in the testes more so than other organs in medaka as well. Additionally, in the present study, exposure to 10 ppm of cadmium
induced apoptosis in the spermatogonia and spermatocytes in the testes. These histopathologic findings suggest that spermatogonia and spermatocytes in medaka are sensitive to cadmium. In mouse testes, the spermatogonia are more sensitive to cadmium than other germ-line epithelial cells.

Several previous studies revealed that cadmium disrupts the hypothalamus-pituitary-gonadal axis in medaka, exposure to 1 µg/l of cadmium in ovo in male medaka was shown to elevate hepatic vitellogenin, an estrogen-dependent glycolipophosphoprotein. In males, the vitellogenin gene is normally silent, although it can be activated by estrogenic exposure. It was assumed that the elevation in hepatic vitellogenin in male medaka was caused by the estrogenic activity of cadmium, because cadmium exhibited estrogenic activity. On the other hand, estrogenic compounds induce apoptosis of testicular germ cells such as spermatocytes and spermatogonial stem cells. In the present study, the observed apoptosis of spermatogonia and spermatocytes could have been due at least in part to the estrogenic activity of cadmium.

In conclusion, a 96 h exposure to 10 ppm of cadmium induced significant apoptosis of spermatogonia and spermatocytes; however, it did not induce edematous, hemorrhagic, or necrotic changes in testicular tissue. Additionally, cadmium exposure did not inhibit the proliferation of spermatogonia or spermatocytes. These histopathologic findings were similar to those reported for rodent testes exposed to a low dose of cadmium. The results of this study suggest that the evaluation of testicular toxicity using medaka is an alternative to the use of rodents. To our knowledge, this is the first report demonstrating histopathologic findings of testicular damage induced by cadmium exposure in medaka.

Disclosure of Potential Conflicts of Interest: We have no conflicts of interest to declare.

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