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Radiation effects on wild medaka around Fukushima Dai-ichi Nuclear Power Plant assessed by micronucleus assay

Kouichi Maruyama[1,*], Bing Wang[2], Kazutaka Doi[3], Koji Ishibashi[4], San'ei Ichikawa[5], Yoshiaki Furuhata[5], Masahide Kubota[5] and Yoshito Watanabe[1]

[1]Department of Radioecology and Fukushima Project, Center for Advanced Radiation Emergency Medicine, Quantum Medical Science Directorate, National Institutes for Quantum and Radiological Science and Technology, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan
[2]Department of Radiation Effects Research, National Institute of Radiological Sciences, Quantum Medical Science Directorate, National Institutes for Quantum and Radiological Science and Technology, 4-9-1 Anagawa, Inage-Ku, Chiba 263-8555, Japan
[3]Center for Radiation Protection Knowledge, National Institute of Radiological Sciences, Quantum Medical Science Directorate, National Institutes for Quantum and Radiological Science and Technology, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan
[4] Tokyo College of Environment, 3-3-7 Kotobashi, Sumida-ku, Tokyo 130-0022, Japan
[5]Japan Wildlife Research Center, 3-3-7 Kotobashi, Sumida-ku, Tokyo 130-8606, Japan
*Corresponding author: Department of Radioecology and Fukushima Project, Center for Advanced Radiation Emergency Medicine, Quantum Medical Science Directorate, National Institutes for Quantum and Radiological Science and Technology, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan.
Tel: +81-43-206-3085; Fax: +81-43- 251-4582; Email: maruyama.kouichi@qst.go.jp
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ABSTRACT

Since the Fukushima Dai-ichi Nuclear Power Plant (F1-NPP) accident in 2011, radiation effects on wildlife in the contaminated areas have been a major concern. The outskirts of the F1-NPP are mainly rural areas, where many rice fields, streams and reservoirs are located. We searched for wild medaka (small aquarium fish) around the F1-NPP and found two wild medaka habitats (S1 and S2). S1 is a stream located 4 km from the F1-NPP, where the ambient dose equivalent rate was 0.4–0.9 μSv/h (2013–14), and S2 is a reservoir located 7.5 km from the F1-NPP, where the ambient dose equivalent rate was 9.8–22 μSv/h (2013–14 and 2017–18). Dosimeters were placed for one day at the locations where the medaka were captured, and the absorbed dose rates were estimated. Radiation effects on wild medaka were examined using micronucleus assay between 2013 and 2018. No significant difference in frequency of micronucleated gill cells was observed among the wild medaka from S1, S2 and our cultivated medaka that were used as a control.

Keywords: medaka; radiation effects; Fukushima; F1-NPP; micronucleus assay; gill

INTRODUCTION

Since the 2011 nuclear accident in Fukushima Dai-ichi Nuclear Power Plant (F1-NPP), radiation impact on wildlife in the contaminated areas has been studied in animals, insects and plants, and accumulating data showing either significant or non-significant radiation effects have been reported. In mammals, hematological changes were observed in wild Japanese monkeys [1]. Moreover, the frequency of chromosomal aberrations in splenic lymphocytes was increased in mice [2, 3]. On the other hand, sperm morphology and spermatogenesis were reported within the normal ranges in livestock bulls [4] and neither was any morphological abnormality of sperm reported in mice [5]. In amphibians, no changes in carotenoid concentrations or morphology in germ cells were observed in frogs [6, 7]. In insects, morphological abnormalities and aberrant color patterns were reported in butterflies [8], whereas the inversion frequency of chromosomes was in the normal range in fruit flies [9]. In plants, the frequency of morphological abnormalities was increased in fir trees [10]. However, these studies have focused on terrestrial wildlife, and few studies on radiation effects have been conducted in fish, although transfer of radionuclides to fish has been well studied in lakes, rivers and seas [11, 12].

Medaka (*Oryzias latipes*), small freshwater fish widely distributed in Japan, are used in studies of radiation biology [13–15]. They have

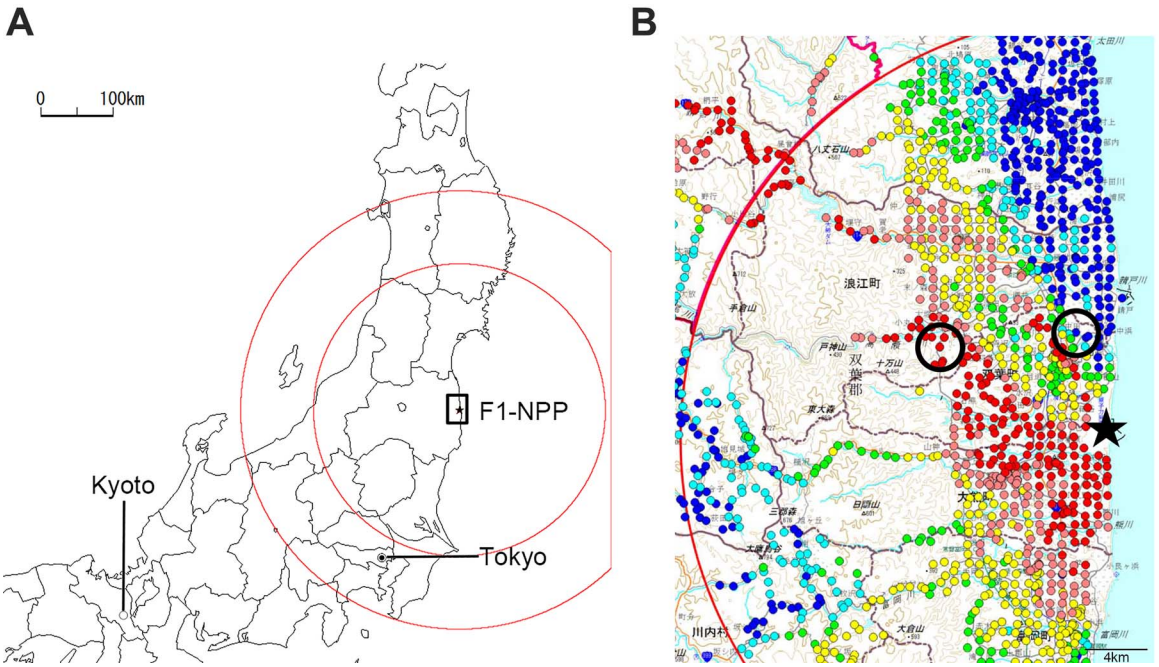

Fig. 1. Location of the sampling sites. The star symbol in the map indicates the location of the F1-NPP. (A) Map of northeast Japan. Small and large circles indicate distances of 200 and 300 km from the F1-NPP, respectively. (B) Enlarged map of areas around the F1-NPP [boxed area in (A)]. The colored spots represent ambient dose equivalent rates 4 or 5 months after the accident: blue, 0–1.0 μSv/h; light blue, 1–1.9 μSv/h; yellowish green, 1.9–3.8 μSv/h; yellow, 3.8–9.5 μSv/h; pink, 9.5–19.0 μSv/h; and red, > 19.0 μSv/h. The small circles, right and left, indicate S1 and S2, respectively. The large red circle indicates a distance of 20 km from the F1-NPP. The scale bar represents 4 km. This ambient dose equivalent map was produced by the Ministry of Economy, Trade and Industry [16].

also been used for ecotoxicity evaluation of chemicals as recommended by the Organization for Economic Co-operation and Development (OECD). Around the F1-NPP, there are many paddy fields, streams and ponds that wild medaka can inhabit. Therefore, we clarified the radiation effects on wild medaka inhabiting areas around the F1-NPP using micronucleus assay, which is an established method for evaluating chromosomal damage induced by ionizing radiation.

MATERIALS AND METHODS

Ethics

All experimental protocols involving the medaka were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institutes for Quantum and Radiological Science and Technology (QST). The experiments were performed in accordance with the QST guidelines for the care and use of laboratory animals.

Sampling of wild medaka

Wild medaka were sampled at 2 sites, S1 and S2 (Fig. 1). S1 is a stream, and wild medaka were captured by a scoop net between 2013 and 2014. S2 is a reservoir, and the wild medaka were captured by a four-armed fishing net between 2013 and 2018. All of the medaka were infected with trichodina. Sampling was supported by the Ministry of the Environment, Japan.

Japanese wild medaka are categorized into 67 mitotypes and 15 subclades based on the cytochrome *b* partial sequence [17]. Following the same procedure, S1 and S2 medaka were predicted to be categorized into the B11 mitotype and B-I subclade (Supplementary Fig. , see online supplementary material).

The ambient dose equivalent rates at a height of 1 m above the ground at the shore of the sampling sites were measured using a NaI (Tl) scintillation detector (Hitachi Aloka Medical TCS-161, Tokyo, Japan).

Measurement of absorbed dose rates

To estimate the absorbed dose rates, Qulxel badge personal dosimeters (Nagase Landauer Limited, Ibaraki, Japan) were used. The dosimeters were wrapped around a rod (Fig. 2A), and the rods were erected at the locations where the wild medaka were captured. In S1 in 2013, two to three dosimeters were set at the surface, bottom and shore for one day (Fig. 2B). In S2 in 2013 and 2014, dosimeters were placed at the surface, middle, bottom and shore for one day (Fig. 2C). Several dosimeters were shielded with thick lead blocks during dose measurements as negative controls. After measurement, all the dosimeters were sent to the provider for dose quantification. The data obtained were the effective doses. According to ICRP pub.74 Table A. 17, the effective dose was converted to the absorbed dose by referring to the energy of the gamma rays emitted by ^{134}Cs and ^{137}Cs [18]. For dosimetry

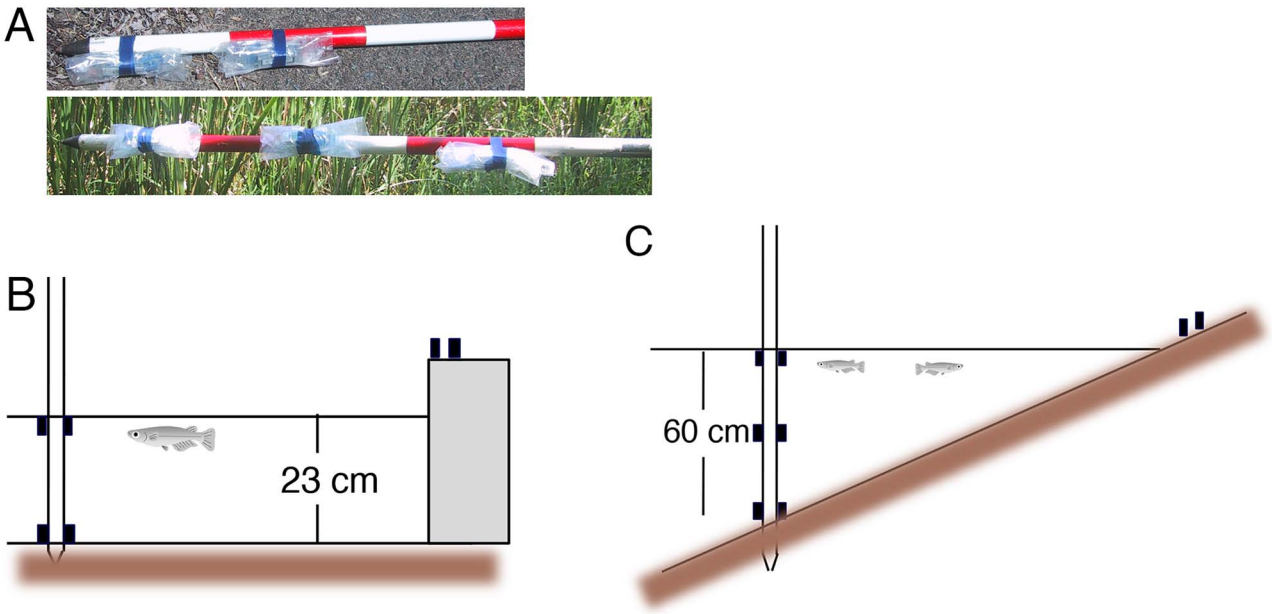

Fig. 2. Schematic drawing for measurement of the dose rate exposure of wild medaka. (A) Images of dosimeters used in S1 (top) and S2 (bottom). (B) Schematic drawing for measuring dose rates in S1. Black boxes represent dosimeters. (C) Schematic drawing for measuring dose rates in S2.

measured in the water, the absorbed dose to water was obtained by multiplying the absorbed dose by the ratio of the mass attenuation coefficient of water to air. The water absorbed dose obtained was used as the absorbed dose of medaka.

Control medaka and irradiation

Our cultivated medaka (Hd-rR inbred strain) were used as both negative and positive controls. Post-hatching larvae were fed Artemia larvae or Super Gold 0 (Oriental Yeast Co., Ltd, Tokyo, Japan), whereas young and adult fish were fed TetraMin (Tetra Werke, Melle, Germany). The fish were maintained under a 14-h light/10-h dark cycle at ~26°C.

As positive controls, cultivated medaka were X-irradiated at a single acute dose of 0.1, 0.5, 1 or 2 Gy. The irradiated medaka were dissected 24 h after irradiation. This irradiation was performed using a 200-kVp X-ray generator (TITAN-320; Shimadzu Co., Kyoto, Japan) at 200 kV and 20 mA with 0.5-mm Cu and 0.5-mm Al filters. The target-to-object distance was 500 mm, and the average dose rate in air was 1 Gy/min.

Micronucleus assay

Micronucleus assay using medaka gills was performed according to Takai *et al.* [19]. Approximately 24 h after capture, the wild medaka were dissected and the gills were collected. The sampled gills were washed in PBS (Nissui Pharmaceutical Co., Tokyo, Japan) and crushed in 200 μL of PBS using a pair of tweezers with flat heads (K-11; KFI Co., Tokyo, Japan). The fragment-free cell suspension was then centrifuged in a 1.5 mL microtube at 800 rpm for 5 min, and the collected cells were subjected to hypotonic treatment with 0.068 M KCl solution for 3 min. The treatment was then terminated by adding a drop of Carnoy's fluid to the tube. The hypotonically-treated cells were collected after centrifugation and fixed with Carnoy's fluid for 10 min. This treatment was performed twice. The fixed cells were centrifuged again and suspended in a 99:1 mixture of methanol and acetic acid (200 μL) for storage until testing. To each of the air-dried preparations, PBS containing 0.004% acridine orange was added, and the stained cell spread was covered with a cover glass. The stained preparations were observed under a fluorescence microscope (BX-53; Olympus, Tokyo, Japan) at a magnification of 400×, and the presence of micronuclei was inspected in gill cells with red-fluorescing cytoplasm. Pictures of the counting area were captured and assembled through an image tiling function using Lumina Vision (Mitani Co, Fukui, Japan). The total number of gill cells was counted in the captured pictures. For each fish, we analyzed between 1004 and 4971 cells (Table 2). Micronucleated cell (MNC) frequency was calculated as the number of MNCs per counted cells.

Statistical analysis

The proportion of MNCs among the number of counted cells was analyzed using the generalized estimating equation approach to account for intra-subject correlations. The logit link function was used to model the probability of a cell being micronucleated and compound symmetry structure was assumed for correlation structure in the same subject. The risk of wild medaka and X-irradiated medaka was presented in terms of odds ratio compared to non-irradiated control medaka.

RESULTS AND DISCUSSION

In the exclusion zone of the F1-NPP accident, wild medaka were sampled at two sites, S1 and S2, which had different contamination

Table 1. Ambient dose rates and equivalent dose rates in water

Place	Date	Ambient dose rate, μSv/h		Equivalent dose rate in water, μGy/h
S1	6-Nov-13	0.9	shore	0.12
			surface (23 cm)	0.12
			bottom (0 cm)	0.13
	7-Aug-14	0.4		
	6-Nov-14	0.4		
S2	6-Nov-13	22	shore	22.25
			surface (60 cm)	7.95
			middle (30 cm)	3.28
			bottom (0 cm)	8.14
	7-Aug-14	15.7	shore	18.6
			surface (60 cm)	7.07
			middle (30 cm)	1.97
			bottom (0 cm)	3.73
	5-Nov-14	12.4		
	5-Sep-17	10.3		
	5-Oct-18	9.8		

levels (Fig. 1). In S1, located ∼4 km north–northwest from the F1-NPP, wild medaka were captured from a stream between 2013 and 2014. This stream is on the side of a road, and its width is narrow and water depth is shallow. The ambient dose equivalent rates were 0.4–0.9 μSv/h in 2013–2014 (Table 1). In 2013, the water depth was 23 cm, and dosimeters were set on the water surface and bottom for 1 day (Fig. 2B). The equivalent dose rates in water were 0.12–0.13 μSv/h, which were much lower than the ambient dose equivalent rate. There was no large difference between the dose rates on the water surface and at the bottom of the stream.

In S2, located ∼7.5 km west–northwest from the F1-NPP, wild medaka were collected from a reservoir between 2013 and 2018. The ambient dose equivalent rates in S2 were higher than those in S1 and decreased from 22 μSv/h in 2013 to 9.8 μSv/h in 2018 (Table 1). The reservoir is large with growing reeds, the water depth is >2 m, and there is a slope beside it. Dosimeters were set on the surface (60 cm), middle (30 cm) and bottom of the water for 1 day where the medaka were captured (Fig. 2C). All of the equivalent dose rates in the measured water were significantly lower than the ambient dose equivalent rate (Table 1). The dose rate in the middle layer (30 cm) was the lowest, which is probably due to water shielding. The dose rate on the surface layer was surprisingly higher, and this may be due to the high radiation from the side slope. Attenuation of all the dose rates was observed from 2013 to 2014.

The micronucleus is a cytoplasmic body with a portion of the acentric chromosome or whole chromosome that is not incorporated into one of the daughter nuclei during cell division, and is a sign of chromosomal damage. Exposure to radiation increases MNC frequency. For medaka, the micronucleus assay technique was established using gill cells [18], which come into direct contact with radioactive cesium in water. In the present study, we adopted this technique to evaluate the radiation effects on wild medaka inhabiting the exclusion zone of the F1-NPP accident.

To confirm the micronucleus assay technique used in this study, MNC frequencies were measured in cultivated medaka (Hd-rR inbred stain), which were non- and X-irradiated for use as negative and positive controls, respectively. As shown in Fig. 3A, the clearly demarcated, yellow-fluorescing small spheres embedded in the red-fluorescing cytoplasm were detected as MNCs. The average MNC frequency was 0.86‰ in non-irradiated medaka (Fig. 3B), which was similar (0.7–0.8‰) to that of other studies [19, 20]. A dose-dependent increase in MNC frequency (1.01‰ at 0.1 Gy, 1.2‰ at 0.5 Gy, 2.14‰ at 1 Gy and 3.02‰ at 2 Gy) was observed in acutely X-irradiated medaka (Fig. 3B). At 0.1 Gy, there was no significant difference from the control, whereas at 0.5 Gy or higher doses, a significant difference from the control was observed.

In S1 and S2, which are in the exclusion zone of the F1-NPP accident, 9 and 21 wild medaka were captured in 2013–2014 and 2013–2018, respectively (Table 2). It is not expected that these captured medaka experienced high dose rates of radiation exposure due to short-lived radionuclides just after the F1-NPP accident in 2011, because the average lifespan of wild medaka is ∼1 year and a few months [21]. In S1 and S2, the average MNC frequencies in the wild medaka were 0.81 and 0.82‰, respectively (Fig. 3B). In S2, the average MNC frequencies in early (2013 and 2014) and late phases (2017 and 2018) were 0.79 and 0.84‰, respectively. The MNCs frequency in wild medaka from S1 and S2 were not statistically different from that in the control cultivated medaka (Fig. 3C).

In this report, no significant effects on micronucleus frequency were observed in wild medaka in radioactively-contaminated areas in Fukushima. In the micronucleus assay, micronucleus frequency may be used as an index for evaluating both the acute effect (genotoxicity) shortly after radiation exposure and the late effect (genomic instability) in the long-term after radiation exposure. Although the detection sensitivity of the micronucleus assay is not as high as gene profiling analysis, it is a useful approach for evaluating phenotype changes, whereas low

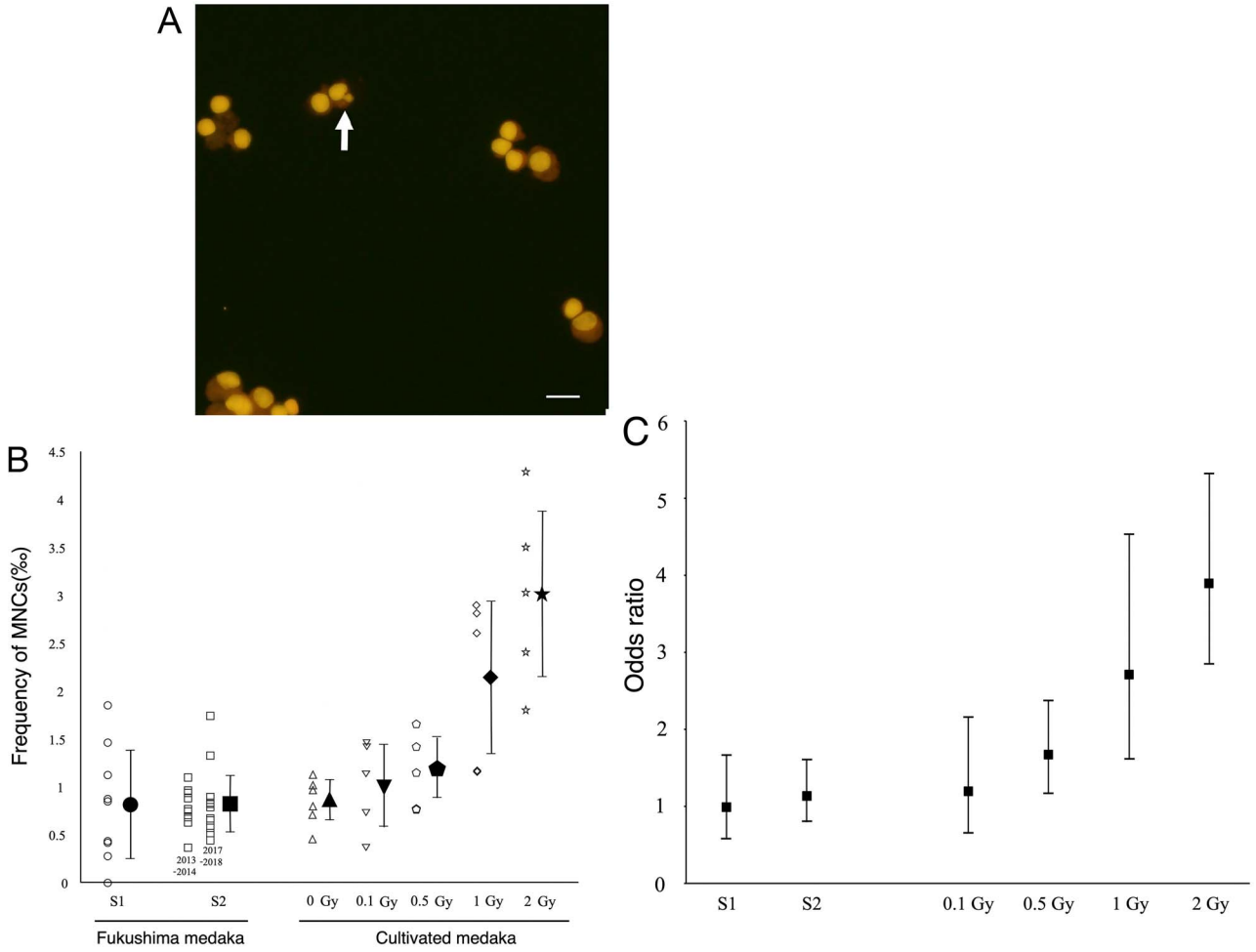

Fig. 3. Micronucleus assay. (A) Acridine orange-stained gill cells of medaka fish. The arrow indicates a micronucleus in a cell. The bar represents 10 μm. (B) Frequency of MNCs in wild and cultivated medaka. Wild medaka were collected from two sites in Fukushima. The cultivated medaka were non- (0 Gy) or X-ray irradiated (0.1, 0.5, 1 and 2 Gy) for use as negative and positive controls, respectively. Each open symbol represents an individual medaka. Closed symbols with vertical bars indicate the average frequency (with standard deviation) of MNCs in each group. (C) Generalized estimating equation approach to account for the intra-subject correlations from the proportion of MNCs. Model-based analysis was used to estimate the risk of MNCs among groups. Odds ratios for medaka in S1, S2, 0.1, 0.5, 1 and 2 Gy irradiation groups compared with medaka without irradiation are given. The vertical line shows the 95% confidence interval of each odds ratio.

doses of radiation often induce transient alteration in gene expressions without phenotype change. As the first approach for studying the radiation effect on wild medaka after the F1-NPP accident, micronucleus assay was adopted in the present study. Micronucleus assay has also been applied for studying long-term irradiation effects and water quality monitoring in rivers [22, 23]. The average lifespan of wild medaka is ~1 year and several months [21]. Therefore, the total absorbed dose would be a maximum of 8.14 μGy/h x 24 h/day x 365 days/year x 1.4 years = 99.8 mGy. The maximum cumulative dose estimated mathematically was about 178 mGy for an individual medaka that hatched in 2011 (when the accident occurred) and survived until 2013 (when it was collected). The micronucleus frequency induced by this dose would be expected to be around the detectable level. However, in the present study, it was estimated based on the body size in length of the collected wild medaka that the majority of fish had hatched after 2011; thus the cumulative dose for the collected medaka in our study was lower due to both the decreased dose rate and the shorter exposure time.

Radiation effects on wildlife in Fukushima have been evaluated by international organizations. UNSCEAR (2017) and IAEA (2015) concluded that exposures to radiation in both marine and terrestrial non-human biota following the F1-NPP accident were generally too low for effects to be observed, although some exceptions had been considered possible because of local variability [24, 25]. Similarly, radiation risks were assessed as negligible for pelagic fish, to which medaka belong, that may inhabit ponds in the exclusion zone within

Table 2. The number of captured medaka and micronucleus cells

Location, Control	Date	Number of captured medaka	Number of counted cells	Average of counted cells	Number of MNCs
S1	6-Nov-13	3	8904	2968	9
	7-Aug-14	3	7263	2421	6
	6-Nov-14	3	11235	3745	5
S2	6-Nov-13	3	6918	2306	6
	7-Aug-14	3	6432	2144	4
	5-Nov-14	3	3918	1306	3
	5-Sep-17	6	12375	2063	12
	5-Oct-18	6	12771	2129	8
0 Gy		7	15760	2251	12
0.1 Gy		5	11396	2279	9
0.5 Gy		5	10658	2131	12
1 Gy		5	9791	1958	19
2 Gy		5	12160	2432	34

Fukushima [26]. Of note, in fish, it was reported that nucleotide substitutions in mitochondrial DNA were observed in salmon collected from a contaminated river in Fukushima [27]. Although the study did not provide information on the dose evaluation or solid evidence showing a causal relationship between radiation exposure and the mutation, their results suggested that nucleotide substitutions in mitochondrial DNA may be a sensitive index for investigating environmental insults to wild fish. The purpose of the present study was to investigate whether there was any significant radiation effect on wild medaka in Fukushima. The micronucleus frequency in wild medaka captured in Fukushima was found to be at a similar level to that of the negative control. Further research is necessary to accurately assess the possible biological effects of the accident on wild medaka using different approaches.

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SUPPLEMENTARY DATA

Supplementary data are available at *RADRES Journal* online.

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