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Effects of chronic γ-irradiation on growth and survival of the Tohoku hynobiid salamander, *Hynobius lichenatus*

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A B S T R A C T

The Tohoku hynobiid salamanders, *Hynobius lichenatus*, were chronically irradiated with γ-rays from embryonic to juvenile stages for 450 days. At 490 μGy h^{-1} or lower dose rates, growth and survival were not significantly affected by irradiation, and any morphological aberrations and histological damages were not observed. At 4600 μGy h^{-1}, growth was severely inhibited, and all the individuals died mostly at the juvenile stage. Chronic LD_{50} was 42 Gy as a total dose. In the liver, the number of hematopoietic cells was significantly reduced in the living juveniles, and these cells disappeared in the dead juveniles. In the spleen, mature lymphocytes were depleted in the living larvae, and almost all the heamtopoietic cells disappeared in the dead juveniles. These results suggest that this salamander died due to acute radiation syndrome, i.e., hematopoietic damage and subsequent sepsis caused by immune depression. The death would be also attributed to skin damage inducing infection. At 18,000 μGy h^{-1}, morphological aberrations and severe growth inhibition were observed. All the individuals died at the larval stage due to a multiple organ failure. Chronic LD_{50} was 28 Gy as a total dose. Assuming that chronic LD_{50} was 42 Gy at lower dose rates than 4600 μGy h^{-1}, a chronic median lethal dose rate could be estimated to be <340 μGy h^{-1} for the whole life (>14 years). These results suggest that, among guidance dose rates, i.e., 4–400 μGy h^{-1}, proposed by various organisations and research programmes for protection of amphibians and taxonomic groups or ecosystems including amphibians, most of them would protect this salamander but the highest value may not on the whole life scale.

1. Introduction

Since 1990s, there have been a number of international initiatives to develop frameworks to provide criteria and a methodological approach for the protection of the environment from ionising radiation (IAEA, 2006; ICRP, 2003, 2008; OECD/NEA, 2007; UNSCEAR, 2011). Frameworks for environmental protection have also been developed on national and regional scales, e.g., in Europe (Howard et al., 2010) and North America (US DOE, 2002). During such development of the frameworks, large knowledge gaps have been identified concerning effects of chronic radiation exposure on non-human species.

Frogs belonging to amphibians are one of the ICRP Reference Animals and Plants (ICRP, 2008), and amphibians are relatively sensitive to acute radiation (UNSCEAR, 2011). For example, the acute LD_{50} values estimated from DNA mass range from 3.1 to 120 Gy for Anura (frogs and toads) and from 2.2 to 6.8 Gy for Caudata (newts and salamanders) (Fuma et al., 2012). On the other hand, amphibians have been identified as a class of organisms for which data concerning effects of chronic radiation exposure are

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especially sparse (Garnier-Laplace et al., 2004). A limited number of chronic irradiation experiments have been made in amphibians (e.g., Panter, 1986; Stark, 2006; Stark et al., 2012).

To our knowledge, there are no data on effects of chronic irradiation on Caudata, which is in general more sensitive to acute radiation than Anura (Conger and Clinton, 1973). In this paper, therefore, the Tohoku hynobiid salamander, *Hynobius lichenatus*, which is one of typical salamanders inhabiting northeast regions of Honshu Island, Japan, was chronically γ-irradiated during the embryonic, larval and juvenile stages, and effects on growth and survival as well as histological changes were investigated.

2. Materials and methods

2.1. Life cycle and radiosensitivity of H. lichenatus

In spring, *H. lichenatus* lays an egg clutch, consisting of two egg sacs, in small ponds or slow-flowing streams surrounded with forests. Eggs hatch in late spring, and larvae emerge. This salamander lives in the aquatic environments from egg to larval stages. In summer, many larvae change to juveniles (metamorphosis). The other larvae spend autumn and winter as they are, and metamorphose in next spring. The metamorphosed juveniles leave the breeding water, and live in terrestrial environments, i.e., under litter and stones in forest floors. They hibernate over the winter period. They attain sexual maturity at several years, i.e., >3 years of age (Yamamoto et al., 2011). Adults live in the same manner as the juveniles. Life span of this species is estimated to be longer than 14 years in natural environments (Yamamoto et al., 2011).

Radiation effects on *H. lichenatus* have not been investigated, and thus the acute LD_{50} is neither determined experimentally nor estimated theoretically. An average LD_{50} of five species of *Hynobius* inhabiting Japan is estimated to be 6 Gy in an acute irradiation regime (Fuma et al., 2012). The acute LD_{50} is therefore estimated to be approximately 6 Gy for *H. lichenatus*.

2.2. Sampling of H. lichenatus eggs

Thirteen egg sacs of *H. lichenatus*, each of which contained 13–33 embryos, were collected on the 11th of May 2012 in a ditch in the mountain forest of Shimogo Town, Fukushima, Japan (N37°15′28.5″ E139°45′13.2″). Embryos were in the gastrula stage (Stage 11, according to development stage of *Hynobius nigrescens*; Iwasawa and Yamashita, 1991). The sampling point is approximately 110 km west of the Fukushima Dai-ichi Nuclear Power Plant, in which a severe accident occurred in March 2011. Based on the airborne monitoring survey data (MEXT, 2012), a radioactive contamination level is considered to be low around the sampling point (on the 7th of May 2012, air dose rate at a height of 1 m: $\leq 0.1\ \mu Sv\ h^{-1}$; deposition of $^{134}Cs + ^{137}Cs$: $\leq 10{,}000\ Bq\ m^{-2}$). Actually, when the sampling was carried out, an air dose rate measured with a NaI(Tl) scintillation survey meter (Hitachi Aloka Medical TCS-161, Japan) was as low as $0.07\ \mu Sv\ h^{-1}$ at a height of 1 m in a forest near the sampling point.

2.3. Rearing and chronic γ-irradiation of H. lichenatus

The salamanders were chronically γ-irradiated at dose rates of 32, 150, 490, 4600 and 18,000 $\mu Gy\ h^{-1}$, which were estimated as described in Section 2.4, from embryonic to juvenile stages. Irradiation was conducted in two rooms, where γ rays were horizontally emitted from ^{137}Cs sources. Irradiation at 32, 150 and 490 $\mu Gy\ h^{-1}$ was conducted in a room where 296 GBq of a ^{137}Cs source equipped with a filter for attenuation of dose rates to 10% of maximum was installed. The salamanders were irradiated at each dose rate in an incubator (i.e., incubators 1, 2 and 3, respectively) whose centre point was at a distance of 7.1, 3.5 and 2.0 m from the γ-ray source, respectively. Irradiation at 4600 and 18,000 $\mu Gy\ h^{-1}$ was conducted in another room where 7.4 TBq of a ^{137}Cs source equipped with a 10% attenuation filter was installed. The salamanders were irradiated at each dose rate in an incubator (i.e., incubators 4 and 5, respectively) whose centre point was at a distance of 3.4 and 1.7 m from the γ-ray source, respectively. The control salamanders were reared in an incubator placed in a non-irradiation room.

Each embryo was separated from the collected egg sacs, and put into 50 mL of aged and aerated tap water in a 100-mL polypropylene container. Initially, these embryos were reared at 15 °C in an incubator with a fluorescent lamp under a 12 h light–dark cycle. Chronic γ-irradiation at 32, 150 and 490 $\mu Gy\ h^{-1}$ was started on the 15th of May 2012 (day 0) by rearing the embryos in the incubators 1, 2 and 3, respectively. At that time, the embryos were in the pre-tail bud stage (Stage 23, according to development stage of *H. nigrescens*; Iwasawa and Yamashita, 1991). Start of chronic γ-irradiation at 4600 and 18,000 $\mu Gy\ h^{-1}$ was delayed, because the irradiation room was not available. The irradiation was started on the 23rd of May 2012 (day 8) by rearing the embryos in the incubators 4 and 5. At that time, the embryos were in the gill formation stage (Stage 39; Iwasawa and Yamashita, 1991). Irradiation was continued during the experiment, i.e., until day 454. At each dose rate, 23–27 embryos were served for observation of effects on survival and growth, and 16–18 embryos were served for histopathological examination and other analysis. Water was renewed once a week.

After hatching (days 15–34), larvae were reared under the same conditions as embryos, but water renewal was carried out twice a week and aquatic oligochaetes *Tubifex tubifex* were given as food twice a week. On day 37, the containers were replaced with 220-mL polycarbonate containers filled with 150 mL of water.

Within 10 days after metamorphosis (days 73–119), the juveniles of *H. lichenatus* began to be reared under the terrestrial conditions in incubators. Each juvenile was reared in a 220-mL polycarbonate (until days 370–377) or 280-mL polypropylene (thereafter) container filled with wet sphagnum moss.

From summer to autumn in 2012 (until day 170) and from spring to summer in 2013 (days 323–454), temperature was maintained at 15 °C. Sphagnum moss was renewed every 10 days. At this renewal, weight of the juveniles was measured, and larvae of moth *Galleria mellonella* were given as food. In addition, sow bugs *Porcellionides pruinosus* and larvae of cricket *Acheta domestica* were given every two weeks.

In winter (days 170–323), the juveniles of *H. lichenatus* were let hibernate. In the first one month, i.e., a pre-hibernation period, temperature was gradually decreased from 15 to 0 °C. In that period, rearing and measurement of body weight were carried out in the same ways as the other seasons. For next two months, temperature was kept at 0 °C to let the juveniles hibernate completely, and renewal of sphagnum moss and measurement of body weight were stopped. Only *P. pruinosus* was given every two weeks. In the last two months, i.e., a post-hibernation period, temperature was gradually increased from 0 to 15 °C. In that period, rearing and measurement of body weight were carried out in the same ways as the other seasons.

The salamanders were reared under a 12 h light–dark cycle during the whole period of irradiation. The containers were randomly moved to the other position in each incubator at feeding and renewals of water or sphagnum moss to ensure equal average radiation exposures.

All experimental protocols involving *H. lichenatus* were reviewed and approved (Experimental Animal Research Plan No. 12-1009-1) by the Institutional Animal Care and Use Committee of