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Radiation tolerance in the tardigrade *Milnesium tardigradum*

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Abstract

Purpose: Tardigrades are known to survive high doses of ionizing radiation. However, there have been no reports about radiation effects in tardigrades under culture conditions. In this study, we investigated tolerance of the tardigrade, *Milnesium tardigradum*, against gamma-rays and heavy ions by determining short-term or long-term survival, and reproductive ability after irradiation.

Materials and methods: Hydrated and anhydrobiotic animals were exposed to gamma-rays (1000 – 7000 Gy) or heavy ions (1000 – 8000 Gy) to evaluate short-term survival at 2, 24 and 48 h post-irradiation. Long-term survival and reproduction were observed up to 31 days after irradiation with gamma-rays (1000 – 4000 Gy).

Results: At 48 h after irradiation, median lethal doses were 5000 Gy (gamma-rays) and 6200 Gy (heavy ions) in hydrated animals, and 4400 Gy (gamma-rays) and 5200 Gy (heavy ions) in anhydrobiotic ones. Gamma-irradiation shortened average life span in a dose-dependent manner both in hydrated and anhydrobiotic groups. No irradiated animals laid eggs with one exception in which a hydrated animal irradiated with 2000 Gy of gamma-rays laid 3 eggs, and those eggs failed to hatch, whereas eggs produced by non-irradiated animals hatched successfully.

Conclusion: *M. tardigradum* survives high doses of ionizing radiation in both hydrated and anhydrobiotic states, but irradiation with >1000 Gy makes them sterile.

Keywords: *Tardigrades,* Milnesium tardigradum, *anhydrobiosis, radiation tolerance*

Introduction

Tardigrades are small invertebrates whose body length ranges from 0.1 – 1.0 mm, and are distributed widely throughout the world under various environmental conditions. Almost all terrestrial tardigrades are known to enter 'anhydrobiosis', a term referring to the ametabolic state induced by dehydration (Keilin, 1959). Anhydrobiotic tardigrades have been shown to tolerate a variety of extreme environmental conditions, such as low and high temperatures (-273°C to $+151^\circ$C), vacuum, high pressure (<600 MPa), and chemicals including alcohols and methyl bromide (Rahm 1921, Seki & Toyoshima 1998, Ramløv & Westh 2001, Jönsson & Guidetti 2001).

Hitherto, only two studies examined tolerance to ionizing radiations in the tardigrades *Macrobiotus areolatus* and *Richtersius coronifer*. Median lethal dose (LD_{50}) of X-rays in *M. areolatus* was about 5000 Gy (May et al. 1964), and that of gamma-rays in *R. coronifer* was about 4700 Gy (Jönsson et al. 2005). Tolerance to X-rays was slightly higher in anhydrobiotic *M. areolatus* than in its hydrated state. On the contrary, hydrated *R. coronifer* was more tolerant to radiation than the anhydrobiotic form,

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suggesting that entering anhydrobiosis is not always effective for protection against radiation in terms of tardigrade lethality. Furthermore, Jönsson et al. (2005) conducted long-term observation of life history (life time, molting, oviposition and hatching) in irradiated *R. coronifer*. Interestingly, they reported that *R. coronifer* was able to lay eggs after irradiation with doses up to 5000 Gy in the hydrated state, and up to 2000 Gy when in anhydrobiosis, although no eggs laid by irradiated animals hatched. These findings indicated that the number of eggs produced after irradiation is higher in hydrated individuals compared to in anhydrobiotic ones.

These reports are important in understanding radiation effects in highly radiation-tolerant organisms, since there have only been limited numbers of investigations using such organisms. In particular, the study of Jönsson et al. (2005) is the first report on the effects of radiation on life history traits in tardigrades. Although that study is an important one, we believe that some improvements in their experimental design are needed. For instance, their animals were not fed after irradiation so it was not clear whether death resulted from the effects of irradiation or poor nutrition, including starvation. In addition, culture temperatures differed between hydrated animals (12°C) and anhydrobiotic ones (20°C). As Jönsson et al. (2005) discussed the difference in life span could be caused by different temperature effects on metabolism, for example. Finally, no eggs produced by *R. coronifer* which had experienced anhydrobiosis hatched. Therefore, we suggest that a better experimental design is needed to evaluate radiation effects in tardigrades more exactly. In the present study, we made the following improvements: (i) irradiated animals were fed, (ii) cultivation was conducted at the same temperature (25°C) for hydrated and anhydrobiotic animals, and (iii) a tardigrade species, *Milnesium tardigradum* was used whose culture methods had already been described (Suzuki 2003). The carnivorous eutardigrade, *M. tardigradum*, used in this study occurs in mosses or lichens which frequently encounter dry conditions. In such habitats, *M. tardigradum* enters anhydrobiosis when water is unavailable. Once water is provided by e.g., rainfall, they absorb water and recover activity. Certain populations of *M. tardigradum* have a thelytokous mode of parthenogenesis. Their life span is <60 days under the continuous rearing environments used by Suzuki (2003).

In the present study, in order to gain additional knowledge about radiotolerance in tardigrades, we compared survival after irradiation with gamma-rays and heavy ions using *M. tardigradum*. This is the first report on post-irradiated effects in *M. tardigradum* under culture conditions.

Materials and methods

Animal preparation

Dry moss, *Bryum* cf. *argenteum*, containing *M. tardigradum* was collected from the vegetation on asphalt pavements in Ibaraki, Japan, and stored under desiccating conditions for <3 months at room temperature, around 25°C, until use. The mosses were immersed for 12–24 h in tap water, and the animals were extracted from rehydrated mosses using a Berman funnels (see Hallas & Yeates 1972). Only animals with active mobility, checked under a stereomicroscope with transmitted illumination (MZ125, Leica, Solms, Germany) were used in the experiment. Medium- and large-sized animals were used for water content measurements, and in experiments of short-term survival after irradiation.

Some individuals were cultured in an artificial rearing system. Animals were reared with water on agar (Wako, Osaka, Japan) (1.5% w/w) in a plastic dish (24 mm diameter), and were fed with unidentified bdelloid rotifers by the method of Suzuki (2003), slightly modified. After several generations in this rearing system, immature animals were used for long-term observation of survival or reproduction after irradiation.

Measurement of water content in hydrated or anhydrobiotic states

Water content in hydrated or anhydrobiotic tardigrades was determined gravimetrically. Fifty active animals or 60 anhydrobiotic ones were weighed with an SE4 electric microbalance (Sartorius, Goettingen, Germany) and re-weighed after heating in an oven at 120°C for 12 h. The weight reduced by heating was considered to be water. In order to prepare anhydrobioic samples, a group of 15 active individuals on a piece of parafilm (Pechiney Plastic Packaging, Inc., Chicago, IL, USA) was desiccated at about 25°C under 85% relative humidity (RH) for over 24 h, and then 0% RH for more than 24 h. RH was controlled by 35% wt./wt. glycerol in water (Invitrogen, Carlsbad, CA, USA) for RH 85% (Johnson 1940) or by silica gel for RH 0%. Four groups of an anhydrobiotic aggregation consisting of 60 individuals were gathered on an aluminum disk with a pin, and were weighed to estimate water content determined as described above.

Irradiation with gamma-rays

Each group containing fifteen hydrated animals suspended in 100 μl distilled water, or of anhydrobiotic animals, was irradiated at room temperature in a 300 μl microtube with gamma-rays (0.2 keV/μm) from ^{60}Co sources at a dose rate of 5.5 to 61.7 Gy/min.

Irradiation with heavy ions

Fifteen hydrated animals suspended in 200 μl of distilled water were placed on a filter paper (1.33 g, diameter 50 mm) in a plastic Petri dish (diameter 50 mm, height 10 mm). For preparation of anhydrobiotic animals, 15 hydrated animals suspended in 100 μl of distilled water were placed on filter paper in the dish and desiccated for >48 h at 0% RH at 25°C. All dishes were covered with a polyimide film (7 μm in thickness, Kapton®, Dupont-Toray, Tokyo, Japan) and tightly sealed with parafilm. The animals were then exposed to ^{4}He (50 MeV, 16.3 keV/μm) delivered from the azimuthally-varying-field cyclotron installed at the Takasaki Ion accelerators for Advanced Radiation Application (TIARA) facility, Japan Atomic Energy Agency (JAEA). The absorbed dose was calculated according to the formula, Dose [Gy] $= 1.6 \times 10^{-9} \times$ linear energy transfer (LET) [keV/μm] $\times$ Fluence [particle/cm^2].

Short-term survival after irradiation

Within 1 h after irradiation dry animals were immersed in distilled water in Petri dishes (24 mm diameter) with 1.5% (w/w) agar on the bottom. Survival was examined at 2, 24 and 48 h after irradiation (hydrated tardigrades) or after rehydration (anhydrobiotic ones). Animals in motion were considered as survivors, and immotile animals, or animals whose body was flaccid, were considered to be dead.

Long-term observation of survival or reproduction after irradiation

M. tardigradum under the culturing system used here was considered to be suitable for the observation of long-term survival and reproductive ability after gamma-irradiation. Animals 2–3 weeks after hatching at the second or third instars in which a developed ovary was not observed were used for these experiments. After gamma-irradiation, the survival, egg laying and hatching of laid eggs were checked daily up to day 8, and thereafter every 4–5 days until day 31.

Data analysis

Unless stated otherwise, 10–15 animals were used in each experiment. Independent experiments were repeated two to four times. Comparisons between groups were made by chi-square test with a Yeats correction, and a *p* value of less than 0.05 was considered to be significant. LD$_{50}$ at 2, 24 and 48 h after irradiation and average life span were estimated by means of linear regression analysis between the survival rate and radiation dose or days after irradiation.

Results and discussion

Short-term survival after irradiation with gamma-rays

We examined the short-term survival of *M. tardigradum* after gamma-irradiation, to compare with results from studies on the other tardigrade species, *M. areolatus* (May et al. 1964), and *R. coronifer* (Jönsson et al. 2005). As seen in Figure 1, *M. tardigradum* showed high survival rates up to 48 h after irradiation with gamma-rays, both in hydrated and anhydrobiotic states as well as the other two species (May et al. 1964, Jönsson et al. 2005). This result supports the concept that terrestrial tardigrades are the most radiation tolerant multi-cellular organisms in terms of short-term post-irradiation survival (Jönsson et al. 2005).

Hydrated and anhydrobiotic animals showed a similar pattern in mean survival at 2 h after gamma-irradiation (Figure 1A), whereas survival rates of hydrated animals were significantly higher than those of anhydrobiotic ones at 24 h and 48 h after irradiation with 4000 Gy and 5000 Gy of gamma-rays (Figures 1B, C). LD$_{50}$ values at 48 h after irradiation were also slightly higher in hydrated animals compared to anhydrobiotic ones (Table I). This survival pattern in *M. tardigradum* was similar to that in *R. coronifer* (Jönsson et al. 2005), but was opposite to that in other anhydrobiotic animals, such as embryos of *Artemia salina* (Iwasaki 1964) and larvae of *Polypedilum vanderplanki* (Watanabe et al. 2006). It is difficult to explain why anhydrobiotic animals are less radiation-tolerant than hydrated ones in the case of *M. tardigradum*. That is so because anhydrobiotic animals with extremely low water contents compared to hydrated ones (Table II) are expected to be damaged less by the indirect action of radiation compared to hydrated animals. This outcome might be the result of a high ability to repair biological damage in hydrated tardigrades, as proposed by Jönsson et al. (2005), although no evidence for this possibility is available at present.

M. tardigradum and *R. coronifer* contain relatively low amounts of trehalose (2.3%, <0.2%, wt./dry wt. respectively) in the anhydrobiotic state (Westh & Ramløv 1991; Horikawa et al. unpublished data), whereas *A. salina* cysts and *P. vanderplanki* larvae contain much greater amounts, about 15% and 20%, wt./dry wt. respectively (Clegg 1965, Watanabe et al. 2002). The presence of different amounts of trehalose may account for the different degrees of radiation tolerance shown by tardigrades and other anhydrobiotic animals, since trehalose has been

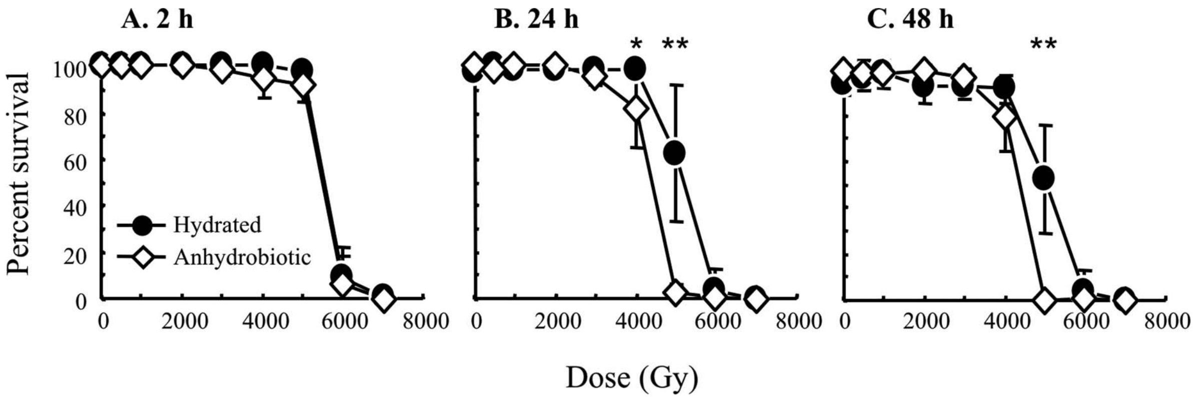

Figure 1. Dose response curves of the tardigrade *M. tardigradum* using gamma irradiaion at doses of 500–7000 Gy, on the basis of short-term survival. Mean survival percent was measured at 2, 24 and 48 h (A–C) after irradiation in hydrated animals or after rehydration in anhydrobiotic ones. Error bars indicate plus and minus one standard deviation. Asterisks indicate significant differences in survival between hydrated and anhydrobiotic animals (chi-square test with a Yeats correction; *$p < 0.05$ %; **$p < 0.001$).

Table I. LD_{50} ± standard error at 2, 24 and 48 h after irradiation of gamma-rays or $^{4}He^{2+}$ in *M. tardigradum*.

Radiation	State	LD_{50} (Gy)		
		2 h-survival[a]	24 h-survival[a]	48 h-survival[a]
Gamma	Hydrated	5500 ± 400	5200 ± 1700	5000 ± 1900
	Anhydrobiotic	5500 ± 500	5400 ± 600	4400 ± 500
$^{4}He^{2+}$	Hydrated	7800 ± 2500	6300 ± 600	6200 ± 1200
	Anhydrobiotic	7900 ± 30000	5400 ± 2200	5200 ± 2900

[a]The values were calculated from the data in Figure 1.

Table II. Water contents of hydrated or anhydrobiotic *M. tardigradum*.

State	N[a]	Weight/ individual (μg)[b]	Water content (%)[b]
Hydrated	10	6.54 ± 0.34	80.73 ± 1.23
Anhydrobiotic	6	1.11 ± 0.17	1.09 ± 0.46

[a]In each experiment, 50 active, hydrated individuals were used, and 60 for anhydrobiotic *M. tardigradum*. Thus, an N of 10 indicates 10 groups of 50 animals; [b]Mean ± standard deviation.

reported to play a protective role against radiation (Yoshinaga et al. 1997).

Long-term survival and reproduction after irradiation with gamma-rays

We examined the long-term survival and reproduction after irradiation of tardigrades in culture. Feeding behavior and molting were also observed in both hydrated and anhydrobiotic animals irradiated with 1000–4000 Gy gamma-rays. All individuals irradiated with 2000–4000 Gy died within 31 days post-irradiation (Table III). Several animals survived for 31 days after gamma-irradiation at 1000 Gy: survival rates were 2.3% for hydrated animals and 2.2% for anhydrobiotic ones, whereas survivals for controls were 13.0% and 37.8%, respectively. The average life span of irradiated animals was shortened in a dose-dependent manner. Shortening of life span in the anhydrobiotic state was similar in *M. tardigradum* and *R. coronifer* based on observations of the latter by Jönsson et al. (2005). However, there was a difference between the two species in the hydrated state; the maximum life span of hydrated *R. coronifer* was longer in the groups irradiated at 1000 and 2000 Gy than in controls (Jönsson et al. 2005). This inconsistency may be related to differences between experimental conditions, such as different temperatures and food supply, but detailed causes responsible for the differences are unclear.

Controls of *M. tardigradum* laid eggs in both hydrated and post-anhydrobiotic states, and only one individual irradiated with 2000 Gy in the hydrated state produced three eggs (Table III). *R. coronifer*, however, laid eggs after irradiation with up to 5000 Gy (Jönsson et al. 2005). This difference in egg-production seems to be the result of using different developmental stages of individuals of the two species at the time of irradiation. That is, we used *M. tardigradum* which had an immature ovary, while Jönsson et al. (2005) probably used mature, egg-possessing *R. coronifer*, because wild individuals were employed in their study. It is therefore conceivable that irradiation at the early stages of the developing ovary inhibits the process of oogenesis. Hatchability of eggs from non-irradiated animals was 81.6 and 82.1% in hydrated and anhydrobiotic states, respectively, whereas no eggs laid by an animal irradiated with 2000 Gy hatched. (Table III). This result indicates that embryogenesis of the next generation was suppressed by irradiation with more than 2000 Gy of gamma-rays in *M. tardigradum*.

This study is the first to examine the effects of radiation exposure in tardigrades under culture conditions. It has been reported that tardigrades are among the most radiation-tolerant multi-cellular organisms with respect to short-term survival (Jönsson et al. 2005). However, our present results show that tardigrades irradiated with high doses of gamma-rays do not achieve their natural life span, and do not produce progeny. Our findings also suggest that high doses of radiation attack biological components via direct action, even in the dry state.

Survival after irradiation with heavy ions

To compare the effects on *M. tardigradum* of different radiation sources, we examined short-term survival following irradiation with high-linear energy transfer (LET) heavy ions. We observed significant differences in survival at 2 h post-irradiation between hydrated and anhydrobiotic animals exposed to 6000, 7000 and 8000 Gy (Figure 2A). At 24 and 48 h after irradiation, significant differences were also observed using 5000 and 6000 Gy (Figures 2B, C). Except for 8000 Gy at 2 h after irradiation, hydrated animals exhibited significantly higher survival than anhydrobiotic ones at all those doses (Figure 2). Survival decreased more rapidly from 2 h to 24 h in both hydrated and anhydrobiotic states after exposure to heavy ions and gamma-rays (Figures 1, 2). It is likely that lethal effects of radiation in *M. tardigradum* occur between 2 h and 24 h after irradiation with heavy ions and gamma-rays.

Table III. Life span and reproductive activity of *M. tardigradum* after gamma irradiation of 0–4000 Gy at the juvenile stage.

State	Dose (Gy)	*n*	24 h-survival (%)[a]	Average life-span (days)[b]	Maximum life-span (days)	Spawning rate (eggs/individual)[c,d]	Hatchability (%)
Hydrated	0	30	86.7	12.2	31	1.27	81.6
	1000	44	65.9	5.6	31	0*	–
	2000	43	74.4	4.6	26	0.07*	0
	3000	45	62.2*	–	26	0*	–
	4000	42	35.7*	–	18	0*	–
Anhydrobiotic	0	45	95.6	26.0	31	0.62	82.1
	1000	45	84.4	10.9	31	0*	–
	2000	44	68.2	5.3	26	0*	–
	3000	45	20.0*	–	13	0*	–
	4000	45	11.1*	–	8	0*	–

[a]Asterisks indicate a significant difference in comparison between the values at the same dose of hydrated and anhydrobiotic cases 5% level (chi-square test with a Yeats correction; $p < 0.05$); [b]Average life span was estimated from the regression line between the survival rate and days after irradiation; [c]Spawning rate was calculated as the number of laid eggs divided by the number of individuals; [d]Asterisks indicate a significant difference in comparison with the control (chi-square test with a Yeats correction; $p < 0.01$).

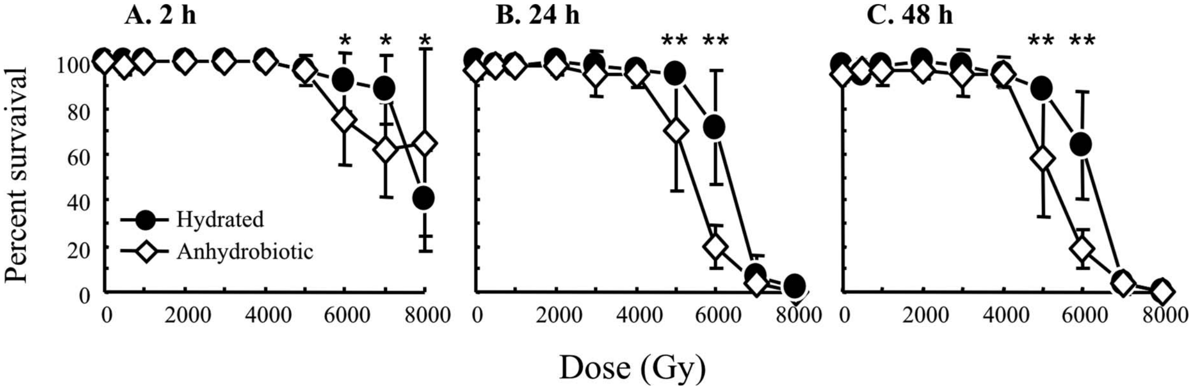

Figure 2. Dose response curves of the tardigrade *M. tardigradum* using ^{4}He irradiation at doses of 500–8000 Gy on the basis of short-term survival. Mean survival percent was measured at 2, 24 and 48 h (A–C) after irradiation in hydrated or after rehydration in anhydrobiotic one. Error bars indicate plus and minus one standard deviation. Asterisks indicate significant differences in survival between hydrated and anhydrobiotic animals (chi-square test with a Yeats correction; *$p < 0.05$%; **$p < 0.001$).

LD_{50} values in hydrated animals were somewhat higher than in anhydrobiotic ones at 24 and 48 h after irradiation (Table I). Furthermore, there was an overall tendency of LD_{50} values for heavy ions (16.3 keV/μm) to be higher than those for gamma-rays (0.2 keV/μm) (Table I), indicating that *M. tardigradum* is more tolerant to heavy ions than that to gamma-rays, in both states. This observation is interesting because high-LET heavy ions are generally thought to be more detrimental than low-LET gamma-rays (Hall 1994).

Further studies are needed to describe and understand the mechanism(s) by which radiation induces biological damage, especially DNA damage, in hydrated and anhydrobiotic tardigrades.

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