Tolerance to proton irradiation in the eutardigrade *Richtersius coronifer* – a nuclear microprobe study

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Abstract

**Purpose:** The tardigrade *Richtersius coronifer* has previously been shown to tolerate very high doses of low linear energy transfer (low-LET) radiation (gamma rays). The purpose of this study was to extend our knowledge on radiation tolerance in this species by investigating the dose-response to high-LET radiation in terms of protons.

**Materials and methods:** Dehydrated tardigrades of the species *R. coronifer* were irradiated with 2.55 MeV (megaelectron-volts) protons at doses ranging from 500 gray (Gy) to 15,000 Gy, to investigate the dose-viability relationship. In addition, a focused proton microbeam was utilised to determine the areal mass distribution, using the ion beam analytical technique STIM (Scanning Transmission Ion Microscopy).

**Results:** The experiment suggests that *R. coronifer* is unaffected by doses of proton irradiation up to 10,000 Gy, but shows very little viability at higher doses. The STIM analysis revealed that the thickness of the dehydrated tardigrades exceeds 150 μm, and that a fraction of the protons may not be fully absorbed.

**Conclusion:** Our results are in line with previous studies of exposure to high-LET radiation in tardigrades, indicating that these animals are equally or even more tolerant to high-LET compared to low-LET gamma radiation. The physiological background to this remarkable result is currently unknown, but deserves investigation.

**Keywords:** tardigrades, anhydrobiosis, radiation tolerance, proton irradiation

Introduction

The millimetre-sized aquatic invertebrates known as tardigrades are tolerant to a number of severe stressors, e.g., a wide range of temperatures, desiccation, pesticides, vacuum and also to high levels of ionising radiation (see reviews in Jönsson 2003, 2007). Earlier irradiation experiments include exposure to both low-LET radiation (gamma rays, Jönsson et al. 2005; Horikawa et al. 2006; May et al. 1964), and high-LET radiation (alpha particles, Horikawa et al. 2006), and in a recent study tardigrades were also exposed to the harsh conditions in space, including cosmic and ultra-violet (UV) irradiation (Jönsson et al. 2008). These studies have revealed that tardigrades are among the most radiation-tolerant animals – LD$_{50}$ (Lethal Dose 50, i.e., the dose at which 50% of the irradiated population have died) values range between 3000 and 6200 Gy (when measured up to 48 h post-irradiation), depending on radiation type, tardigrade species and whether the animals were desiccated or hydrated during irradiation. These LD$_{50}$ values are roughly 1000 times higher than the LD$_{50}$ value for humans, which is around 4 Gy (Anno et al. 2003). The evolutionary background to the extreme radiation tolerance in desiccation-tolerant animals is still largely unknown, but it is hypothesised to be a side-effect of mechanisms connected to desiccation tolerance (Jönsson 2003). Also some other desiccation-tolerant animals show a high tolerance to radiation (*Artemia franciscana* (Crustacea), *Polypedilum vanderplanki* (Insecta), *Adineta vaga*, *Philodina rossola* (Rotifer)) (Su et al. 1990; Watanabe et al. 2006a,b; Gladyshev and Meselson 2008). Radiation studies in these organisms are of interest from several perspectives. First, to the extent that the physiological and biochemical system underlying tolerance to...
desiccation and radiation is identical, studies on radiation tolerance can identify the nature and important components of such system, thereby improve our knowledge on adaptations to desiccation. Second, understanding mechanisms of extreme tolerance to radiation is of considerable interest in health research, and may ultimately contribute to improved treatment technologies against some diseases. For instance, in both radiation biology and research on cancer and ageing an understanding of the molecular systems of DNA repair is central. Thirdly, the usefulness of desiccation and radiation-tolerant animals in astrobiological research has recently been emphasised (Jönsson 2007; Horikawa et al. 2008). Of particular interest in exposures to the space environment are tolerances to UV radiation and protons, the latter of which represents the major part of the cosmic radiation in space (Horneck et al. 2006).

Low-LET and high-LET radiations are expected to affect biological tissue differently, with more damage expected from high-LET radiation due to higher deposition of energy and being more densely ionising. Horikawa et al. (2006), however, reported similar or lesser effects from irradiation with alpha particles compared to gamma rays in short-term survival of the tardigrade Milnesium tardigradum. The purpose of this study was therefore to investigate if another tardigrade species, Richtersius coronifer, show high tolerance to high-LET radiation. Our study reports the first data on the effects of proton microbeam irradiation on tardigrade viability.

**Materials and methods**

**Specimen collection and sample preparation**

We used the parthenogenetic eutardigrade Richtersius coronifer in our experiment. This species is one of the most studied tardigrades in respect to desiccation and radiation tolerance (e.g., Westh and Ramlov 1991; Jönsson and Rebecchi 2002; Jönsson et al. 2005). Moss with populations of *R. coronifer* were collected in the field at Öland, South-Eastern Sweden, 2–3 weeks before preparation of the experimental samples and stored dry at room temperature. The moss was rehydrated and extracted over night in plastic funnels with tap water, and the extraction was kept at room temperature until desiccation. Tardigrades of medium to large body size (ca. 0.5–1.0 mm body length) were chosen for the experiments, and specimens were assigned to replicate and treatment groups in a random fashion. Single tardigrade specimens were placed within polyvinyl chloride (PVC) rings (height = 1 mm, inner diameter = 2 mm) glued to ca. 15 mm × 15 mm stainless-steel nets (each with five PVC rings) with mesh size 80 μm, and desiccated in a 10-inch desiccator (Scientific Equipment, Houston, Texas, USA) at 94.5% relative humidity and 20°C for 2–3 days over a saturated potassium nitrate (*KNO₃*) salt solution. Relative humidity was monitored by a hair hygrometer (Lambrecht GmbH, Göttingen, Germany, accuracy 2.5% RH), inside the desiccator. This procedure ensured that the animals had reached an equilibrium hydration with the surrounding air and had entered an anhydrobiotic state (Wright 1989). Before irradiation, the open end of the PVC-rings with desiccated tardigrades was covered by 6 μm thick Mylar (Goodfellow Cambridge Ltd, Huntingdon, UK), using Vaseline to keep the Mylar attached to the ring.

**Post-irradiation procedures**

The samples were hydrated in tap water about 24 h after irradiation, and viability was recorded 4, 24 and 48 h after rehydration. The animals were recorded as viable when showing coordinated leg movements.

**Microbeam irradiation experiment**

The proton microbeam irradiation experiments were carried out at the Lund Nuclear Microprobe (NMP), using 2.55 MeV protons. Two types of irradiation experiments were carried out – one designed to deliver a precise dose to the tardigrades and a second to characterise their areal mass distribution. The precise dose was achieved by using the beam deflector to deliver a ms (millisecond)-range ‘time window’ of protons within a rectangular area. In this experiment, a defocused beam being approximately 150 × 150 μm² in size was used. All irradiation experiments on dehydrated tardigrades were carried out in vacuum (at a pressure of 10⁻⁶ millibar), and a pre-trial revealed no difference in survival between animals irradiated in air and in vacuum. In this way, additional uncertainties in the dose calculation due to energy loss of the beam in air could be eliminated. The total time the animals spent in vacuum varied between 30 and 45 min. Before reaching the tardigrade the beam had to penetrate the 6 μm thick Mylar foil covering the animals, which caused an energy loss of 100 keV (kiloelectronvolts) – thus the remaining energy of the protons as they impacted on the tardigrade was 2.45 MeV. When calculating energy losses, the animals were modelled as organic matter (Watanabe et al. 2006a), with a density of 1 g/cm³, and the dehydrated animals were initially assumed to have a thickness of 200–250 μm and a dry weight of 3 μg (Westh and Ramlov 1991).

With these prerequisites, the dose was calculated according to: \(\text{LET} = 14 \text{MeV} \mu\text{m}^{-1}\) – with an assumed animal thickness of 250 μm, all energy, i.e., 2.45 MeV, will be deposited in the body as the range of
2.45 MeV protons in organic matter is around 100 μm. This gives an absorbed dose per proton of 1.3 × 10^-4 Gy and if the desired dose is, e.g., 500 Gy, this will correspond to a desired proton beam charge of 0.6 pC (picocoulomb). To deliver such a small charge, a high voltage beam deflector controlled by a timer, set to up to a few hundred ms, was used to allow the beam to hit the sample only for the preset, short time interval. The beam current was measured in between irradiations, either just before the sample or on the sample, and the average beam current was approximately 50 pA (picoampere).

Nine different experimental groups were chosen where the tardigrades in each group received a different dose. The selected doses were 500, 1000, 2000, 4000, 6000, 8000, 10,000, 12,000 and 15,000 Gy. In addition to these experimental groups, we included a control group which was sham-irradiated, i.e., that was left in the experimental chamber under vacuum for 30 min without receiving any dose. Each experimental group consisted of 15 animals and all experiments were carried out twice (two trials). A few specimens could not be irradiated due to a peripheral location on the stainless-steel net, and these were removed from the subsequent analysis. The final analysis therefore included 11–15 specimens/exp. group/trial.

**Ion beam analysis of tardigrades: Determination of energy loss**

The ion beam analysis technique STIM (Scanning Transmission Ion Microscopy) (Overley et al. 1988) was used to determine the energy loss of the beam in the animals, to find the areal density of the animals and thus to improve the dose calculation. This analysis established how deep into the body the radiation penetrates and what portion of the radiation was deposited in the dehydrated animals. STIM measures the energy loss of the transmitted ions, from which the areal mass of the irradiated target (in this case the dehydrated tardigrade) can be calculated, given that the composition of the sample is known. Here, in contrast to the previous irradiation experiment, a focused beam was scanned over the sample, and two-dimensional maps showing how the energy loss varied across the sample were obtained. The data was analysed using the GeoPIXE software (Ryan et al. 2005; Pallon et al. 2009).

**Statistical analysis**

Our initial dose-response analyses with logistic regression failed to reach a significant Goodness-of-fit, and we therefore analysed variation among dose levels using the Chi-square test and Fisher’s Exact test (SYSTAT 10.0, Systat Software, Inc. Chicago, Figure 1. Dose-responses of the tardigrade *R. coronifer* irradiated with a proton microbeam. The animals were irradiated in a desiccated state and the Figure shows the proportion of viable (= with coordinated leg movements) animals 4 h (a), 24 h (b), and 48 h (c) after the animals were rehydrated, and for two trials. The means for each dose and trial were calculated from dichotomous data (viable, non-viable) of 11–15 specimens. Statistical tests were based on combined dose-groups (see Methods). For all post-rehydration times and for both trials, the overall Chi-square test was significant (P < 0.001, df = 4). In pair-wise comparisons of combined dose-groups (Fisher’s exact test), dose 12 + 15 kGy deviated significantly from all lower (combined) dose groups (P ≤ 0.002 in all comparisons, df = 1). Among the lower (combined) dose-groups, significant differences were found only for the 4 + 6 kGy, which differed from the 0 + 0.5 kGy and 1 + 2 kGy groups in the second trial (P < 0.05).
USA). Due to low expected frequencies dose-groups were combined into the five groups: 0 Gy + 0.5 kGy, 1 kGy + 2 kGy, 4 kGy + 6 kGy, 8 kGy + 10 kGy, 12 kGy + 15 kGy. For analyses of general differences over these combined dose-groups, the Chi-square test was used, while in pairwise comparisons we use Fisher’s Exact test. LD50 values were estimated from a fitted four-parameter logistic function (Sigmaplot 10.0, Systat Software, Inc. Chicago, USA):

\[
Viability = 0.032 + \frac{0.71}{1 + \left(\frac{Dose}{10.5}\right)^{11.8}}
\]

using pooled data from both trials for each dose.

Results

Dose-viability relationship

The viability of controls and proton-irradiated tardigrades after 4, 24 and 48 h post-rehydration is shown in Figure 1. There was a significant overall difference in viability over (combined) dose groups (\(\chi^2 = 48.1, df = 4, P < 0.001\)). This was mainly due to significantly lower viability in the highest dose groups (12 + 15 kGy), while we found few statistical differences among the lower dose groups (see caption of Figure 1). Thus R. coronifer seemed to be unaffected, in terms of short-term viability, by proton irradiation up to 10 kGy, but strongly affected at higher doses. LD50 at 24 h post-rehydration was estimated at 10.24 kGy.

Ion beam analysis of tardigrades: Determination of energy loss

The energy loss of the proton beam was measured in four dehydrated tardigrades, considered to be a random sample of medium-large-sized tardigrades – a typical STIM 2D map is shown in Figure 2a. In this map, a traverse was selected across the sample (indicated by the green rectangle), and in Figure 2b the energy loss as a function of position along the traverse is shown. From these measurements it can be concluded that for the majority of the protons, the assumption that all of the proton’s energy is deposited in the tardigrade is true. However, depending on the shape of the individual tardigrade, some of the protons may exit the tardigrade without depositing all of the energy. This means that we may have overestimated the absorbed dose by not more than 10%, with only small individual variations depending on the size and shape of a given tardigrade. In Figure 2c, a mean value (including standard deviation) of the traverses of the analysed tardigrades is shown, along with an illustration of the approximate position of the proton beam during irradiation. If a more accurate dose determination is required, a mean dose correction of 10% could therefore be applied. In addition, the STIM analysis revealed that the protons penetrated about 150 \(\mu\)m into the tardigrades, which means that the thickest portion (the central part) of dehydrated tardigrades exceeds 150 \(\mu\)m in thickness.

Discussion and conclusion

This investigation indicates that the tardigrade R. coronifer has a high tolerance to proton microbeam irradiation, and also suggests that this tardigrade is less affected by proton irradiation compared to gamma rays. Biological effects of high-LET radiation are expected to be the result of direct hits (i.e., ionisation/excitation) of atoms or biomolecules, especially DNA, while effects of low-LET radiation mainly originate from indirect action of radicals, generated through interactions with in particular H2O (Hall 1994). In general, high-LET radiation imposes more damage to living cells and tissues at a given dose, compared to low-LET radiation, due to a higher density of ionisation. This fact is usually expressed in terms of the concept of radiation weighting factor (WR), which represents a recent standardisation of the biological effects of different radiation types, where low-LET radiations have been set to WR = 1, and protons (>2 MeV) to WR = 5 (International Commission on Radiological Protection [ICRP] 1990). An alternative concept, but less straight-forward to measure in practice, is the relative biological effectiveness (RBE) of the radiation, which describes the biological effect of a radiation type (e.g., protons) relative to another type (often gamma ray). The RBE varies with LET, and has a maximum at the density of ionisation where the average distance between two ionising events matches the diameter of the DNA double helix, thus giving the highest probability of causing a DNA double-strand break by the passage of one single particle. For protons, this occurs at an LET around 20–30 keV/\(\mu\)m, depending on what endpoint is studied (Belli et al. 1989, 1998) and for alpha particles at an LET of 100 keV/\(\mu\)m (Hall and Kei 2003).

From the radiation weighting factor for protons we clearly expected a stronger negative effect on tardigrade survival of protons compared to previous irradiations with gamma rays. However, viability tended to decline only above 10 kGy of proton irradiation, while a previous study showed that the corresponding point after gamma irradiation was 2 kGy (Jönsson et al. 2005). Thus, R. coronifer seems to have a higher short-term tolerance to high-LET radiation relative to low-LET gamma-rays. In both studies the dose-response showed a marked shoulder, indicating the existence of a threshold.
level above which the damage is too high for the animal to handle. This contrasted with general expectations that high-LET radiation should give rise to dose-response with a weak plateau/shoulder pattern (Hall [1994]), see also Easter and Hutchinson [1961] for a study in *Artemia*). The physiological basis of this pattern is still unknown, but capacity limits of the DNA repair system are likely to be involved. Qualitatively similar results in respect to low-LET (gamma-rays) and high-LET radiation were reported by Horikawa et al. (2006) in their study of the tardigrade *Milnesium tardigradum*, where they used alpha particles (\(^{4}\)He, 50 MeV, 16.3 keV/\(\mu m\)) instead of protons. However, the difference in LD\(_{50}\) between the gamma and alpha irradiation was much smaller in the study by Horikawa et al. (2006), and at 24 h post-rehydration the values were identical (5.4 kGy, note that these values were estimated from linear regressions, while the dose-responses were obviously non-linear). The LD\(_{50}\) value at 24 h post-rehydration in *R. coronifer* was 3.0 kGy for gamma-rays (Jönsson et al. 2005), considerably lower than the value (10.24 kGy) for protons in the current study. In terms of relative biological effectiveness (RBE), defined as the ratio between a reference dose (in this case the dose of gamma rays, from \(^{137}\)Cs) and a test dose (here the dose of protons) that is required to produce the same response, this gives a value of RBE of 0.29 for *R. coronifer*, compared to 1.0 (with gamma rays, from \(^{60}\)Co, as reference) in *M. tardigradum* (Horikawa et al. 2006). RBE for protons/gamma radiation is generally expected to be in the range of 1–17, depending on endpoint studied (National Council on Radiation Protection and Measurements [NCRP] 1990). In desiccation-tolerant cysts of *Artemia franciscana*, Gaubin et al. (1979) reported RBE-values ranging between 1.5 and 2.3 for proton/gamma radiation.

A third species of tardigrades, *Ramazzottius varieornatus*, has also been exposed to high-LET (\(^{4}\)He, 50 MeV, 16.3 keV/\(\mu m\)) irradiation, but only at one dose, 4 kGy (Horikawa et al. 2008). Survival of this species after irradiation in a desiccated state was about 90%. Thus, studies on three different desiccation-tolerant tardigrade species have documented a high tolerance to high-LET irradiation, suggesting that this is a general characteristic of desiccation-tolerant tardigrades. Also in the desiccation-tolerant chironomid insect *Polypedilum vanderplanki*, desiccated larvae had similar tolerance to high-LET and low-LET radiation. Estimated values of LD\(_{50}\) was 4649 Gy after \(^{4}\)He irradiation (Watanabe et al. 2006a) compared to 4400 Gy after irradiation with gamma-rays (\(^{60}\)Co) (Watanabe et al. 2006b), giving an RBE-value 48 h post-rehydration of 0.93 (Watanabe et al. 2006a). Thus, the remarkable tolerance to both low-LET and high-LET radiation found in tardigrades seems to extend also to some other desiccation-tolerant animals. Desiccation-tolerant cysts of *Artemia franciscana* have also been the subject of several irradiation studies. Interestingly, these crustacean embryos seem to be more vulnerable to proton irradiation than to gamma irradiation, with a shorter plateau and steeper slope after the shoulder in the dose-response curve (Gaubin et al. 1979). The study by Gaubin et al. (1979) also showed a pronounced effect with advanced developmental endpoint. Thus radiation effects were lowest when observed at emergence and highest for nauplius survival. Similar effects were shown by Su et al. (1990) after irradiation by X-ray and krypton ions. Desiccation-tolerant rotifers are also very tolerant to low-LET (gamma) radiation (Shimada and Egami 1985; Gladyshev and Meselson 2008), but there are no reports of exposure to high-LET radiation for this animal group.

The mechanisms of radiation tolerance in tardigrades and other desiccation-tolerant animals are currently unknown, but the involvement of efficient DNA repair mechanisms seems likely (Jönsson 2007; Watanabe et al. 2007; Gladyshev and Meselson 2008). Comparing samples from different phases of the desiccation cycle, Watanabe et al. (2007) showed that tolerance to high-LET radiation correlate positively with levels of the disaccharide trehalose in larvae of *P. vanderplanki*. Trehalose has also been shown to protect plasmid DNA against ionising radiation (Yoshinaga et al. 1997). However, a functional link between radiation tolerance and trehalose in desiccation-tolerant animals has yet to
be verified. Also, compared to cysts of *Artemia franciscana* and larva of *P. vanderplanki*, which have 15–20% trehalose in the dry state (Clegg 2001; Watanabe et al. 2007), tardigrades have low levels of trehalose (<3% dw, Westh and Ramloëv 1991; Hengherr et al. 2007) and bdelloid rotifers do not have trehalose at all (Lapinski and Tunnacliffe 2003; Caprioli et al. 2004). Nevertheless, these different animal groups have similar capacities to survive desiccation and radiation.

The LET of the protons in this study are comparable with the LET of the alpha particles in the studies by Horikawa et al. (2006, 2008). However, due to the very different energies of the ions in the two experiments, the protons are practically completely stopped in the tardigrades whereas the alpha particles have a range that by far exceeds the thickness of the tardigrades. Due to the fact that ions deliver most of their energy at the end of their path, in the proton case, the dose has predominantly been deposited in the ‘bottom’ of the animal, i.e., on the opposite side of the animal compared to the beam entrance. Since we were not able to record the exact body orientation of single tardigrade specimens, we cannot evaluate the location of energy deposition at the tissue level. Also, it is not known if tardigrades contain any particularly radiation-sensitive or tolerant parts, but such variation in radiation sensitivity within the body could influence the results of this kind of radiation studies. More elaborate methods of specimen preparation may allow for such detailed analyses. Our study focused on acute lethality and did not evaluate long-term effects on survival or reproduction of proton irradiation. As mentioned above for *Artemia* cysts, observed radiation effects could depend on which developmental stage is used as endpoint. Our study did not use animals undergoing early developmental changes, only adult specimens. However, although monitoring of survival for 48 h after rehydration probably provides a good prediction of long-term survival, the surviving irradiated tardigrades could well have been sterile or unable to produce viable offspring. The unexpectedly high short-term tolerance to proton irradiation suggests that such studies on fitness effects should be undertaken, as well as studies on induced DNA damage. The extent to which tardigrades are able to repair extensive damage to DNA has not yet been studied, but the existence of an effective repair system has been suggested based on reported high radiation tolerance (Jönsson 2007). In conclusion, our study has shown a surprisingly high short-term survival after proton irradiation in *R. coronifer*, adding to the accumulating evidence of a remarkable radiation tolerance in tardigrades.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**


