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Gamma Radiation Tolerance and Protein Carbonylation Caused by Irradiation of Resting Cysts in the Free-living Ciliated Protist *Colpoda cucullus*

Ryota SAITO, Ryota KOIZUMI, Tatsuya SAKAI, Taiga SHIMIZU, Taiki ONO, Yoichiro SOGAME

National Institute of Technology Fukushima College, 30 Nagao Kamiarakawa Taira Iwaki Fukushima, 970-8034 Japan

Abstract. The ciliate *Colpoda cucullus* forms resting cysts to survive unfavorable environmental stresses. In this study, we have shown that *Colpoda* resting cysts survived exposure to a gamma radiation dose of 4000 Gy, although vegetative cells were killed by 500 Gy. After 4000 Gy irradiation, more than 90% of resting cysts and approximately 70% of dry cysts could excyst to form vegetative cells. In both cases, the excystment gradually increased after the induction of excystment. In addition, we also showed that protein carbonylation level was increased by gamma irradiation, but decreased by incubation in the cyst state. These results indicated that cell damage was repaired in resting cysts. *Colpoda* probably developed tolerance to gamma radiation by forming resting cysts as a strategy for growth in terrestrial environments, as part of contending with the stress due to reactive oxygen species caused by desiccation.

Key words: *Colpoda*, cyst, gamma radiation, cell repair, protein carbonylation

INTRODUCTION

Encystment is a reversible cell differentiation process involving dramatic morphological changes (Benčaťová and Tirjaková 2017, 2018a, b). In protists, encystment has been found in many species, sometimes with different functions. Some protists form reproduction cysts as a part of their regular life cycle, while other protists form resting cysts as a strategy to survive unfavorable environments (Verni and Rosati 2011). Resting cyst formation is part of the typical protist life cycle under cryptobiosis (Gutiérrez et al. 1990), when metabolic activity becomes barely measurable or stops in a reversible manner (Keilin 1959; Clegg 2001).

Colpoda is a common terrestrial protist ciliate: its process of encystment and the tolerance of its cysts to stress have been well studied. Resting cyst formation in *Colpoda* can be promoted by an increase in the Ca^{2+} concentration (Yamaoka et al. 2004) and is accompanied by dramatic morphological changes (Gutiérrez et al. 2003). When encystment is induced, the cells begin to swim spirally, turn round, and stop (Asami et al. 2010), with a rounded cell shape (Kida and Matsuoka 2006). The cells are then surrounded by a cyst wall (Gutiérrez et al. 2003; Kida and Matsuoka 2006; Funadani et al.

Address for correspondence: Yoichiro Sogame, National Institute of Technology Fukushima College, 30 Nagao Kamiarakawa Taira Iwaki Fukushima, 970-8034 Japan; E-mail: sogame@fukushima-nct.ac.jp; gamegamesogamail@gmail.com; TEL: +81-246-46-0875.

2010) and vegetative cell structures, such as cilia and mitochondria, disintegrate (Kida and Matsuoka 2006; Funatani et al. 2010). As a result of resting cyst formation, *Colpoda* species have a high tolerance to various environmental stresses, including desiccation (Taylor and Strickland 1936), freezing (Uspenskaya and Lozina-Lozinski 1979), high and low temperature (Taylor and Strickland 1936), ultraviolet irradiation (Uspenskaya and Lozina-Lozinski 1979; Matsuoka et al. 2017), and acid treatment (Sogame et al. 2011a).

Gamma radiation harms organisms (Richer et al. 2016), with damage to nucleic acids, proteins, and lipids (Halliwell and Gutteridge 1999; Close et al. 2013; Azzam et al. 2012), mediated by reactive oxygen species (ROS) produced by radiolysis of water (Azzam et al. 2012). Some multi-cellular organisms have been reported to have an extreme tolerance to radiation as the result of cryptobiosis. For example, the resting eggs (cysts) of some bdelloid rotifers can hatch after exposure to 1120 Gy of gamma radiation (Gladyshev and Meselson 2008). Similarly, brine shrimp (*Artemia*) resting eggs (cysts) have been reported to have extreme tolerance to gamma radiation: 20% of resting eggs were able to hatch after exposure to 6750 Gy (Gaubin et al. 1985), although the adults were rendered sterile (Suquire, 1970; Gaubin et al. 1985). Also, wet larvae of the African sleeping chironomid *Polypedilum vanderplanki* were killed by 2000 Gy of gamma radiation. However, with cryptobiosis (anhydrobiosis) the larvae could survive 7000 Gy of radiation (Watanabe et al. 2006).

Protozoan cysts, including *Colpoda* species cysts, have been shown to tolerate various environmental stresses as described above, and we reported bioassay data showing that cell damage due to irradiation was repaired in *Colpoda cucullus* resting cysts (Sogame et al. 2019a). However, detailed information on tolerance to gamma radiation in free-living protozoan cysts has not been reported, except for a study of cysts of the parasitic protist *Giardia lamblia*, which have been reported to survive 250–2000 Gy of radiation, although they lost their infectivity (Sundermann and Estridge 2010). Therefore, the aim of this study was to investigate the gamma radiation tolerance of cysts of the free-living single-cell protist ciliate *C. cucullus*.

MATERIALS AND METHODS

Cell culture and induction of encystment and excystment

Colpoda cucullus strain R2TTYS cells (Sogame et al. 2019b) were cultured in glass flasks at 25°C. The culture medium was an infusion of dried rice leaves (0.05% w/v) supplemented with 0.05% Na_2HPO_4 (w/v, final conc.) with bacteria (*Klebsiella pneumoniae* strain NBRC13277) as a food source. The bacteria were cultured on agar plates containing 1.5% agar, 0.5% polypeptone, 0.5% yeast extract, and 0.5% NaCl. *C. cucullus* cells were cultured for 1 d, then collected by centrifugation (1500 g for 1 min) and used for experiments.

Encystment and excystment were induced essentially as described by Sogame et al. 2011a. Encystment was induced by suspending *C. cucullus* cells at high cell density (> 10^4 cells/ml) in encystment-inducing medium [1 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$]. Excystment of *Colpoda* cysts was induced by replacing the medium with excystment-inducing medium [0.2% (w/v) infusion of dried rice leaves supplemented with 0.05% Na_2HPO_4 (w/v, final conc.)].

Gamma irradiation

Vegetative cells, wet cysts, and dry cysts were irradiated with a number of doses of gamma radiation [i.e. 500 Gy (0.5 Gy/min), 1000 Gy (0.9), 2000 Gy (1.9), 3000 Gy (2.8), or 4000 Gy (3.7)] using an RE2022 with cobalt 60 (42 TBq) as the radiation source (Toshiba, Tokyo, Japan), located in the National Agriculture and Food Research Organization (NARO). For irradiation, vegetative cells (3–6 h after excystment) were transferred to petri dishes and the cell concentration was adjusted to 10^3 cells/ml. To obtain wet cyst samples, encystment was induced in cells in a petri dish and the cells were incubated for more than 1 week. To obtain dry cysts, wet cyst samples were air-dried for 1 week. The effect of irradiation on vegetative cells was determined by cell viability, calculated as: Percent cell viability = (number of viable cells after irradiation / number of viable cells before irradiation) × 100. The concentration of viable cells before irradiation was adjusted to 10^3 cells/ml. The number of viable cells after irradiation was determined by direct counting. The number of cells in 100 µl (5% of sample) was determined by dividing the sample into 10 aliquots and counting the cells using a microscope. The LD_{50} of *Colpoda* vegetative cells was determined by a Questgraph LD_{50} calculator (AAT Bioquest Inc., 2020) based on cell viability. The effect of irradiation on wet cysts and dry cysts was determined as the value of excystment after irradiation. Viable cysts excyst after the induction of excystment, but nonviable cysts do not. Therefore, excystment value was calculated as: Percent excystment = (number of excysted cysts / number of excysted and non-excysted cysts) × 100. Samples of excysted and non-excysted cells (> 100 cells) were directly counted using a microscope. In Fig. 4, the viability of each type of cell is shown as a relative value (with the non-irradiated control of each cell type = 100%), because the viability of control dry cysts was significantly lower than that of control vegetative cells and wet cysts. The significance of differences among samples was evaluated by the Mann-Whitney U test.

Analysis of oxidative protein damage

Protein oxidation (carbonylation) was studied using an OxyBlot Protein Oxidation Detection Kit (Merck KGaA, Tokyo, Japan) following the manufacturer's instructions. Protein samples were extracted from wet cyst samples as described by Sogame et al., 2011b. The protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) as described in the manufacturer's instructions. The protein samples were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel, with proteins from 4 x 10^5 cells in each lane, as described by Sogame et al. 2011b. The protein bands were transferred onto a Clear Blot Membrane-P plus (ATTO Corporation, Tokyo, Japan), using an AE-6677 semi-dry blotting system (ATTO Corporation) for 1 h at 30 mA, with three types of blotting buffer: 300 mM Tris containing 20% methanol (v/v), 25 mM Tris containing 20% methanol (v/v), and 25 mM Tris-borate buffer, pH 9.5, containing 20% methanol (v/v). For immunoblotting, the membranes were incubated overnight at 4°C in a solution containing 80 mM Na_2HPO_4, 1.5 M NaCl, 20 mM KH_2PO_4, 30 mM KCl, 0.5% Tween-20 (v/v) (PBST), and supplemented with 1% skim milk. The blots were immunostained with rabbit anti-DNP antibody (Merck KGaA) for 1 h at 25°C, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG (Merck KGaA) for 1 h at 25°C. The antibodies were dissolved in PBST. The blots were then treated with an enhanced chemiluminescence (ECL) detection kit (ECL Western Bloting Detection Reagents, GE Healthcare Co. Ltd., Backinghamshre, UK). Carbonylated proteins in the immunoblots were detected and their signal intensities were analyzed by the Chemi Doc MP Imaging System with Image Lab imaging software (Bio-Rad Laboratories, Inc., Tokyo, Japan). After western blot analysis, the blots were stained with 0.1% (w/v) Coomassie brilliant blue R250 (CBB) in a solution of 40% methanol (v/v) and 1% glacial acetic acid (v/v), and destained with a 50% methanol solution (v/v). The protein density in each lane was analyzed by the Chemi Doc MP Imaging System (Bio-Rad Laboratories, Inc.).

RESULTS

Gamma irradiation of *C. cucullus* wet cysts

The excystment of *C. cucullus* wet cysts, that had been non-irradiated or irradiated with 500–4000 Gy, was measured as a function of time after induction of excystment and is shown in Fig. 1. At 3 h after induction of excystment (Fig. 1a), 95.89% of non-irradiated wet cysts and 53.89% of 500 Gy irradiated wet cysts were excysted, < 10% of 1000 and 2000 Gy irradiated wet cysts were excysted, and < 1% of 3000 and 4000 Gy irradiated wet cysts were excysted. At 6 h after induction of excystment (Fig. 1b), 99.51% of 500 Gy irradiated cysts were excysted, which was statistically the same as excystment of non-irradiated cysts ($p > 0.05$). In addition, 94.43% of 1000 Gy irradiated cysts and 80.12% of 2000 Gy irradiated cysts were excysted, although < 10% of both were excysted at 3 h after induction of excystment. In contrast, < 1% of wet cysts irradiated at 3000 and 4000 Gy were excysted at 3 h after induction of excystment and about 10% were excysted at 6 h after induction of excystment. However, by 9 h after the induction of excystment (Fig. 1c), > 98% of non-irradiated and 500 Gy irradiated wet cysts and > 90% of 1000 and 2000 Gy irradiated wet cysts were excysted. In addition, 73.98% and 57.98% of 3000 and 4000 Gy irradiated wet cysts, respectively, were excysted at 9 h after induction of excystment. Excystment of all samples subsequently increased gradually and almost 100% were excysted at 36 h after induction of excystment (Fig. 1d).

In vivo protein carbonylation assays of irradiated wet cysts

In vivo gamma radiation-induced oxidative protein damage in wet cysts was analyzed by western blot analysis using a protein oxidation detection kit (OxyBlot, Merck) (Fig. 2). Non-irradiated cysts (Fig. 2, Non-IR) and 4000 Gy irradiated cysts (Fig. 2, IR) were analyzed to investigate cell damage due to irradiation. In addition, cysts incubated for 12 h after 4000 Gy irradiation (Fig. 2, IR incubated) were analyzed to investigate cell repair of radiation damage in resting cysts, as has been reported by Sogame et al. 2019a. Protein carbonylation of the 31–72 kDa proteins in 4000 Gy irradiated cysts was 4.7 times higher than in non-irradiated cysts: compare the "Non-IR" and "IR" lanes in the "Carbonylated protein" panel in Fig. 2. However, protein carbonylation was suppressed by incubation after irradiation and was only 1.9 times higher in cysts incubated after irradiation than in non-irradiated cysts: compare the "IR" and "IR incubated" lanes in the "Carbonylated protein" panel in Fig. 2. In these experiments, changes in the level of protein carbonylation was not due to different amounts of protein in the samples, because the amount of protein was similar in all three CBB-stained lanes (Fig. 2, Protein panel).

Gamma irradiation of *C. cucullus* dry cysts

The excystment of non-irradiated and 500–4000 Gy irradiated dry cysts was measured as a function of time after induction of excystment. At 3 h after induction of excystment (Fig. 3a), < 5% of non-irradiated and 500 Gy irradiated dry cysts were excysted, and < 1% of 1000–4000 Gy irradiated dry cysts were excysted. At 6 h after induction of excystment (Fig. 3b), approxi-

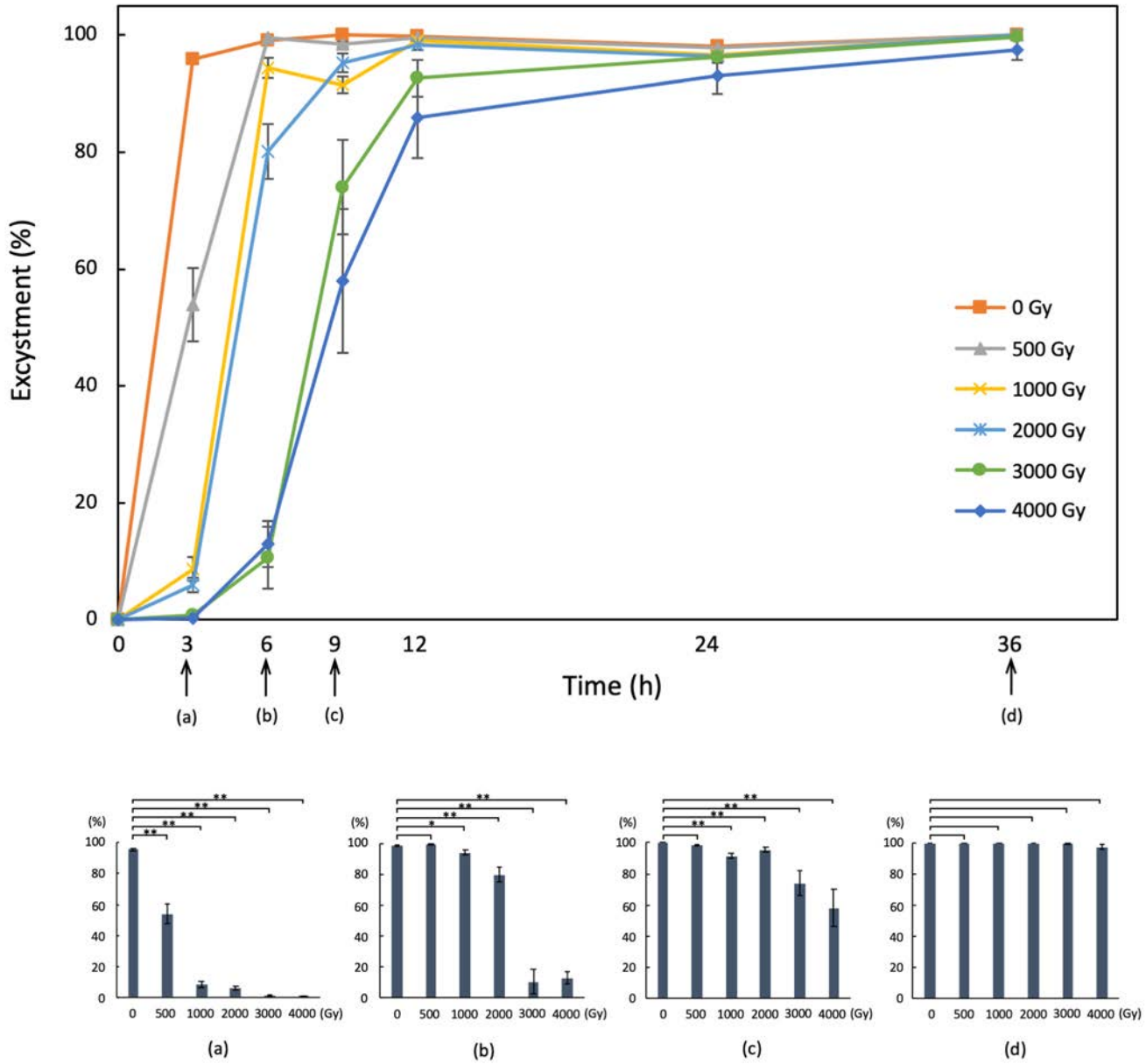

Fig. 1. Excystment of *Colpoda* wet cysts, after gamma irradiation at 0 (non-irradiated), 500, 1000, 2000, 3000, and 4000 Gy, as a function of time after the induction of excystment. The points and bars mark the means and standard errors, respectively, of six measurements at each dose. The excystment mean ± SE at 3, 6, 9, and 36 h after induction of excystment is shown in (a), (b), (c), and (d), respectively. The column heights and attached bars in (a) to (d) are the means and standard errors, respectively, of six measurements. Asterisks and double asterisks indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney U test).

mately 30% of non-irradiated and 500 Gy irradiated dry cysts and 11.32% of 1000 Gy irradiated dry cysts were excysted, but < 5% of 2000–4000 Gy irradiated dry cysts were excysted. The number of excysted cells then gradually increased for all samples from 24 h after induction of excystment to 96 h after induction of excystment. At 24 h after induction of excystment (Fig. 3c), approximately 80% of non-irradiated cysts and 500 Gy irradiated cysts, 60.11% of 1000 Gy irradiated cysts, and approximately 40% of 2000–4000 Gy irradiated cysts were excysted. Finally, at 96 h after induction of excystment (Fig. 3d), all samples were > 65% excysted: non-irradiated, and 500 and 1000 Gy irradiated dry cysts were approximately 90% excysted, 2000 Gy

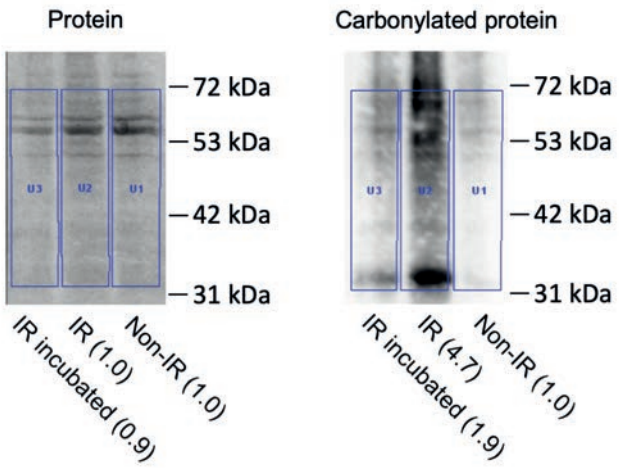

Fig. 2. Analysis of proteins (Left panel) and protein carbonylation by ECL (Right panel) from non-irradiated and 4000 Gy irradiated cells. The samples in the lanes were from non-irradiated cells (Non-IR), 4000 Gy irradiated cells (IR), and cells incubated for 12 h after 4000 Gy irradiation (IR incubated). The protein bands and ECL signals were measured and are shown in parentheses for each lane relative to the Non-IR sample.

irradiated dry cysts were 72.02% excysted, and 3000 and 4000 Gy irradiated cysts were approximately 70% excysted.

We noted that, in this study, excystment of 500 Gy irradiated dry cysts tended to be higher than that of non-irradiated dry cysts from 9 h after the induction of excystment to the end of this experiment, although this difference was not statistically significant ($p > 0.05$).

Effect of gamma radiation on the viability of *C. cucullus* vegetative cells, wet cysts, and dry cysts

The effect of different doses of gamma radiation (500, 1000, 2000, 3000, and 4000 Gy) on the viability of *C. cucullus* vegetative cells, wet cysts, and dry cysts was compared (Fig. 4). The viability (excystment) data for wet and dry cysts at 36 h and 96 h after induction of excystment are shown in Figs. 1d and 3d, respectively. The viability of irradiated vegetative cells was also measured at these radiation doses. The data were expressed relative to the viability of cysts and cells that were not irradiated (Fig. 4). These results indicated that *C. cucullus* wet and dry cysts had extreme tolerance to gamma radiation of 500–4000 Gy compared to vegetative cells with significant differences among the samples ($p < 0.01$).

With regards to vegetative cells, approximately 50% of vegetative cells were killed by 500 Gy irradiation, most were killed by 2000 and 3000 Gy, and all were killed by 4000 Gy: the LD_{50} of vegetative cells was 815 Gy. However, > 95% of wet cysts and > 70% of dry cysts survived doses > 2000 Gy (Fig. 4): the survival of wet cysts was greater than that of dry cysts ($p < 0.01$).

DISCUSSION

This is the first report, to our knowledge, describing the extreme tolerance of resting cysts of the free-living single-cell protist *C. cucullus* to gamma radiation: relative to non-irradiated cysts, 97.44% of wet cysts and 75.39 % of dry cysts could survive after 4000 Gy irradiation. It has been reported that the cysts of *G. lamblia*, a single cell parasitic eukaryote, appeared to be viable after exposure to 2000 Gy gamma radiation, based on propidium iodide (PI) staining (Sundermann and Estridge 2010). However, the authors of that study noted that PI was not a reliable indicator of cyst viability. The problem may be that it is difficult for PI to penetrate cysts, even if the cell membrane is damaged as described for *Colpoda* (Sogame et al. 2019b). It is difficult to determine the survival of irradiated cysts of parasitic protists because of the difficulty in measuring their infectivity. However, cell viability of *C. cucullus* could be readily determined in this study by measuring excystment. In other studies, oocysts and tissue cysts of *Toxoplasma gondii*, a single cell parasitic eukaryote, were reported to be killed by exposure to 250 Gy and 400 Gy radiation, respectively (Dubey et al. 1996). In contrast, in this study *C. cucullus* resting cysts showed extremely high tolerance to gamma radiation compared to those organisms.

In spite of the tolerance to gamma irradiation of *C. cucullus* resting cysts, about half of *C. cucullus* vegetative cells were killed by 500 or 1000 Gy irradiation in this study. There are similar reports with regard to vegetative cells: *Cyanophora paradoxa* and *Glaucocystis nostochinearum*, species of Glaucophyte, showed no tolerance to 1000 Gy irradiation (Potts 1999), although *G. lamblia* trophozoites were not completely killed by 1000 Gy irradiation (Lenaghan and Sundermann 2003). Our present findings and these reports indicate that the gamma radiation tolerance of *C. cucullus* was cyst specific; i.e., a property of resting cysts rather being species-specific.

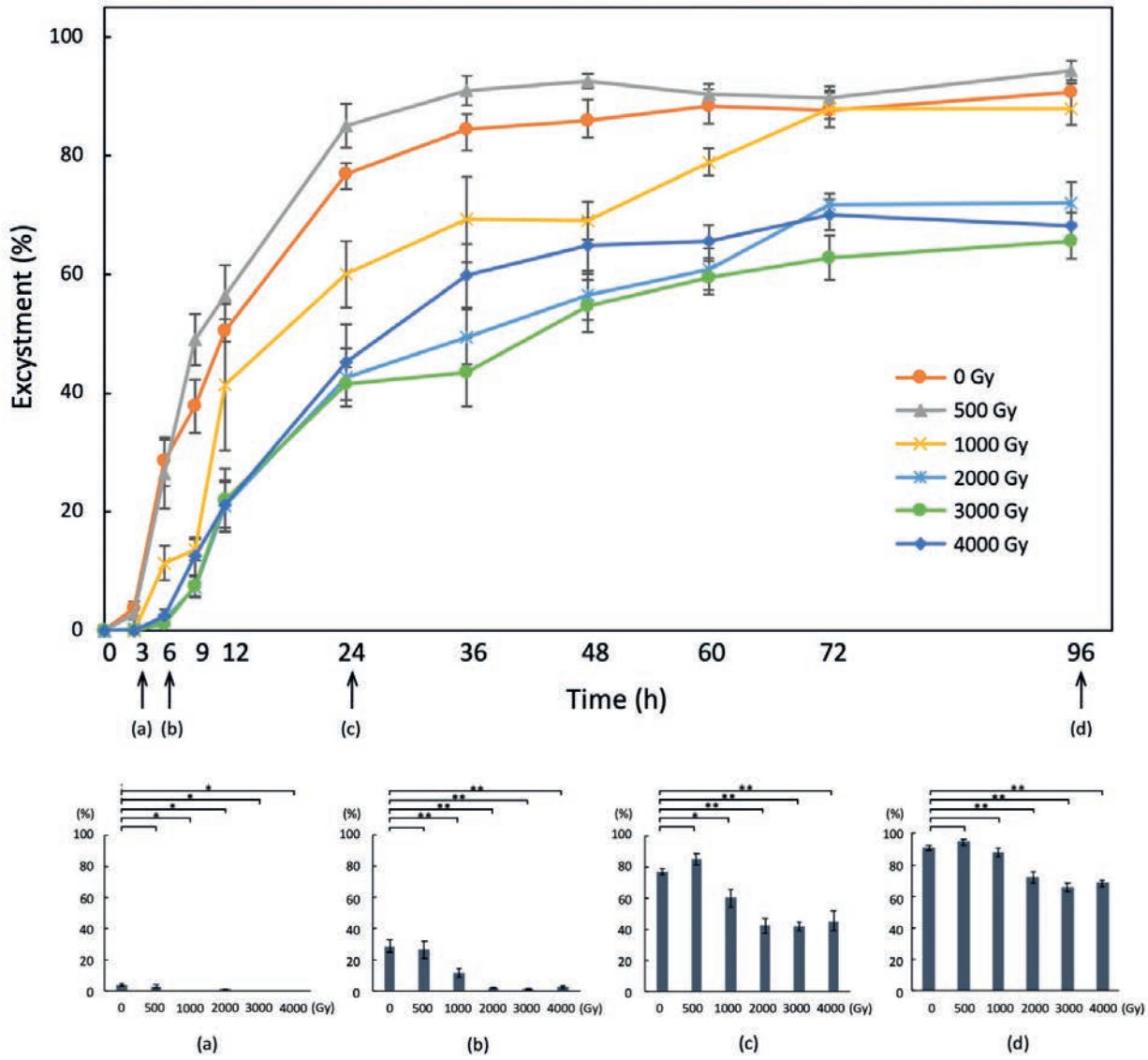

Fig. 3. Excystment of *Colpoda* dry cysts, after gamma irradiation at 0 (non-irradiated), 500, 1000, 2000, 3000, and 4000 Gy, as a function of time after induction of excystment. The points and bars mark the means and standard errors, respectively, of six measurements at each dose. The excystment mean ± SE at 3, 6, 24, and 96 h after the induction of excystment is shown in (a), (b), (c), and (d), respectively. The column heights and attached bars in (a) to (d) are the means and standard errors, respectively, of six measurements at each dose. Asterisks and double asterisks indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney U test).

Gamma radiation consists of high energy photons produced by nuclear events (Richter et al. 2016). The effects of gamma radiation on microorganisms (Jung et al. 2017) can be direct or indirect (Silindir and Ozer 2009). Absorption of gamma radiation by cells directly disrupts molecular structures, resulting in chemical and biological changes in physiological processes (Azzam et al. 2012). Gamma radiation can also act indirectly through radiolysis of water, producing ROS that damage nucleic acids, proteins, and lipids (Azzam et al. 2012). This causes molecular damage in cells (Close et al. 2013; Azzam et al. 2012) and is considered a major stress factor for cells, including protists (Slaveykova et al. 2016). Protein oxidation, such as protein carbonylation (Median and Regnier 2010), is one of the significant results of ROS stress and has been reported to increase after irradiation (Sukharev et al. 1997; Maisonneuve et al. 2009). Carbonylated proteins are toxic

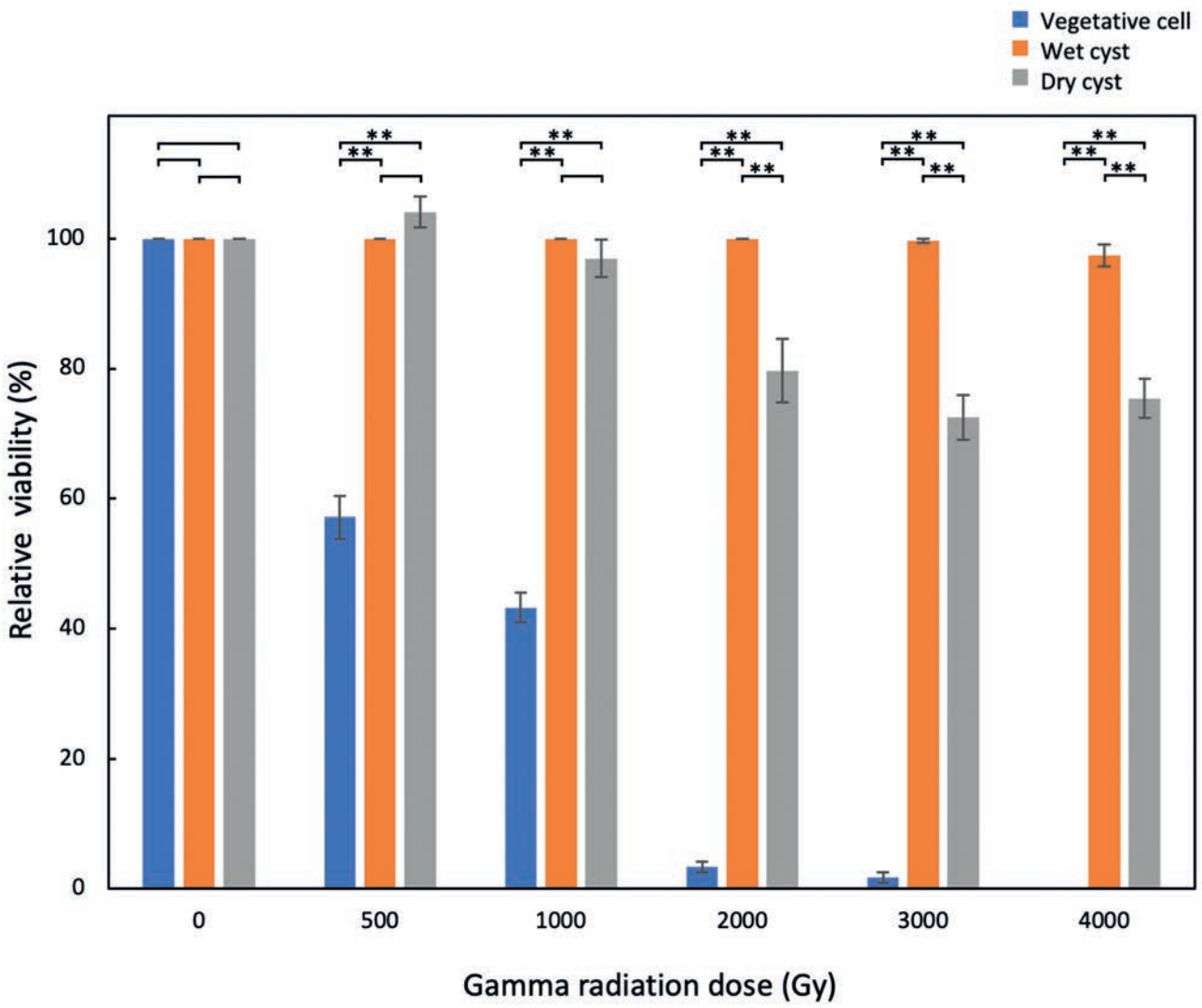

Fig. 4. Relative viability of *Colpoda* vegetative cells, wet cysts, and dry cysts after gamma radiation doses of 0 (non-irradiated), 500, 1000, 2000, 3000, and 4000 Gy. The column heights and attached bars are the means and standard errors, respectively, of six measurements at each dose. Double asterisks indicate a significant difference at $p < 0.01$ (Mann-Whitney U test).

if they are not properly degraded and removed (Dalle-Donne et al. 2006).

In this report we showed that 4000 Gy of gamma radiation increased *in vivo* protein carbonylation in *C. cucullus* wet cysts. This result indicated that gamma irradiation induced ROS stress, thereby producing protein carbonylation and cell damage. However, excystment of 4000 Gy irradiated wet cysts gradually increased as a function of time after induction of excystment and was the same as that of non-irradiated cysts at 36 h after induction of excystment. This result suggested that the damage due to gamma radiation was repaired during excystment. It has been reported that, when cells were induced to excyst, metabolic activity promptly recovered because expression of the DEAD-box RNA helicase was elevated at 10 min after induction of excystment (Sogame et al. 2013). Therefore, after induction of excystment, irradiated cysts can repair their radiation damage and gradually revert to vegetative cells. Cell repair after irradiation has also been reported in the hyperthermophilic archaeon *Phyrococcus furiosus* in the active state under favorable culture conditions (DiRuggiero et al. 1997).

However, we have reported bioassay data showing that cell damage caused by irradiation can be repaired

in resting cysts (Sogame et al. 2019a). Interestingly, *Colpoda* resting cysts repair damage caused by irradiation even when their metabolic activity becomes barely measurable. In this study, we showed that the level of protein carbonylation was reduced by incubation in the cyst state. This is first report showing repair of cell damage due to gamma irradiation in resting cysts at the molecular level. As noted above, cell repair after irradiation has been reported in *P. furiosus*. However, the repaire in *P. furiosus* cause in the active state under favorable culture conditions (DiRuggiero et al. 1997). However, in this study, we showed repair of cell damage due to gamma irradiation in *C. cucullus* resting cysts with unmeasurable metabolic activity. This result suggested that metabolic activity in resting cysts can be elevated by stress to repair cell damage, although there can also be repair in resting cysts with low metabolic activity.

Gamma radiation can also cause serious damage to DNA through ROS stress (Imlay and Linn 1988; Azzam et al. 2012). Therefore, both protein and DNA damage can probably be repaired in resting cysts. In that case, a factor similar to RAD51 may be activated to help repair DNA damage, as reported in *Tetrahymena thermophila* (Campbell and Romero 1998).

If organisms are dehydrated, cell damage due to ROS stress is likely to decrease due to the lack of intracellular water and, therefore, cell radiation tolerance should increase. In fact, the radiation tolerance of *P. vanderplanki* has been reported to increase under anhydrobiosis, which removed almost all the water in the organism (Watanabe et al. 2006). However, the relative viability of *C. cucullus* in the hydrated state (wet cysts) at gamma irradiated at >2000 Gy was higher than in the dehydrated state (dry cysts) (Fig. 4), perhaps because dehydration also caused ROS stress (França et al. 2007). In agreement with these results, the excystment of non-irradiated dry cysts (90.76%) was slightly less than that of non-irradiated wet cysts (100.00%) in this study (Fig. 1d, 3d). However, the relative viability (i.e., excystment) of 2000–4000 Gy irradiated dry cysts was significantly less than that of wet cysts, although there may have also been desiccation stress in the dry cysts. The cell damage in wet cysts probably could be repaired, but repair in dry cysts would not be possible due to the lack of water, except after induction of excystment. However, when dry cysts were induced to excyst, they could rehydrate and repair could start. Hence, the excystment of dry cysts gradually increased, although it was delayed compared to wet cysts.

In this study we showed that wet cysts and dry cysts of *C. cucullus* R2TTYS were tolerant to gamma radiation at doses up to 4000 Gy. Ciliate protists, such as *C. cucullus*, may have acquired radiation tolerance to contend with ROS stress caused by desiccation, which may have been a strategy to adapt for survival in terrestrial environments. We also showed that there was cell repair in resting cysts, which suggested that radiation tolerance in resting cysts may have been a result of cell repair.

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