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Gamma rays induce DNA damage and oxidative stress associated with impaired growth and reproduction in the copepod *Tigriopus japonicus*

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ABSTRACT

Nuclear radioisotope accidents are potentially ecologically devastating due to their impact on marine organisms. To examine the effects of exposure of a marine organism to radioisotopes, we irradiated the intertidal copepod *Tigriopus japonicus* with several doses of gamma radiation and analyzed the effects on mortality, fecundity, and molting by assessing antioxidant enzyme activities and gene expression patterns. No mortality was observed at 96 h, even in response to exposure to a high dose (800 Gy) of radiation, but mortality rate was significantly increased 120 h (5 days) after exposure to 600 or 800 Gy gamma ray radiation. We observed a dose-dependent reduction in fecundity of ovigerous females; even the group irradiated with 50 Gy showed a significant reduction in fecundity, suggesting that gamma rays are likely to have a population level effect. In addition, we observed growth retardation, particularly at the nauplius stage, in individuals after gamma irradiation. In fact, nauplii irradiated with more than 200 Gy, though able to molt to copepodite stage 1, did not develop into adults. Upon gamma radiation, *T. japonicus* showed a dose-dependent increase in reactive oxygen species (ROS) levels, the activities of several antioxidant enzymes, and expression of double-stranded DNA break damage genes (e.g. *DNA-PK*, *Ku70*, *Ku80*). At a low level (sub-lethal dose) of gamma irradiation, we found dose-dependent upregulation of *p53*, implying cellular damage in *T. japonicus* in response to sub-lethal doses of gamma irradiation, suggesting that *T. japonicus* is not susceptible to sub-lethal doses of gamma irradiation. Additionally, antioxidant genes, phase II enzyme (e.g. *GSTs*), and cellular chaperone genes (e.g. *Hsps*) that are involved in cellular defense mechanisms also showed the same expression patterns for sublethal doses of gamma irradiation (50–200 Gy). These findings indicate that sublethal doses of gamma radiation can induce oxidative stress-mediated DNA damage and increase the expression of antioxidant enzymes and proteins with chaperone-related functions, thereby significantly affecting life history parameters such as fecundity and molting in the copepod *T. japonicus*.

1. Introduction

Gamma radiation (also ionizing radiation, IR) occurs naturally due to radioactive decay of radioisotopes and atmospheric radiation. However, anthropogenic factors such as nuclear power plant accidents can lead to massive environmental exposure to gamma radiation. As an example, the Chernobyl nuclear accident in 1986 had diverse repercussions on the environment, economy, and public perception of science. The nuclear power plant accident in Japan in 2011 raised great human and environmental health concerns because nuclides were released into the coastal marine environment and transported to surrounding areas by sea currents (Kim et al., 2012b; Nair et al., 2013). However, it is unclear to what extent marine organisms are affected by nuclear contamination, and virtually nothing is known about the effects of exposure of humans to seafood-mediated radiation (Fisher et al., 2013).

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IR consists of alpha, beta, and gamma rays according to the energy state generated during decay processes. Gamma radiation is particularly well known, as it is the most energetic form of electromagnetic radiation with the highest frequency and shortest wavelength. Several researchers have reported that gamma rays have significant adverse effects on organisms ranging from DNA damage to community level perturbations (Daly and Thompson, 1975; Kovalchuk et al., 1999; Zaka et al., 2004). One of the most serious consequences of exposure to gamma radiation is double-stranded breaks (DSBs) in genomic DNA. Karran (2000) demonstrated that DSBs arise through the direct action of IR, and other researchers have examined gamma radiation-induced DNA damage, chromosomal aberrations, and mutations in human blood cells, the pale grass blue butterfly, and fish larvae (Sudprasert et al., 2006; Hiyama et al., 2013; Rhee et al., 2013). Rate of induction of DSBs by gamma rays ranged from approximately 0.004–0.007 DSBs/Gy/Mb across species such as the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and mammalian cell lines (Bonura and Smith, 1976; Rensik and Martin, 1976; Prise et al., 1998). In Chernobyl, Dubrova et al. (1996) reported increased germline mutations in children, suggesting that IR has strong residual effects in populations. Organisms have an innate defense mechanism to protect against IR-induced damage. For example, DSB rejoining through nonhomologous end-joining (NHEJ) and the aid of DNA-dependent protein kinases (DNA-PKs), Ku70, and Ku 80, as well as DNA ligase, proliferating cell nuclear antigen (PCNA), and other proteins protects organisms from IR-induced DNA damage. Thus, these proteins are potential biomarkers of DNA damage and/or repair mechanisms in diverse organisms (Bhaskaran et al., 1999; Blas-Machado et al., 2000; Jalal et al., 2011; Rhee et al., 2013).

Regarding the adverse effects of gamma rays at the cellular level, Cassidy et al. (2007) demonstrated that gamma rays induce the production of ROS, which can damage cells, by Compton effects. Barzilai and Yamamoto (2004) mentioned that gamma-induced ROS cause severe damage to cellular macromolecules, especially DNA. Rhee et al. (2012) examined the effects of gamma irradiation-induced ROS in the embryos of the hermaphroditic killifish (*Kryptolebias marmoratus*) by evaluating the in vivo endpoints of growth impairment and reproduction and the in vitro parameters of antioxidant enzyme activities and expression patterns of diverse genes associated with cellular defense mechanisms. However, most studies of the effects of gamma radiation on aquatic organisms have focused on effects at either the molecular or individual levels (Gilbin et al., 2008; Mothersill et al., 2013). No study to date has reported the effects of gamma radiation on zooplankton which is the most widespread marine organisms.

The copepod *Tigriopus* sp. is one of most abundant marine invertebrates worldwide, and is a promising model species for ecotoxicological studies (Raisuddin et al., 2007). Copepods link producers and consumers in the marine food web. Among copepods, harpacticoida, which consists of about 3000 species, play an important role as prey for benthic and sessile organisms, as they inhabit the surfaces of sediments and rocks (Raisuddin et al., 2007). *Tigriopus japonicus* is a particularly promising model species because of the ease of maintenance of this species under laboratory conditions and the short life cycle of this organism (about 2–3 weeks). Furthermore, there is a massive mRNA database for *T. japonicus* as well as genomic data, which allows evaluation of specific molecular mechanisms underlying physiological responses by examining gene expression patterns (Lee et al., 2010).

In this paper, we examined the biochemical and molecular responses of *T. japonicus* to gamma radiation-induced damage to fill in the knowledge gap between physiological changes and molecular effects. We also investigated molecular defense mechanisms induced by gamma radiation by evaluating several in vivo endpoints such as mortality, fecundity, and molting in the copepod species *T. japonicus*.

2. Materials and methods

2.1. Culture and maintenance of T. japonicus

The copepod *T. japonicus* was maintained under controlled incubation conditions, which comprised a 12 h light/12 h dark photoperiod and an incubation temperature of 25 °C. Salinity of the culture medium was 30 psu, which was achieved with Tetramarin Salt Pro® (United Pet Group, Inc., Cincinnati, OH, USA) for marine organisms. Copepods were fed the green microalgae *Tetraselmis suecica* as a food source once a day. Species identity was confirmed by sequence analysis of the mitochondrial DNA cytochrome oxidase I (*CO1*) gene, which is a barcoding gene for invertebrates (Jung et al., 2006).

2.2. Gamma radiation effects on mortality

To calculate the estimated lethal dose, we irradiated 10 ovigerous female *T. japonicus* with several doses of gamma radiation (0–800 Gy, 10 individual × 3 replicate for each group). For gamma irradiation, we used Gammacell® 1000 Elite using Cesium 137 as the radiation source (MDS Inc., Ottawa, ON, Canada) and temperature (25 °C) was maintained during irradiation. After gamma radiation (about 2 Gy/min), we placed *T. japonicus* in a controlled incubator, and monitored mortality under a stereomicroscope for 7 days. Experiments were conducted in triplicate.

2.3. Effects of gamma radiation on fecundity and molting to copepodite stages

To examine the effects of gamma radiation on fecundity and molting, we irradiated ovigerous female and just-hatched nauplii with different doses of gamma rays and then measured fecundity and evaluated molting to adult stage. We exposed 10 of the gathered ovigerous females and 10 nauplii to 0, 50, 100, 150, 200, 300, and 400 Gy gamma irradiation to evaluate its effects on fecundity and the molting process to adult, respectively. To obtain just-hatched nauplius stage individuals, we initially isolated ovigerous female using a 200 μm sieve, incubated them in fresh seawater for 12 hr, and gathered just-hatched nauplii by removing ovigerous female using the 200 μm sieve. Number of hatched nauplii and their rate of growth to adults were measured for 10 days and 20 days, respectively. The hatched nauplii were counted from 10 individuals. For growth rate, average days required for growth from both nauplius to copepodite and copepodite to adult were measured with 30 nauplii (10 individual × 3 replicate) from each dose.

2.4. Measurement of ROS levels

To analyze gamma radiation-induced oxidative stress, we exposed adult *T. japonicus* (approximately 250 individuals) to gamma radiation (0, 50, 100, 150, and 200 Gy). Intracellular ROS were measured as described by Kim et al. (2011). Briefly, samples were homogenized with a Teflon pestle in a buffer containing 0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, and 0.4 mM PMSF (pH 7.4). To remove debris, homogenized samples were centrifuged at 10,000 × *g* for 20 min (4 °C). Supernatant were reacted with H_2DCFDA and fluorescence was measured at 485 nm for excitation and 520 nm for emission (Thermo Scientific Co., Varioscan Flash). Total protein content in the supernatant was determined to normalize the ROS contents using the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

2.5. Measurement of antioxidant-related enzyme activities

To assess glutathione-related enzymes activities, we exposed adult *T. japonicus* (approximately 300 individuals) to five different doses of gamma radiation (see Section 2.4). To measure the activities of GR (EC 1.8.17) and GPx (EC 1.11.1.9), we used a glutathione reductase (GR) assay kit and glutathione peroxidase (GPx) cellular activity assay kit (Sigma–Aldrich Co., St. Louis, MO, USA), respectively. Activity of glutathione *S*-transferase (GST; EC 2.5.1.18) was measured as described by Regoli et al. (1997) with minor modifications. Activities of each antioxidant enzyme were calculated by the reduced absorbance using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience). Total protein content was determined using the Bradford method (Bradford, 1976).

2.6. Messenger RNA expression of p53, DNA repair genes (Ku70, Ku80, DNA-PK), PCNA, antioxidant genes, and chaperone genes

To measure the expression patterns of DNA repair genes (*Ku70*, *Ku80*, *DNA-PK*) in addition to *p53* and *PCNA*, we irradiated *T. japonicus* (approximately 200 individuals) with sublethal doses of gamma radiation (150 and 200 Gy) and measured mRNA expression 20, 40, 60, 180, and 360 min after irradiation. To evaluate the expression patterns of antioxidant genes and chaperone genes for 1 h, we irradiated *T. japonicus* with four doses of gamma radiation (50, 100, 150, 200 Gy). Total RNA was extracted using TRIZOL® reagent (Invitrogen, Paisley, Scotland, UK) according to the manufacturer's instructions. Quantity and quality of total RNA was checked at 230, 260, and 280 nm using a spectrophotometer (Ultrospec 2100 Pro, Amersham Bioscience). To synthesize cDNA for real-time quantitative RT-PCR (real-time qRT-PCR), 2 μg of total RNA and oligo $(dT)_{20}$ primer were used for reverse transcription (SuperScript™ III RT kit, Invitrogen, Carlsbad, CA, USA).

Real-time qRT-PCR was conducted under the following conditions: 94 °C/4 min; 40 cycles of 94 °C/30 s, 57 °C/30 s, 72 °C/30 s; and 72 °C/10 min using SYBR Green as a probe (Molecular Probe, Invitrogen). To confirm amplification of specific products, melting curve cycles were run using the following conditions: 95 °C/1 min; 55 °C/1 min; 80 cycles of 55 °C/10 s with 0.5 °C increase per cycle using real-time qRT-PCR F or R primers (Supplementary Table 1). The *T. japonicus 18S rRNA* gene was used to normalize expression levels between samples. All experiments were performed in triplicate. Fold change relative to control level was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

All results are expressed as mean values. Normal distribution and homogeneity of variances of data were checked by Levene's test. Data were analyzed using one-way ANOVA, followed by Tukey's honesty significant difference test ($P < 0.05$). Pearson's correlation was used to analyze the relationship between mRNA expression levels and antioxidant enzyme activities. All statistical analyses were performed using SPSS® version 21 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Mortality due to gamma radiation

Mortalities were not observed until 4 days after irradiation for all doses, as shown in Fig. 1. However, 5–6 days after gamma irradiation, mortality was significantly increased for copepods treated with 600 and 800 Gy.

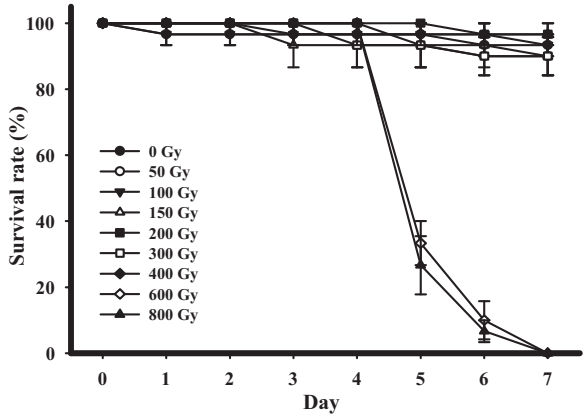

Fig. 1. Mortality of *T. japonicus* after exposure to gamma radiation (0, 50, 100, 150, 200, 300, 400, 500, 600, and 800 Gy) for 7 days. Error bars indicate mean ± SE. Sample size for each group was 10 individuals and experiments were conducted in triplicate.

3.2. Effects of gamma radiation on fecundity and molting to copepodite stages

Fecundity was measured by counting the number of nauplii hatched daily from ovigerous female for 10 days. Ovigerous *T. japonicus* not treated with radiation produced about 8 nauplii per day, but in the gamma-irradiated groups, fecundity decreased in a dose-dependent manner (Fig. 2), suggesting that high doses of gamma radiation adversely affect the fecundity of ovigerous *T. japonicus*. Fifty Gy- and 100 Gy-irradiated ovigerous *T. japonicus* have hatched nauplii every other days but in over 150 Gy irradiated groups the fecundity was decreasing day after day. Particularly, in groups irradiated with over 150 Gy radiation, ovigerous *T. japonicus* produced less than 1 nauplius/individual/day.

After gamma radiation, we assessed the molting process to adult over a period of 20 days. As shown in Fig. 3, molting of just-hatched nauplius was significantly retarded by gamma radiation in a dose-dependent manner. In untreated nauplii, it took about 6 days to develop from nauplius stage 1 to copepodite stage 1, and subsequent molting steps to adult took about 9 days. However, upon gamma radiation, the molting process of *T. japonicus* to the next representative developmental stages (e.g. copepodite stage 1 and adult) took 7 days and 10 days, respectively. Particularly, in groups

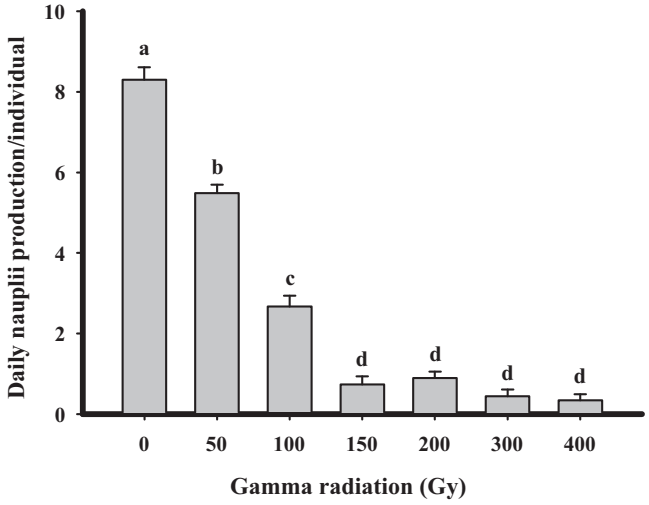

Fig. 2. Effect of gamma radiation on the daily fecundity of ovigerous females (number of nauplii per individual per day). Ten ovigerous females were used for each dose. Significant differences were analyzed by ANOVA (Tukey's post hoc test; $P < 0.05$).

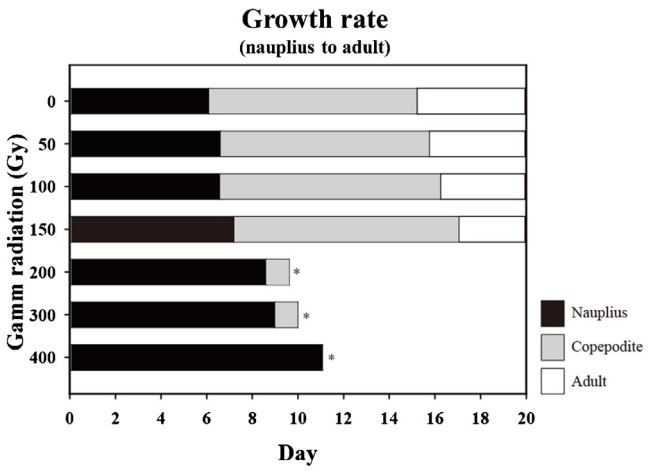

Fig. 3. Effect of gamma radiation on the molting of *T. japonicus* from nauplius to adult. Asterisk indicates 100% mortality of nauplius and copepodite.

irradiated with 200 and 300 Gy radiation, *T. japonicus* did not survive to copepodite stage 2, and in the 400 Gy-irradiated group, molting of nauplii to copepodite stage 1 was not successful.

3.3. Gamma radiation-induced ROS and antioxidant enzyme activities

To assess whether gamma radiation increased oxidative stress levels in *T. japonicus*, we analyzed the level of intracellular ROS and the activities of antioxidant enzymes (Fig. 4). ROS level increased dose-dependently in response to gamma radiation. Activities of the antioxidant enzymes GST, GR, and GPx also showed a dose-dependent in activity in response to increasing doses of gamma radiation in *T. japonicus*.

3.4. Expression patterns of p53 and DNA repair genes

To investigate the DNA damage response to gamma irradiation (150 and 200 Gy), we examined expression of *p53*, three DNA repair genes, and *PCNA* over 360 min. *p53* gene expression showed a bell-shaped response over time depending on the radiation dose (Fig. 5). *p53* was expressed at higher

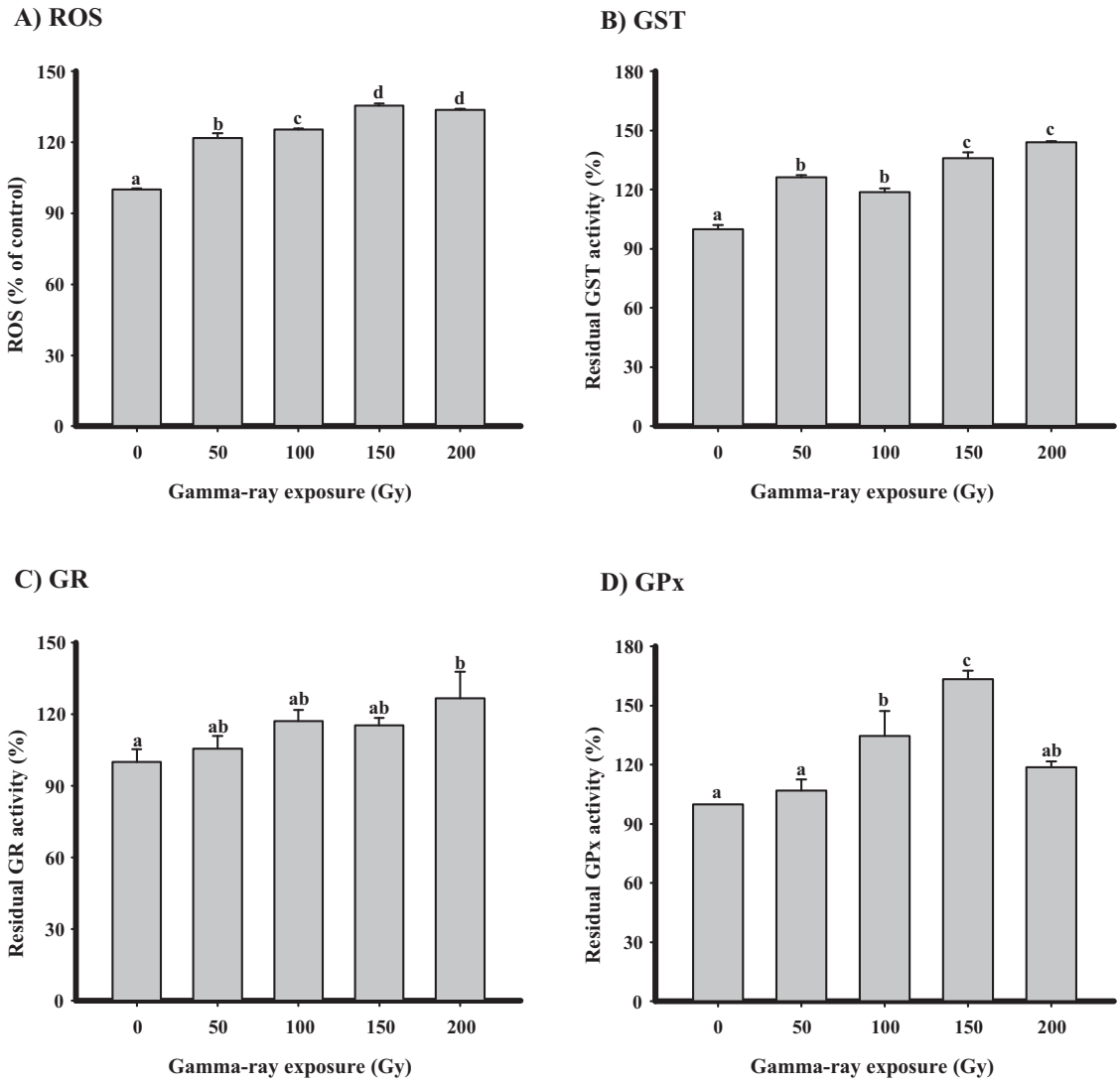

Fig. 4. Effects of different doses of gamma radiation (0, 50, 100, 150, 200 Gy) on ROS generation and antioxidant enzyme activities. (A) Reactive oxygen species (ROS), (B) glutathione *S*-transferase (GST), (C) glutathione reductase (GR), and (D) glutathione peroxidase (GPx) in *T. japonicus* (Tukey's post hoc test; $P < 0.05$). Data are means ± SD of three replicates of exposed group.

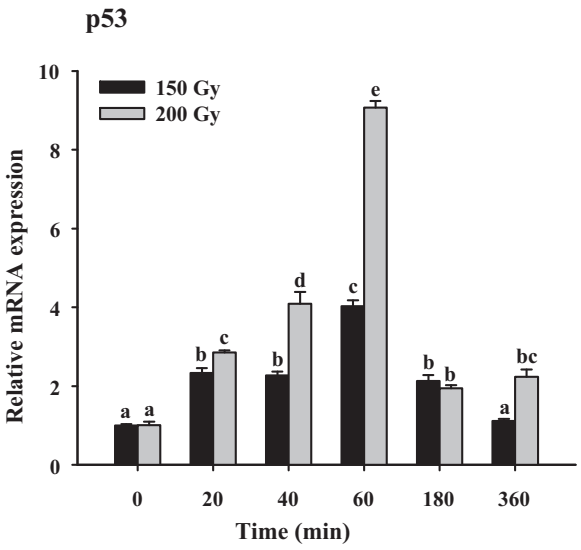

Fig. 5. Effect of gamma radiation (150 and 200 Gy) on *TJ-p53* gene expression over time. Values are means of three replicate samples and data are shown as means ± SD. Significant differences were analyzed by ANOVA (Tukey's post hoc test; $P < 0.05$).

levels in 200 Gy gamma-irradiated *T. japonicus* than 150 Gy-irradiated *T. japonicus*, indicating greater DNA damage in the 200 Gy-irradiated group. DSB repair genes and *PCNA* showed a similar pattern of expression to *p53* (Fig. 6), but expression levels differed among genes according to time and radiation dose.

3.5. Expression patterns of antioxidant and chaperone genes

We examined expression patterns of 13 antioxidant and chaperone genes after exposure of *T. japonicus* to 1 h of gamma radiation (Fig. 7). Transcript levels of all antioxidant and chaperone genes increased significantly in response to gamma radiation. Of the genes encoding antioxidant enzymes, transcript levels of *TJ-GPx* increased most significantly in response to radiation doses of 100, 150, and 200 Gy. mRNA expression levels of *TJ-GPx* and *TJ-GST-delta E(1)* also showed a good correlation with the corresponding enzyme activities (Pearson correlation coefficients of $r = 0.96$ and $r = 0.91$, respectively; $P < 0.05$). Expression of *TJ-Hsp20.7* was 30-fold upregulated by gamma radiation.

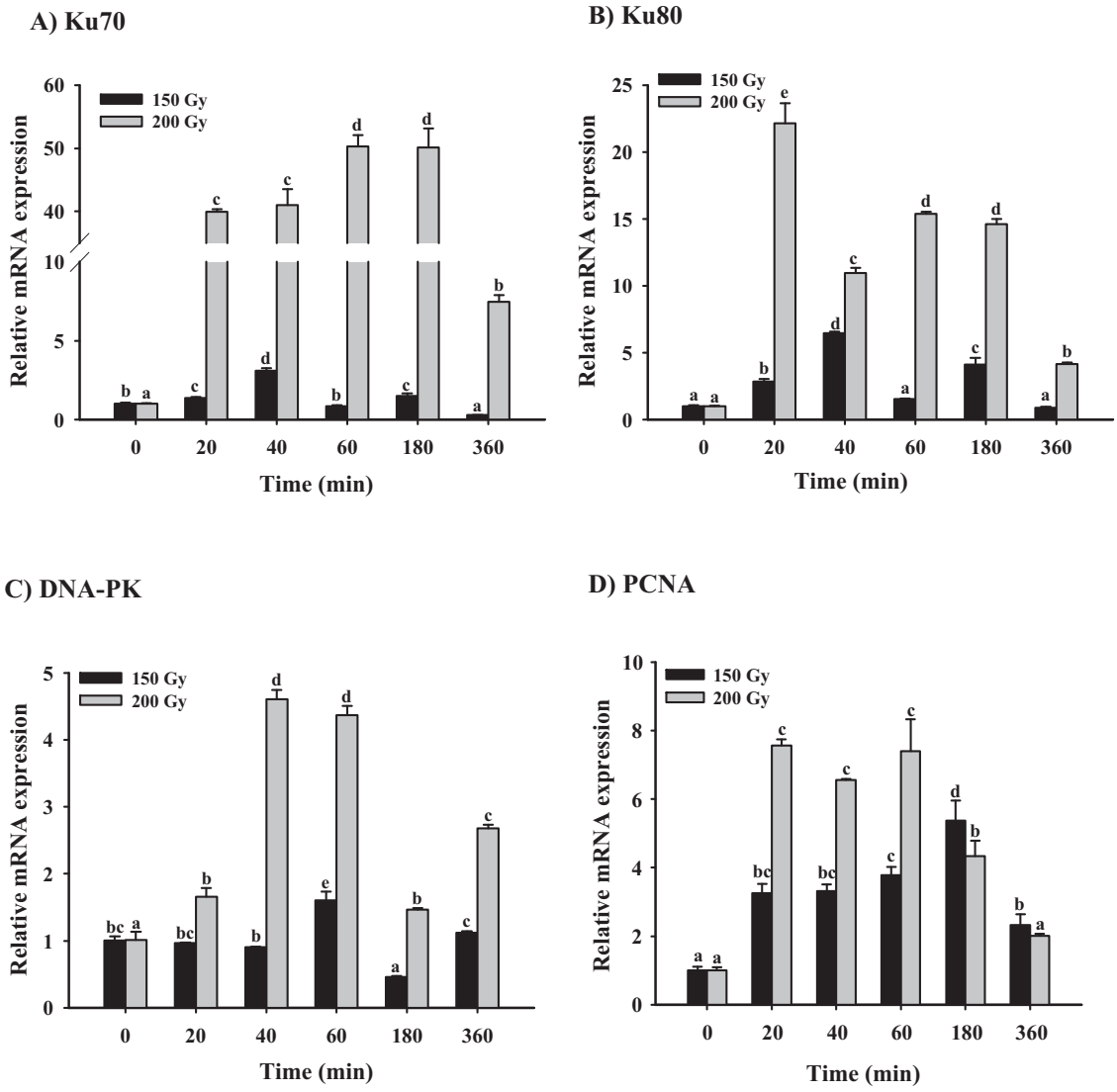

Fig. 6. Effects of gamma radiation (150 and 200 Gy) on (A) *TJ-Ku70*, (B) *TJ-Ku80*, (C) *TJ-DNA-PK*, and (D) *TJ-PCNA* gene expression over time. Values are means of three replicate samples and data are shown as means ± SD. Significant differences were analyzed by ANOVA (Tukey's post hoc test; $P < 0.05$).

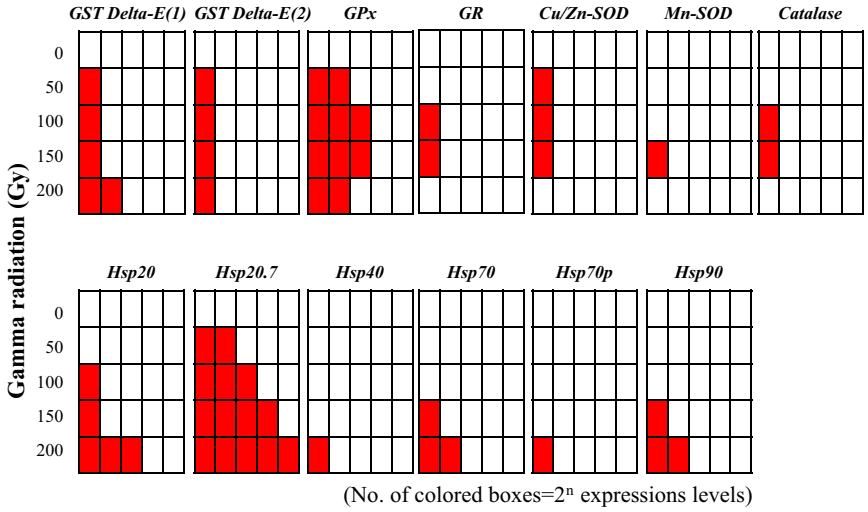

Fig. 7. Expression patterns of antioxidant and chaperone genes in *T. japonicus* for different gamma radiation doses (0, 50, 100, 150, and 200 Gy).

4. Discussion

The recent nuclear power plant disaster in Fukushima in 2011 raised concern about the marine environment due to the coastal location of this nuclear power plant; in fact, huge amounts of nuclides spilled out directly into the Pacific Ocean (Kameník et al., 2013; Kumamoto et al., 2013). It is important to determine the effects of radionuclides on marine organisms to estimate and monitor the risks of IR to protect and ensure marine and human health (Rieder and Cole, 1998; Kuhne et al., 2009; Rhee et al., 2012). However, there is limited information about the effects of radiation on invertebrates, despite their essential role in marine ecosystems; most studies of gamma radiation-induced effects have focused on vertebrates such as fish and mammals (Cooper et al., 2000; Cassidy et al., 2007; Rhee et al., 2012). Thus, a better understanding of gamma radiation-induced effects on aquatic invertebrates is necessary to be able to estimate the potential impacts of gamma radiation on marine ecosystems.

To obtain information about gamma radiation-induced mortality, we irradiated the intertidal copepod *T. japonicus* with gamma rays (0–800 Gy) to estimate the lethal dose (LD50-96 h), but were not able to calculate this value as there was no mortality within this time interval. Won and Lee (2014) reported that the LD50-96h of the cyclopoid copepod *Paracyclopina nana* was 172 Gy under gamma irradiation. The rotifer *Brachionus koreanus*, one of most primitive eukaryotes, did not die even when exposed to 1200 Gy gamma radiation (unpublished data). This lethal dose value is much higher than that (about 2 Gy at 30 days after radiation) at which lethal effects are observed in mammals (Myers, 1989), suggesting that different species have different susceptibilities to gamma radiation. *T. japonicus* has greater tolerance to several anthropogenic and natural stresses such as metals, organotins, and ultraviolet B than the calanoid copepod *P. nana* and other copepods (U'ren, 1983; Barka and Pavillon, 2001; Lee et al., 2007; Hwang et al., 2010; Mohammed et al., 2010; Kim et al., 2012a; unpublished data, Supplementary Table 2). Resistance of *T. japonicus* to gamma radiation can potentially be exploited to determine how gamma radiation affects molecular-level processes.

Ovigerous female *T. japonicus* generally produce about eight individuals per day. However, fecundity was significantly reduced in a dose-dependent manner after gamma ray irradiation, as shown in Fig. 2. Three days after over irradiation with 150 Gy gamma radiation, ovigerous female *T. japonicus* were not able to hatch nauplii and even dropped egg clutches. After dropping their egg clutches, some female did develop a new egg sac, but the dropped egg sac never hatched. Female harpacticod and cyclopoid copepods store spermatophores to overcome mate-limiting conditions (Titelman et al., 2007; Liken, 2010; Thorp and Covich, 2010). Titelman et al. (2007) demonstrated that copepods develop new egg sacs using one spermatophore over a period of 2–2.5 months. *T. japonicus* females were able to develop new egg sacs without males in about 3 weeks. Our results suggest that upon gamma radiation, sperm stored in females and eggs are adversely affected, thereby impairing reproduction. Won and Lee (2014) also found that gamma radiation (20–100 Gy) impaired reproduction by reducing the rate of egg production and delaying molting in the planktonic copepod *P. nana*, suggesting that gamma radiation has a negative effect on reproductive mechanisms in copepods. In addition, a high dose of gamma radiation limited the respawning of female *P. nana*. In the water flea *Daphnia magna*, Gilbin et al. (2008) reported that gamma radiation (31 mGy/h) resulted in reproductive failure. Dubrova et al. (1996) reported a high frequency of germline mutations in children born in the Chernobyl area. These types of germline mutations increase the risk of hereditary disease (Byrne, 1999). Taken together, our results indicate that gamma radiation reduced fecundity and impaired reproduction in ovigerous female *T. japonicus*. Accumulation of germline mutations has been shown to change population structures of communities in specific niches (Larkin et al., 2002). In fact, community structure is an important determinant of the health of an ecosystem, as the stability of community structure is defined as its ability to return to equilibrium after perturbation by anthropogenic or natural events (Heip and Herman, 1985). Reduction in marine products, such as fish, along the Fukushima coastal area after the nuclear power plant accident indicates that nuclide leaching into the ocean adversely affected marine ecosystem health.

Growth rate (i.e. molting) in copepods also affects community structure (Larkin et al., 2002). Upon irradiation with 50–150 Gy gamma rays, ovigerous female *T. japonicus* showed a delay in molting and severe reproductive impairment (e.g. failure to reach to copepodite 1 or 2 stages) (Fig. 3). We found that the nauplius stage was more sensitive to gamma radiation than adult ovigerous females (data not shown). Streffer (1995) demonstrated that humans, rats, and mice are most sensitive to radiation damage during the early fetal period. Hiyama et al. (2013) reported that early developmental stages of the pale grass blue butterfly were susceptible to IR and showed a disproportionate increase in DNA for metamorphosis. Thus, growth retardation (i.e. delayed molting) from nauplius to copepodid and copepodid to adult in response

to gamma radiation suggests that gamma radiation perturbed the molting process in *T. japonicus*. Rhee et al. (2012) reported a delay in the growth rate of hermaphroditic fish embryos exposed to gamma rays (2.5–10 Gy). *T. japonicus* irradiated with 200 and 300 Gy gamma radiation were not able to grow to copepodite stage 2 within 9 days, while the group irradiated with over 400 Gy of gamma radiation were not even able to molt to copepodite stage 1, implying that gamma radiation over 200 Gy is deleterious to *T. japonicus*, although this is a sublethal dose. Regarding the relationship between retarded growth rate and changes in energy balance, Verslycke et al. (2004) and Gilbin et al. (2008) reported that a reduction in cellular energy to detoxify damage induced by chlorpyrifos exposure in the mysid *Noemysis integer* and gamma radiation-induced damage in *D. magna* resulted in improved survival. Exposure of several aquatic organisms such as *Daphnia*, shrimp, and fish to various environmental stresses (e.g. pesticides, metals, IR) has been shown to be positively correlated with retarded growth, reduced body mass, and reduced respiration rate (Coen and Janssen, 1997; Verslycke et al., 2004; McKim and Benoit, 2011). Thus, delayed molting in gamma irradiated-*T. japonicus* is likely to be closely associated with a change in the amount of energy allocated to detoxification, DNA repair, and global defense mechanisms versus growth.

Gamma radiation can generate ROS directly by interacting with water in cells; the ROS can then damage DNA (Ward, 1981; Kang et al., 2012). In gamma-irradiated *T. japonicus*, we found a gamma dose-dependent increase in intracellular ROS as well as activities of the antioxidant enzymes GST, GPx, and GR, which are involved in detoxification. Rhee et al. (2013) showed that irradiation of the fish, *K. marmoratus*, with a sublethal dose of gamma radiation resulted in generation of ROS, and that this was directly related to increased expression of DNA repair genes and activity of several antioxidant enzyme activities. Mukherjee et al. (2010) demonstrated low mortality and enhanced SOD and CAT enzyme activities after exposure of the copepod *Mesocyclops hyalinus* to gamma radiation (2–10 Gy), suggesting that antioxidant enzymes are induced as part of the detoxifying mechanism in response to sublethal toxicity, as we found for *T. japonicus*. Karowicz-Bilińska et al. (2002) showed a correlation between oxidative stress indices and intrauterine growth retardation in pregnant women.

We also measured mRNA expression of the *p53* gene over 0–360 min in response to two critical sublethal doses (150 and 200 Gy) of radiation. *p53* transcript levels were higher in the 200 Gy-irradiated group than the 150 Gy-irradiated group (Fig. 5), and this increase in expression of *p53* was associated with greater perturbation of the molting process in *T. japonicus*. Okaichi et al. (2008) found a differential increase in *p53* expression in human tumors depending on the mutated hotspots, suggesting that *p53* plays a crucial role in cellular responses to IR. Expression of *p53* in *T. japonicus* was controlled by the degree of gamma ray irradiation.

The reproductive impairment and growth retardation effects of gamma radiation are likely due to gamma ray-induced DSBs. IR generates single- and double-stranded breaks, base-free sites, and other modification of DNA bases (Ward and Kuo, 1976). However, organisms have self-repair mechanisms to repair DNA damage, and many studies have focused on gamma ray-induced damage and DNA repair defense mechanisms (Hiyama et al., 2013; Rhee et al., 2013). In gamma-irradiated *T. japonicus*, we measured expression patterns of *Ku70*, *Ku 80*, and *DNA-PK*, as the proteins encoded by these genes are key components of the non-homologous end joining (NHEJ) pathway and DSB repair pathway (Fig. 6). In *T. japonicus* irradiated with 200 Gy gamma rays, expression of *TJ-Ku70*, *-Ku80*, and *TJ-DNA-PK* genes was significantly increased, implying an increase in repair of gamma ray-induced DSBs. Mahaney et al. (2009) suggested that NHEJ is one of main DNA repair mechanisms for IR-induced DNA damage in relation to DSBs in human cells. Our findings on the same patterns of expression in gamma-irradiated *T. japonicus* lend support to the hypothesis that Ku70 and Ku80 heterodimers can form heterotrimers with DNA-PK to repair DSBs in DNA. We also measured the expression of *PCNA* to examine cellular proliferation and the status of DNA repair, because *PCNA* fills DNA gaps generated by radiation and is a marker of cell survival (Rastogi et al., 2010). Kelman (1997) and Torres et al. (2004) reported that regulation of *PCNA* by p53 is important to identify DNA damage and evaluate IR-induced genetic instability.

Antioxidant genes with antioxidant enzyme activities were induced by exposure of *T. japonicus* to gamma rays, although no clear correlation between increased expression and enzymatic activity was discernable. Nie et al. (2006) also reported discrepancies between mRNA expression profiles and enzyme activities, which they attributed to translational efficiency. However, the activities of GPx and GST in gamma-irradiated *T. japonicus* showed a good correlation with mRNA expression of the genes encoding these proteins. Particularly, *T. japonicus GPx* was induced 11-fold relative to the control, implying that the protein encoded by *TJ-GPx* plays a pivotal role in controlling the level of glutathione in gamma-irradiated *T. japonicus*. HSPs are key chaperone proteins in cells. In gamma-irradiated *T. japonicus*, we evaluated the expression of six *Hsp* genes (*Hsp20*, *Hsp20.7*, *Hsp40*, *Hsp70*, *Hsp70p*, and *Hsp90*). Of these, *TJ-Hsp 20.7* was expressed at 30-fold higher levels in *T. japonicus* exposed to 200 Gy irradiation than control *T. japonicus*. Hsp70 and Hsp90 are considered to be the major proteins that protect cells from stress-induced damage. However, in human cells, Baek et al. (2000) showed that small Hsps had a protective role in radiation-induced apoptosis. Rhee et al. (2013) and Kim et al. (2010) reported a significant increase in *Hsp 27* expression upon gamma and UV-B radiation of the fish *K. marmoratus* and the rotifer *B. koreanus*, respectively, suggesting that small Hsps may repair cellular damage caused by sublethal doses of radiation.

Taken together, gamma radiation induces oxidative stress in *T. japonicus*; the resulting cellular damage is repaired through induction of global defense mechanisms such as DNA repair, antioxidant activities, and increased expression of chaperone genes. We observed a causal correlation between in vivo parameters (reproductive impairment and molecular perturbations) and oxidative stress and DNA damage in the intertidal copepod *T. japonicus* in response to exposure to gamma radiation. Our findings provide insight into how gamma radiation may affect population and community structure through reduced fecundity and retardation of molting processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.04.005.

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