

Thermal stress increases oxidative DNA damage in coral cell aggregates

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Abstract. We have established an experimental system to study the response of coral cells to stresses using coral cell aggregates (tissue balls). The objective of this study was to evaluate the use of tissue balls for studies of oxidative DNA damage in corals cells under thermal stress. Tissue balls prepared from dissociated cells of *Pavona divaricata* were exposed to high temperature (31°C) in the presence or absence of an antioxidant or to normal temperature (25°C). DNA damage of coral cells was investigated using a Comet Assay (alkaline single-cell gel electrophoresis). The comet tail length, which indicates the degree of DNA damage, was significantly longer in coral cells from tissue balls exposed to 31°C than those from the controls (25°C) or those treated at 31°C in the presence of an antioxidant, 10 mM mannitol. The present results suggest that coral cells suffer oxidative DNA damage under thermal stress. It was not clear whether DNA damage occurred in algal cells as algal DNA was retained within the cell wall and the comet tail was not observed. This study also showed that tissue balls provide us with a good experimental system to study the effect of stress and various chemical reagents on coral cells.

Key words: Coral, Comet assay, DNA damage, Bleaching

Introduction

Coral reefs are under threats due to increasing sea surface temperature (SST) that cause coral bleaching. A number of laboratory and field studies have reported the link between high temperature stress and coral bleaching (Hoegh-Guldberg and Smith 1989; Fitt et al 1993; Lesser 1997; Hoegh-Guldberg 1999). High temperature stress has been proposed to produce damage of the algal photosynthetic apparatus, which leads to production of reactive oxygen species (ROS) (eLesser 1997; Jones et al. 1998; Bhagooli and Hidaka 2004; Yakovleva and Hidaka 2004) Although cells have an enzymatic antioxidant system to scavenge the harmful ROS (Downs et al. 2002; Lesser and Farrell 2004), the excess ROS cause damage in cellular components such as protein carbonilation (Downs et al. 2002; Richier et al. 2005), lipid peroxidation (Richier et al. 2005) and DNA degradation (Lesser and Farrell 2004). Recent studies showed that apoptotic or necrotic death of host cells are involved in cnidarian bleaching (Lesser and Farrell 2004; Richier et al. 2006; Dunn et al. 2007). On the other hand, zooxanthellae expelled by coral hosts during bleaching exhibit cell degeneration and apoptotic or necrotic death (Franklin et al. 2004; Strychar et al. 2004). However, until now the direct

information about the effect of thermal stress on the DNA level in reef-building corals is very limited.

We have established an experimental system to study the response of coral cells to stress treatment using coral cell aggregates (tissue balls) (Nesa and Hidaka, submitted). Dissociated coral cells aggregate to form spherical bodies, which rotate by ciliary movement. These spherical bodies (tissue balls) stop their rotation and become disintegrated when exposed to stress. The relationship between the survival time and zooxanthella density of tissue balls showed a negative correlation at 31°C, while no significant correlation between the survival time and zooxanthella density of tissue balls was found at 25°C (Nesa and Hidaka, 2009). The results support the hypothesis that zooxanthellae become a burden for host corals under thermal stress probably via production of harmful substances such as reactive oxygen species (ROS).

In this study the use of tissue balls for the study of oxidative DNA damage of coral and algal cells under thermal stress was evaluated. We used the comet assay (single cell gel electrophoresis) to detect DNA damage in individual target cells (e.g., Avishai et al. 2003; Rinkevich et al. 2005).

Material and Methods

Collection and Maintenance of coral specimens

Small colonies of *Pavona divaricata* were collected from the reef at Bise, northern Okinawa. Corals were then brought to the Nishihara campus of the University of the Ryukyus and were maintained in an aquarium provided with a subgravel filter at 26°C under 150 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ on a 12:12h light/dark cycle for up to 2 weeks prior to use.

Preparation of tissue balls

Pavona divaricata fragments were rinsed gently with 0.22 μm filtered seawater (FSW) and then coral tissue was removed from the skeleton using a WaterPik (Teledyne, WP-70J). To remove the mucus produced during the isolation procedure, coral slurry was filtered through 180 μm nylon mesh. The slurry was then homogenized with a homogenizer and filtration through 40 μm nylon mesh was performed. The resulting homogenate was centrifuged at 1200 rpm for 5 min, and then at 1000 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in FSW using a vortexer. The suspension containing dissociated coral cells and zooxanthellae was incubated in a 24-well plate. In each well 1 ml of dissociated coral-zooxanthella cell suspension was incubated with 1 ml 0.22 μm FSW for one night at room temperature. After overnight incubation, dissociated coral cells and zooxanthellae became aggregated and achieved a spherical shape. These spherical bodies (tissue balls) started to rotate by ciliary movement. After one night tissue balls were transferred to a clean petridish with fresh FSW. Healthy (rotating) tissue balls of similar size were chosen under a stereomicroscope (Nikon-SMZ-10) and put separately in each well of a 96-well plate containing 280 μl FSW. These preparations were allowed to recover at room temperature for 3-6 hours before the stress experiments. Although tissue balls contained various numbers of zooxanthellae, the density of zooxanthellae in the tissue balls was not determined in this study.

Treatments of tissue balls

In the first series of experiments, tissue balls were exposed to normal (25°C) or high (31°C) temperature in filtered seawater (FSW) for 10 h. Two 96-well plates, each with 25 tissue balls, were prepared and one plate was placed in an incubator (Yamato, Program Incubator IQ820) set at 25°C and the other plate was placed in another incubator (Sanyo, MIR-152) set at 31°C. In both incubators, the plates were illuminated using a 19W fluorescent light and the light intensity measured by a light meter (LICOR, LI-250) was 35 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$. In the second series

of experiments, tissue balls were exposed to normal (25°C) temperature in FSW or high (31°C) temperature in FSW containing 10 mM mannitol, a scavenger of hydroxyl radicals. In both series of experiments, the experiment was repeated 3 times.

The comet assay

To detect possible DNA damage in coral cells, we used a Comet Assay kit (Trevigen). In the comet assay, cells were embedded in agarose on a microscopic slide. Cells were lysed to remove cytoplasm and most nuclear protein, leaving supercoiled DNA as 'nucleoids'. During electrophoresis DNA is attracted to the anode, but if breaks are present, a "comet tail" of DNA extending from the nucleoid is seen. The size and shape of the comet reflects the extent of DNA damage.

After exposure to thermal stress, tissue balls were placed into 1 ml of ice cold 0.01M PBS containing 20 mM EDTA and dissociated into single cells by pipetting for two minutes. Cells were collected by centrifugation (1,500 g, 1 min) and re-suspended in 300 μl of 0.01 M PBS containing 20 mM EDTA. 50 μl of the cell suspension was mixed with 500 μl low melting agarose (LMA). 75 μl of this mixture was added onto a comet slide and homogeneously spread using a pipette tip. The slides were placed in a refrigerator (4°C) for 20 min. For coral cell lysis, slides were then immersed in a lysis solution containing 1% sodium lauryl sarcosinate (provided with the kit) for 30 min at 4°C. Slides were immersed in alkaline solution containing 200 mM EDTA (pH 13) at room temperature for 20 minutes. Slides were then placed on a horizontal electrophoresis apparatus. TBE electrophoresis buffer was added to the electrophoresis tray to cover the slides. Electrophoresis was conducted at 20-22 volts and 0.01 mA for 10 min at room temperature. After electrophoresis, slides were immersed in 70% EtOH for 5 min. DNA staining was performed by adding 50 μl SYBR Green I (SYBR Green I 10 μl + 1 X TE buffer 200 μl) to each circle of slides. All steps were performed under dimmed light to prevent additional DNA damage during the procedure.

For zooxanthella cell lysis, we used a modified Comet Assay following Erbes et al. (1997). Slides were immersed in alkaline lysing solution containing ionic detergent (0.1% Sodium Dodecyl Sulfate) for 10 min at room temperature. Slides were immersed in alkaline electrophoresis solution for 5 min at 4°C. Slides were then placed on a horizontal electrophoresis apparatus. Alkaline electrophoresis solution was added to the electrophoresis tray to cover the slides. Electrophoresis was conducted at 20-22 volts and 0.01 mA for 10 min at 4°C. After

electrophoresis, slides were immersed in Tris buffer for 5 min.

Image analysis

Samples were visualized and photographed on a fluorescence microscope (Nikon OPTIPHOT-2) at 20 X or 40 X using a digital camera (Nikon Digital Sight DS-LI). Comet tail lengths were measured by using Image J (1.40) software. At least 50 comets were measured in each condition in each of three replicated experiments. Comet tail length is easy to measure and still is a good parameter of DNA damage in sample cells (reviewed by Lee and Steinert 2003).

Statistical analysis

The experiment was repeated three times and the average comet tail length in each condition was used as the statistical unit. A statistical program, Stat view for windows (5.0.1) was used for data analysis. A Mann Whitney U-test was performed to test the significant differences of the comet tail length among the treatment groups. A p value <0.05 was considered to be significant.

Results

DNA damage in coral cells

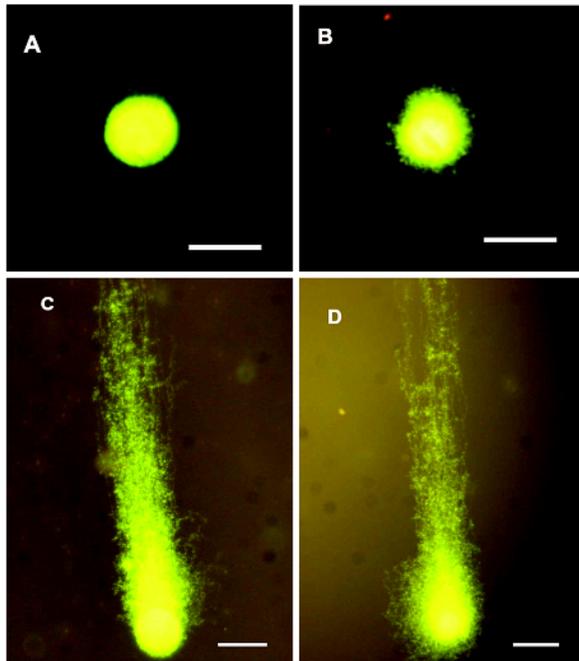


Figure 1: Fluorescence photomicrograph of nuclei of coral cells after alkaline single cell electrophoresis (Comet Assay). A and B, nuclei of coral cells from the control tissue balls kept at 25°C. C and D, nuclei of coral cells from tissue balls exposed to high temperature (31°C). Scale bar = 10 μ m

The nuclei of coral cells from the control tissue balls (25°C) was circular without a tail (Fig. 1A) or with a very small tail (Fig. 1B), while the nuclei of cells from the tissue balls exposed to high temperature (31°C) showed comets with a long tail (Fig. 1C, D).

In this study, we measured comet tail length as an indicator of DNA damage of cells within the tissue balls exposed to different stress conditions. The comet tail length of high temperature-treated coral cells was significantly longer (Fig. 2) than that of the control cells (Mann Whitney U test, $p < 0.05$).

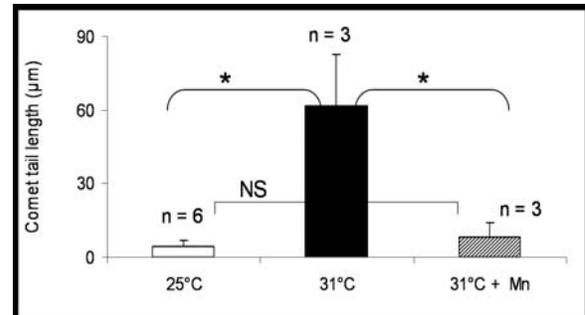


Figure 2: Comet tail length of coral cells from tissue balls exposed to different treatment conditions; open column represents the control (25°C), closed column represents high temperature (31°C) treatment and striped column represents high temperature (31°C) treatment in the presence of 10 mM mannitol.

The coral cells from tissue balls exposed to high temperature (31°C) in the presence of 10 mM mannitol showed significantly shorter comet tail length (Fig. 2) than those from tissue balls exposed to high temperature in the absence of mannitol (Mann Whitney U test, $p < 0.05$). There was no significant difference in the comet tail length between the cells from tissue balls exposed to high temperature in the presence of mannitol and those from the control tissue balls. The comet tail length was $4.3 \pm 2.8 \mu$ m ($n=6$), $61.7 \pm 21.2 \mu$ m ($n=3$), and $8.0 \pm 6.0 \mu$ m ($n=3$) for the control, high temperature-treated tissue balls, and high temperature-treated tissue balls in the presence of the antioxidant, respectively.

DNA damage in algal cells:

The analyzed images of comet assays for zooxanthellae using alkaline lysing solution containing ionic detergent revealed that most of the zooxanthellae from both control and HT treatments showed a distinct head without a tail, or with a small tail (Fig. 3). The comet tail length of algal cells was $0.14 \pm 0.19 \mu$ m ($n=3$) and $0.36 \pm 0.18 \mu$ m ($n=3$), for the controls (25°C) and high temperature (31°C) treated tissue balls, respectively. No significant difference was found in the length of comet tails of zooxanthellae between the control and HT treatment (U-test, $p=0.275$).

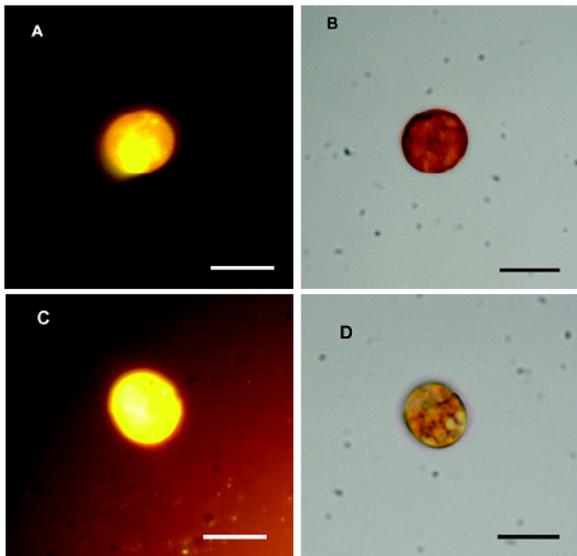


Figure 3: Fluorescence emitted by zooxanthella cells treated with different conditions (A, C); A and B represent control (25°C), and C and D represent high temperature (31°C) treatment. B and D are light microscopy images. Scale bar = 10 μ m

Discussion

The present results clearly showed that coral cells suffer DNA damage under thermal stress. The addition of an antioxidant, mannitol, decreased the extent of DNA damage in tissue balls exposed to high temperature (31°C). This shows that tissue balls suffer oxidative DNA damage probably due to ROS produced by symbiotic algae under thermal stress. Addition of mannitol extended the survival time of *Pavona divaricata* tissue balls suggesting that this antioxidant is effective in protecting cellular integrity of corals (Nesa and Hidaka, 2009).

Apoptotic death of host cells (Richier et al. 2006; Dunn et al. 2007) or algal cells (Strychar et al. 2004) might be involved in cnidarian bleaching. While apoptosis results in DNA fragmentation, DNA damage may lead to apoptosis. Rinkevich et al. (2005) reported that UVB irradiation increases DNA breaks of coral and algal cells. It is likely that the DNA damage detected in the present study is due to direct action of ROS produced by symbiotic algae under thermal stress. Further study using inhibitors or inducers of apoptosis and other cell death pathways may be helpful to understand the death pathway of host and algal cells under thermal stress. The present results also suggest that tissue balls provide us with a useful experimental system for studies of cell death under stress conditions.

In the present study, nuclei of algal cells from tissue balls exposed to high temperature did not show a typical comet shape with a long tail. It is contrary to our expectations as symbiotic algae might be a source of ROS (Nesa and Hidaka, 2009). If ROS

are produced in the chloroplast of the symbiotic algae, algal DNA is most likely to suffer oxidative damage. Rinkevich et al. (2005) detected DNA damage in isolated algal cells caused by UVB irradiation using the Comet Assay. It is not clear whether algal DNA did not suffer oxidative damage in our experiments, or DNA was actually fragmented but fragmented DNA was confined within a rigid cell wall. The modified Comet Assay, which uses an alkaline lysis solution, was applicable to the single-cell alga *Chlamydomonas*. But it is still to be confirmed whether the modified Comet Assay is applicable to *Symbiodinium* spp. Further studies using a transmission electron microscope might be helpful to understand the damage in algal cells under thermal stress.

Rinkevich et al. (2005) found that the holobiont (isolated coral cells containing symbiotic algae) is more sensitive to UVB radiation than isolated coral cells without symbionts or isolated algal cells. Their finding that symbiotic cnidarians are highly sensitive to ROS produced by UVB is consistent with other recent studies; tissue balls with higher densities of zooxanthellae had shorter survival times than those with lower densities of zooxanthellae under thermal stress (Nesa and Hidaka, 2009), and coral larvae with zooxanthellae had lower survivorship and higher levels of oxidative cellular damage than larvae of the same species that lack zooxanthellae (Yakovleva et al. 2009). Experimental systems using planula larvae, tissue balls, and isolated cells have their own advantages and disadvantages, but they might provide useful information on the role of symbionts in cellular damage of the holobiont under stress conditions.

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