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Genetic Variation in Response to Global Warming in a Coral Reef Species, *Porites lobata*

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Abstract
Climate change due to global warming is one of the worst environmental disasters in the world, which affects all ecosystems and has led to increasing degradation of coral reefs. The increase of sea surface temperature is inversely related to the resistance of corals and is directly associated with their bleaching. High temperature disrupts the symbiotic relationship between coral and algal symbiont and results in coral bleaching. To evaluate the adaptation of corals to heat stress, in this study, we investigated the thermal stress effect on the expression of genes involved in programmed cell death (PCD), cysteinyl aspartate proteases 3 (will be mentioned as *Caspas3* hereafter) and anti-apoptotic pathway, B-cell lymphoma 2 (will be mentioned as *Bcl2* hereafter) in *Porites lobata* (Dana, 1846). Corals were incubated at 25°C for 2 weeks (adaptation period) and then exposed to 34°C (heat shock) for 24 and 48 hours. Then, the expression of genes was measured using real-time PCR. The results revealed that both genes were up-regulated at 24 hours after heat induction. *Bcl-2* expression (anti-apoptotic gene) was induced at 24 hours and was down-regulated at 48 hours. In contrast, *Caspase3* (apoptotic gene) continued to be expressed up to 48 hours. These results might indicate that coral cells are headed towards bleaching and death with increased temperature. The results of this study, regarding the observed expression patterns, can clarify the response of different genes to a thermal stress in coral reefs. The exposure of corals to acute conditions with high temperatures presented the behavior of the desired genes in the studied conditions.
Keywords: Environmental deterioration, Apoptosis, *Bcl-2*, *Caspase3*, Real-time PCR, Thermal stress, Global change

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1. Introduction

Stony corals are Cnidarians that have an obligated symbiotic relationship with algal photosynthetic symbionts of the genus *Symbiodinium* (1). Bleaching of coral reefs is closely associated with excessive thermal stress via disturbances in coral-algae symbiosis (2). It has been considered as the most serious threat to reefs in recent years (3). Bleaching of coral reefs adversely affects their health, growth, and reproduction and endangers their structure and function (4,5). Due to global warming and temperature increase, the frequency and scale of mass bleaching have increased over the last 40 years in different corals of the world (6) as well as the Persian Gulf (7) (Fig. 1).

Since the environmental impact of global warming has been gradual, one expects to see adaptive responses for corals that can be mirrored at the physiological level (8). However, the thermal stress has had some irreversible effects on corals around the world through endangering both the hosts and the symbionts as they enter programmed cell death (PCD) (9). PCD, also known as apoptosis, in corals was first reported by Cikala et al (10). Apoptosis leads to widespread inflammation and genetic instability (11), increase in colony mortality and tissue necrosis, bleaching, discontinuity of skeletal growth, and decrease in larval survival, synthesis of proteins, and thickness of epithelial tissue (12). Evidence on some apoptotic genes suggests that PCD in corals is similar to that in vertebrates (13). The relevance between coral bleaching and the disease outbreak indicates that the inherent immune system of coral (as the host) is influenced by bleaching and long-term changes continue even after the end of tough conditions (14). Observations also indicate that apoptosis occurs simultaneously in

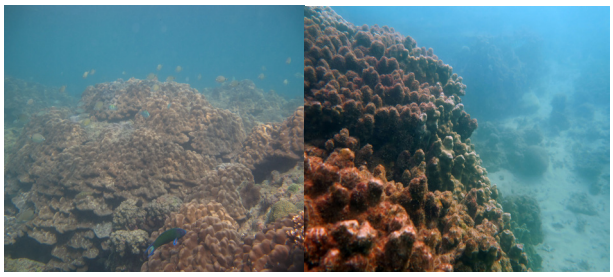

Fig. 1. Coral Reefs of the Persian Gulf (Qeshm Island) after a Temperature Rise in Summer, Left: 2015, Right: 2017.

symbionts and hosts under environmental stress (9).

Apoptosis is a mediated pathway that leads to the elimination of specific cells. This highly conserved process plays roles in biological and physiological functions of multicellular organisms. Its importance is obvious in developmental processes such as metamorphosis (15), symbiosis (16), tissue homeostasis, health, growth (17), response to stress, and defense against pathogens as well (18). Apoptosis is a complicated and pivotal biological process, which causes organisms to remove undesirable cells through disease or even homeostasis (19). Cikala et al (10) realized the apoptosis process in corals for the first time.

Bioinformatic evidence on some apoptotic genes suggests that coral apoptosis is similar to apoptosis in vertebrates (13). In apoptotic cascade, caspases, a family of cysteine proteases, are vital mediators of the inflammatory response and cell death through either formation of apoptotic bodies or cell destruction (20). In a counter-effective action, cells benefit from some anti-apoptotic genes, some of which act as oncogenes (21), which suppress PCD, mainly through the control of mitosis (22). *Bcl-2* is an oncogene that has been proven to act as an apoptosis regulator (20,23,24). Considering that stresses influence a wide variety of biological, physiological, and molecular aspects of an organism, and immunity is the first system which organisms employ when they face a tension, in the current study, the effect of thermal stress on *P. lobata* was studied by the expression analyses of the representative of apoptotic (*Caspase3*) and anti-apoptotic (*Bcl-2*) genes in order to find out how high temperature can change the expression of genes involved in the immune system.

2. Materials and Methods

2.1 Sampling and Tissue Isolation

The samples of *P. lobata* were collected from the Persian Gulf, located in the southeast of Qeshm Island (26° 55.623' N. 56° 15.377' E), through SCUBA diving at a depth of 6 m. Small portions of *P. lobata* reef were prepared by graver and hammer. The live specimens were transferred to the simulation system at the Persian Gulf and Oman Sea Ecological Research Institute, Bandar Abbas, Iran in February 2019. Samples were adapted to laboratory conditions for two weeks at 25°C, a constant salinity of 35 psu, continuous water flow, and a 10 hour (light):14 hour (dark) photoperiod. The corals were fed once during the day with phytoplankton and once during the night with zooplankton (Deep Ocean, Iran). The tissue isolation from control specimens was carried out at 25°C in four biological replicates at the end of the 2-week adaptation period. The water temperature of treatment tanks increased to 34°C. It took almost 12 hours to increase the water temperature to 34°C. Tissue isolation from treated samples was performed at 24 and 48 hours after temperature adjustment in four replicates (totally 12 specimens). The tissues were put in cryovials containing RNAlater (Yekta Tajhiz, Tehran, Iran). They were kept at 4°C for 24 hours, snap-frozen in liquid nitrogen, and stored at -70°C.

2.2. RNA Isolation and cDNA Synthesis

RNA was isolated from tissues (100 mg) using Trizol (Ambion, Invitrogen Co., US) according to the manufacturer's protocol. Quality and quantity of RNA samples were checked by 1.5% (w/v) agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Scientific, US), respectively. DNase Kit (SinaClone, Tehran, Iran) was used to remove DNA residuals. RNA (1 µg) was reverse transcribed into cDNA using cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer 's protocol.

2.3. Quantitative Polymerase Chain Reaction (qPCR)

Real-time PCR was carried out on cDNA samples in two technical replicates for each biological sample (totally 24 specimens) using gene specific primers (Table 1) in a 10 µL reaction volume. The reaction was conducted as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles of [denaturation at 95°C for 30 seconds, annealing

Table 1. Characteristics of the Primers Used in the Study

Primer	Forward	Reverse	Tm (°C)	Amplicon Size
βAct	5′- ATCATGAAGTGCGATGTGGA - 3′	5′- GGAGCAATGATCTTGATCTTCA - 3′	55.25 56.53	151
Caspase3	5′- TTATGATGCATTCATTTTCTCCA – 3′	5′ – GCTTGGAAGAAAAACATCTTTGG – 3′	53.52 57.08	150
Bcl-2	5′- ACACTTTTGCCGCAGTGG - 3′	5′ – TCGTTGAAGATAAAATCCACCA - 3′	55.97 54.66	171

at 60°C for 30 seconds, and final extension at 72°C for 20 seconds]. *Beta-actin* (*βAct*) gene was used as the reference gene or internal control to normalize the data and Livak and Schmittgen (25) method was used to analyze the relative expression of genes.

The efficiency of the primers was determined using serial dilution test. Amplification efficiency (E) was determined for each target gene using the equation $E(\%) = (10^{1/slope} - 1) \times 100$, where the slope was estimated by plotting the Ct over the serial dilutions of cDNA. The expression of target genes relative to the reference genes was calculated by the formula $2^{-\Delta\Delta CT}$ to analyze the relative changes in gene expression. The prepared cDNA was serially diluted (1, 1/3, 1/9, 1/27 and 1/81).

The results of qRT-PCR showed relative mRNA expression level and normality was tested by Kolmogorov-Smirnov test. Differences in the expression of genes between different thermal treatments were analyzed by the one-way analysis of variance (ANOVA), followed by a Tukey's multiple range test for multiple comparisons at the significant level of $P<0.05$. All statistical analyzes were performed using SPSS and SigmaPlot software packages.

3. Results and Discussion

Continuous exposure of the samples to heat shock for 48 hours resulted in bleaching of coral samples. It means that the color of coral samples faded after 48-hours of exposure to 34°C compared to the ones at the control temperature (25°C). The bleaching of samples indicates the disturbance in symbiotic relationship between algal symbionts and corals. However, the corals did not completely bleach until the end of the heat shock induction. This event was obvious in nature due to the climate change and thermal stress on coral reefs during the warm seasons. Therefore, global warming has led to bleaching and then death of many coral reefs in the Persian Gulf in 2017 (Fig. 1).

The results showed that gene expression levels were different in the whole experiment between *Caspase3* and *Bcl-2* although the expression of genes was not significant ($P>0.05$) compared to the reference gene. Fig. 2 (A and B) indicates that the genes were both up-regulated at 24 hours after heat shock; however, the expression of *Bcl-2* was at the maximum level and higher compared to *Caspase3* at 24 hours. It seems that the gene expression increased from the initial time of the experiment to 24 hours after heat stress induction in both genes. Interestingly, the up-regulation of *Caspase3* continued until 48 hours after heat stress induction (Fig. 2A), while the expression of *Bcl-2* sharply decreased at 48 hours (Fig. 2B).

Understanding the relationship between stress and immune system pathway can reveal the details of pathways involved in fighting a stress (26). Coral bleaching not only disrupts the symbiotic relationship between algae and coral but also affects different aspects of the physiology and ecology of corals (4,26,27). Studying coral responses to environmental changes through common tools such

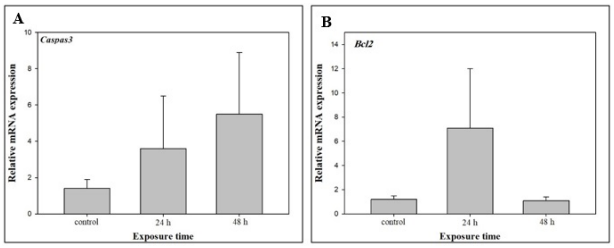

Fig. 2. Expression of the Apoptotic Gene *Casp3* (A) and Anti-apoptotic Gene *Bcl-2* (B) in a Coral Reef Species (*Porites Lobata*) in Response to Increased Temperature at 24 and 48 hours after Heat Shock Compared to Control Phase

as transcriptomic and genomic techniques has revealed many facts about biological pathways. However, the environmental impacts on their immune system have remained vague (28,29). The levels of gene expression in bleached colonies provide evidence for some processes affecting corals (12). The immune system of corals has a vital role in resistance against stressors and this fact would improve predictions about the future status of corals (29,30,31). It is reported that apoptosis is active in various cellular processes in Hydra and bleaching in the anemone *Aiptasia pallida* (9, 32). However, the evolutionary origin of these pathways is not clear yet.

Gene analysis has shown that pathways related to the immune system such as apoptosis are suppressed during and after bleaching (12). The relationship between coral bleaching and its immunity is complicated (14). Bleaching of corals affects the molecular system including the expression of different genes, especially inherent immune genes, and these reactions can continue up to one year after the event. This continuity of gene expression variations is shown at the whole transcriptome level, but the expression levels might be different (12). According to a study by Pinzón et al (12) on bleaching of a coral reef species (*Orbicella faveolata*) due to natural thermal stress, in a symbiotic relationship, the host and symbiont can have different responses to bleaching, and the host immune system might be suppressed even one year after a bleaching event. They showed that the *casp8* expression was upregulated in bleached colonies of *O. faveolata* and it was downregulated after recovery of corals and obviously there were differences in expression of genes in bleached and unbleached colonies; less expression level of *Casp8* gene was observed in white colonies as well (12). Studies on *O. faveolata* under thermal stress have shown that components of *Casp8* pathway were suppressed in bleached colonies. Primary disruption of apoptosis in bleached colonies is likely to be due to bleaching control mechanisms. When the apoptosis is blocked, bleaching decreases (17,33). Although a mechanism for reduction of bleaching is conducted in corals, continuous downregulation of apoptosis could have an immunosuppressive effect almost one year after bleaching (12). Bleached colonies of *O. faveolata* have a

higher prevalence of disease than unbleached colonies during and after bleaching (4). Environmental stressors suppress the immune system in other invertebrates as well (34). Depending on the intensity of apoptosis in the stressed specimen, its gene expression may vary in different tissues. In the sea anemone, for example, the expression of genes in endoderm was higher compared to ectoderm. This may be due to the specialized function of Caspase in different tissues. This gene is more expressed in endoderm-dependent processes even before symbiosis (13).

The difference in gene expression between unbleached and bleached colonies indicated that there must be dysfunction in the complement systems in bleached colonies that causes the immune system to become less active or inactive. The less active component system indicates the lack, reduction or suppression of immune system. The expression level of *Casp8* has been lower in completely bleached corals (12).

Inducing the heat shock of 33°C to 34°C in a soft coral species (*Aiptasia pallida*) showed that activity of *Caspas3* in tensioned samples increased by 45% compared to the control samples. It is noteworthy that *Casp* gene in *A. pallida* is homologous to the secondary structure of *Caspase3* in vertebrates, and heat stress induces apoptosis in the sea anemone species (*Aiptasia* sp.) (9,17). It was shown that proteins of *Bcl-2* family allow the assembly of apoptosome by modulating the release of cytochrome c from mitochondria while the apoptosome contains caspase-9 (35). Observations of inhibitory treatments indicate that *Caspase* activity can be inhibited by gene silencing (17). Most of *Bcl-2* family domains are present in the apoptotic pathway (e.g., Bak and Bax) of vertebrates and they are clearly seen in sea anemones as soft corals and hard corals. Although many *Bcl-2* proteins of corals appear to be orthologues of *Bcl-2* specific proteins in mammals, the relationships of anti-apoptotic proteins in *Acropora* are unclear (35,36). In the current study, considering that *Casp8* and *Bcl-2* act in the opposite way in the apoptosis of cells, the expression trend of both was logically justifiable.

Studies have shown that the frequency of apoptotic cells in the host initially increases with enhancing thermal stress and then decreases. The reduction of apoptotic cells coincided with an increase in necrotic cells in corals. Examining more than 35 000 cells has shown that programmed death and necrosis would increase in response to heat stress over time (9). Thermal stress stimulates the pathways of cell death, which vary among different cells in extent and volume. The studies showed that the apoptosis rate increased in endodermal cells of *A. pallida* under heat shock of 33.5±0.5°C during the first 18 hours and decreased afterwards (9). This may be due to the high sensitivity of endodermal cells to environmental stresses. It means that the expression level of genes increased in the early stages of stress. The constant stress endangers the cell health and causes an increase in the expression level of *caspase* gene, thereby leading to cell death.

Since coral bleaching could be a drastic ecological event and a significant threat for coral reef ecosystems, understanding this process through adaptation and evolution of the cell death process is crucial (37). These observations show that the physiological tolerance of the holobiont as well as the reflection of different genetic compositions over time can define the overall condition of a colony (38,39).

This study indicated that the apoptotic pathway is present in *Porites*. Finding the apoptotic pathway of cnidarians can provide a better understanding of this pathway in higher vertebrates and mammals. Concurrent histological examinations in future studies may reveal a broader view of the mechanisms of apoptosis in corals as defense mechanisms.

4. Conclusion

In this study, coral reefs were used as indicators of environmental deterioration due to global warming. The expression of two genes associated with the apoptotic pathway (*Casp3* as an apoptotic gene and *Bcl-2* as an anti-apoptotic gene) in the coral reef species *Porites lobata* under the thermal stress was investigated at two distinct times (24 and 48 hours) using a high temperature of 34°C. The results of this study revealed that the expression patterns of these genes are consistent with the functions of these genes. The high expression level of gene *Casp3* at 48 hours confirms the down-regulation of gene *Bcl-2* at this time in response to stressful conditions in which leads to decreased resistance and death of *P. lobata*. The investigation of physiological pathways through molecular and genomic approaches could be the subject of future studies for interested researchers.

Conflict of Interest Disclosures

The authors declare that there is no conflict of interests.

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