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Technical note

The effect of chilling and cryoprotectants on hard coral (*Echinopora* spp.) oocytes during short-term low temperature preservation

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Abstract

Understanding chilling sensitivity and chilling injury of coral oocytes, in the presence and absence of a cryoprotectant, is important in developing cryopreservation protocols, as well as for short-term storage and transport (e.g., for species conservation). The objective of this study was to investigate the chilling sensitivity of hard coral (*Echinopora* spp.) oocytes and the effectiveness of methanol (as a cryoprotectant) in protecting these oocytes during short-term, low temperature preservation. Oocytes were exposed to 0.5, 1, or 2 M methanol at 5, 0, or −5 °C for 1, 2, 4, 6, 8, 16, or 32 h, and their quality determined based on adenosine triphosphate (ATP) content. Methanol at 0.5 M was the most effective means to reduce chilling-induced reduction in ATP concentrations. Coral oocytes can be stored at room temperature for 4 h in filtered nature seawater with no detrimental effect on oocyte quality; however, in the present study, oocyte survival was extended for 8 h by addition of methanol in low concentrations (0.5 or 1 M) at low temperatures (5 and 0 °C). These findings should enhance conservation efforts and facilitate low-temperature transport of endangered and threatened coral species.

Keywords: Coral; Oocyte cryopreservation; Cryoprotectant; Chilling sensitivity; Thermal injury

1. Introduction

Coral reefs are at risk worldwide, as most coral species are very fragile and susceptible to damage by humans. Increased global warming and ocean acidity due to increasing carbon dioxide concentrations, along with overfishing and pollution, has caused massive damage to coral reefs, and delayed their recovery.

It is important that conservation efforts focus on coral conservation before population diversities are severely depleted. One of the most promising *ex situ* conservation techniques to preserve coral populations is to cryopreserve their germ cells. Although cryoprotective agents are important for low temperature preservation of cells, they usually have some toxicity [1], which generally can be reduced by lowering exposure temperature [2]. Cryopreservation has been successfully applied to coral sperm [3], but chilling sensitivity (100% mortality) occurred when coral larvae at room temperature were chilled (−11 °C) for 5 min [4]. In studies of the cryobiology of coral oocytes conducted in our laboratory [5–7], hard coral oocyte viability (determined using an ATP assay) was 72.2 ± 4.4%, following

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treatment with 1 M methanol [5]. However, in our previous studies, there were no significant differences in ATP concentration in *Junceella fragilis* oocytes after exposure to 1 vs 2 M methanol treatment, although mitochondrial membrane potential of oocytes was reduced and the conventional mitochondrial distribution pattern was lost [6]. Furthermore, we also determined that gorgonian coral oocytes had substantial cooling tolerance at 5 and 0 °C, but were very sensitive to chilling at −5 °C, with dramatic decreases in ATP after 4 h of chilling [7]. High sensitivity to chilling is also an obstacle affecting fish oocyte cryopreservation [8]. It is important to determine whether hard coral oocytes are also sensitive to chilling. The objective of the present study was to investigate the chilling sensitivity of hard coral (*Echinopora* spp.) oocytes and the effectiveness of methanol (as a cryoprotectant) in protecting these coral oocytes from injury during low temperature storage.

2. Materials and methods

2.1. Coral collection

Coral oocytes (*Echinopora* spp.) were collected when spawning occurred in April and May, by scuba diving from the reef slopes at a depth of 3 to 6 m in Kenting National Park, Nanwan, Taiwan (21°56′ N, 120°44′ E), as previously described [6]. Oocytes were collected at night by use of a vacuum suction technique using a disposable plastic 60 ml syringe with 5-mm diameter plastic tubing to ensure capture of the oocyte during coral spawning events [5]. Oocytes were immediately transported back to the laboratory and kept in an aquarium containing filtered seawater (maintained at 25 °C).

2.2. Effect of cryoprotectants on oocyte chilling sensitivity

This study investigated the effects of chilling (in the presence or absence of methanol) on oocytes. Hard coral oocytes were exposed to 5, 0, or −5 °C in solutions containing 0.5, 1, or 2 M methanol for 1, 2, 4, 6, 8, 16, or 32 h. Control oocytes were incubated in filtered natural seawater (salinity, 35‰) at room temperature (25 °C), 5, 0, or −5 °C. Cryoprotectants were added and removed in a single step in filtered natural seawater. Oocytes in each test tube (1.5 ml) containing cryoprotectant solutions were placed in a low temperature bath (5, 0, or -5 °C). After chilling, oocytes were warmed in a water bath at 25 °C, washed twice in filtered natural seawater, and then their viability was determined using an ATP assay [9], as described in the next section. Unless otherwise stated, all chemicals used in the study were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.3. ATP assay

Mitochondria play a vital role in oocyte energy metabolism by providing ATP for gamete maturation and embryo development. The concentration of ATP is a direct marker of metabolic activity and energy status of oocytes [9]. Therefore, an ATP assay was used to assess oocyte viability. Following chilling, coral oocyte ATP concentrations were measured with the ApoSENSORTM Cell Viability Assay Kit (BioVision, Cambridge BioScience, UK). Briefly, 10 oocytes were treated with 200 μL of Nuclear Releasing Reagent for 5 min at room temperature (with gentle shaking). Then, 220 μL of Enzyme Reconstitution buffer was added to the vial of ATP Monitoring Enzyme, resulting in a yellow-green milky-like solution. Thereafter, 1 μL of the ATP monitoring enzyme was added into the oocyte lysate and ATP content of the oocyte was measured in 1 min using a luminometer (Berthold Lumat LB 9,507, Berthold Technologies, Wildbad, Germany).

2.4. Statistical analysis

Each treatment in the experiment contained three replicates and experiments were repeated at least three times. After ensuring homogeneity of variance with the Levene test ($P > 0.05$) and normality with the one-Sample Kolmogorov-Smirnov test ($P > 0.05$), a two-way ANOVA was applied to test for the effects of treatments, time and their interaction on ATP content Individual mean differences were determined with post-hoc tests and differences were considered statistically significant for P'0.05. All data were analyzed with SPSS software (Version 17.0; SPSS, Inc., Chicago, IL, USA) and presented as mean ± SEM across the three replicates.

3. Results

The effects of varying concentrations of methanol after chilling at 5, 0, or −5 °C on ATP concentrations in oocytes are shown (Fig. 1). Methanol at 0.5 M was the most effective concentration to reduce chilling sensitivities. Howevever after just 1 h chilling at −5 °C, the ATP concentrations of oocytes in 0.5 and 1 M methanol were significantly different from those in 2 M methanol or control groups (Fig. 1b, 1c, and 1 d). No ATP was released from oocytes after 4 h chilling in 2