Cryobiology 75 (2017) 60–67

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Cryopreservation of *Bufotes viridis* embryos by vitrification

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ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form
30 December 2016
Accepted 8 February 2017
Available online 11 February 2017

Keywords:
Vitrification
Cryoprotectants
Bufotes viridis
Blastula
Jelly coat
Toxicity

ABSTRACT

Loss of biodiversity among amphibians is a current concern. Our hypothesis is that the embryos of amphibian species at risk of extinction could be cryopreserved by vitrification, using methods which have proved successful with fish oocyte. To test this hypothesis, samples of four cryoprotectants - methanol (MeOH), dimethyl sulphoxide (Me2SO), propylene glycol (PG) and polyethylene glycol (PEG), some singly, some in combination, were plunged in liquid nitrogen for 5 min to find the best solution for vitrification. To find the least toxic of these solutions, blastulae and stage G17 embryos of *Bufotes Viridis*, a typical amphibian, were exposed to solutions at different concentrations (0.5–10 M) for different lengths of time (15–30 min), with and without their normal protective jelly coats. In each case the number of survivors, which reached stage G25 was counted. Finally a series of embryos was vitrified in liquid nitrogen using the most efficient and least toxic cryoprotectants.

Propylene glycol had the best vitrification characteristics, but MeOH vitrified at higher concentrations. The optimum regime, with the least toxic ctyoprotectants, consisted of 1M Me2SO for 15 min and a combination of 15% $PEG_{(w/v)}$ + 3M PG + 2M Me2SO for 3 min, with the jelly coat intact, followed by vitrification. This gave a survival percentage of 87.6% immediately after vitrification. Methods designed for cryopreservation of fish embryos make a good starting point for cryopreservation of the embryos of amphibian.

1. Introduction

Decline in species and genetic biodiversity, is occurring all over the world in the 21st century [2–5,19,27]. Among amphibians there has been a 50% decline in population and 32% of species are in danger of extinction [27]. In some areas the decline of amphibians is greater than that of other vertebrate taxa [2,4,15].

Conservation of natural ecosystems helps to preserve amphibians in-situ, but may be difficult to control [1,5,25].

Cryobanking is an alternative preservation strategy. Storage of embryos for extended periods of time at a relatively low cost, and the ability to suspend the growing embryo at specific stages of development are among important benefits of cryopreservation for research and educational purposes. Significant progress has been made in the cryopreservation of bacteria, spermatozoa and microlecithal eggs, but more studies are needed to develop a successful cryopreservation technique for the meso- and macrolecithal eggs of amphibians and fish.

Cryopreservation by vitrification (rapid cooling) is among the more effective cryopreservation techniques, because cell and tissue injury is less. The embryos are first immersed in a mild dehydrating solution to reduce intra-cellular water. The remaining water in the cells is then replaced with cryoprotectants that will not create large crystals when cooled down [24]. Finally, the specimens are vitrified by sudden cooling in liquid nitrogen.

During the last three decades, research has been concentrated on the cryopreservation of fish eggs and embryos [16,17,30], but there are some reports on the cryopreservation of amphibian eggs and embryos. Frisbie et al. [8], tried to supercool *Rana sylvatica* embryos at different stages of development, up to −5 °C. They found some embryos survived cooling for a few hours. Guenther et al. [13] and Kleinhans et al. [18] investigated factors that influence intracellular ice formation (IIF) in oocytes (stage I &II) of the *Xenopus* frog. Lawson et al. [20] cryopreserved early embryos of the marsh frog, *Limnodynastes peronii*and and recovered a few live cells following cryopreservation of both gastrula and neurula. Uteshev

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http://dx.doi.org/10.1016/j.cryobiol.2017.02.003
0011-2240/

et al. [28] using embryos of *Rana temporaria* and *Bufo bufo*, found 10% dimethyl sulfoxide (Me2SO) and 10% saccharose were the best cryoprotectants for recovering live stem cells.

In this study we examine and repeat several of the more successful cryopreservation methods. *Bufotes viridis* was selected as an example of a typical amphibian, whose egg is among the range of small size amphibian eggs. It was proposed that due to the small size, and lower yolk density, there will be a reduced potential barrier for cryopreservation diffusion and that there will be an increased chance of success.

Our hypothesis is that the method of cryopreservation, which has been proved successful in some extent for fish will also prove satisfactory for the toad, *Bufotes viridis*. To test this hypothesis, we aim:

1) To confirm the vitrification characteristics of several cryoprotectant solutions.
2) To test the successful solutions for toxicity to the *Bufotes* embryo at different stages, different strengths and for different lengths of time.
3) To test toxicity for embryos with and without their normal protective jelly coat.
4) To test the use of the most successful solution in vitrification in liquid nitrogen

2. Material and methods

2.1. Egg collection

Bufotes viridis is a terrestrial toad and lays its eggs in long strands. *Bufotes viridis* spawns were obtained from amplected pairs collected from ephemeral ponds in a semi-aired area (Bajgah, 52° 35′ E, 29° 43′ N) 10 km from Shiraz city, Fars, Iran. Spawns were transferred to polypropylene containers filled with de-chlorinated and aerated tap water. Mixed clutches of eggs (three clutches in total-mean clutch size = 6643 ± 1855) from different parents were used for all experimental treatments. Before starting the experiments, eggs were left in lab temperature to equilibrate with environment temperature. The experiment was performed on two stages of development: Intact blastula and stage G17 (newly hatched larva) according to Gosner's (1960) table [11].

2.2. Experiment 1: vitrification characteristics test

Vitrification characteristics were tested in the absence of the embryo. We suggested that all cryoprotectants were equally suitable for vitrification and the method was used to test individual and combination solutions. To determine the vitrification characteristics of individual cryoprotectants, 11 different concentrations of the solutions (0.5–10M) were loaded in cryovials (Greiner, Germany). Each cryovial was then plunged into liquid nitrogen for 5 min and after warming in a water bath (26 ± 1 °C) the degree of ice formation in the solutions was assessed. Ice formation makes the solution opaque [29]. Based on the results of the vitrification characteristic of individual cryoprotectants, six combination cryoprotectants (V_1-V_6) were suggested (Table 1). Then the same procedure as for individual cryoprotectants was carried out. There were three replicates for each concentration.

2.3. Experiment 2: cryoprotectant toxicity test

We hypothesised that increasing the concentration of cryoprotectants both individually and in combination, would not have any effect on their toxicity for either blastula or stage G17 embryos.

The level of cryoprotectant toxicity was determined by exposing the blastula and embryo at stage G17 (n = 100 for each stage and each concentration) to different concentrations of individual cryoprotectants (0.5–10 M) for 15 and 30 min and of combination cryoprotectants (V_2,V_4,V_6) for 5, 10 and 15 min all in laboratory temperature (22 °C ± 2.0). We applied most of these times and concentrations with slight modifications based on the protocols that were introduced by Zhang and Rawson (1996) and Guan (2009) in zebra fish. After treatment, embryos were washed three times in de-chlorinated water and transferred to special container to develop. Embryos were allowed to continue their development until stage G25, and the survival percentage and total size at stage G25 were analyzed and compared with the results found from tadpoles, which had developed in normal condition as controls. No cryoprotectant was applied to the eggs in the control group. There were three replicates for each concentration and the control group. Before and after experiments the growing eggs were situated in identical conditions of food (when growing larvae started feeding - boiled lettuce leaves), light (12h. light, 12h. darkness), temperature (20 °C ± 2.0) and pH (7.71 ± 0.15).

After carrying out the two tests above, the maximum concentration of cryoprotectant, which had no negative effect on embryo survival was determined. Minimum concentration of the cryoprotectant that is still active was also estimated, and might give a better result.

2.4. Experiment 3: vitrification characteristic and cryoprotectant toxicity test in dejelly blastula

Our preliminary suggestion was that removing the normal jelly coat would make it easier for cryoprotectants to enter the cell and therefore give good results during vitrification at lower concentration. The hypothesis to test is that “removing the jelly coat will make no difference to the toxic effect of different cryoprotectants”.

In order to examine whether the normal jelly coats have a significant role in development and in response to different cryoprotectants (toxicity test), other groups of eggs (n = 100 for each concentration) were de-jellied. Eggs at blastula stage were de-jellied by two methods. In the mechanical method [20] the jelly layer was removed by rolling the egg on a filter paper to absorb free water around the egg, and then when the jelly stuck to the paper we removed the jelly layers from the egg by forceps while the vitelline envelope remained around the embryo. In the chemical method, eggs were de-jellied using cysteine solution. Eggs were slowly rolled in 2% w/v cysteine solution at PH 8.1 for 5 min. During this procedure jelly layers were easily removed while the blastula remained within the vitelline layer [20].

The developmental patterns of dejelly eggs were compared with the patterns were found in normal jelly eggs.

2.5. Experiment 4: vitrification process

The hypothesis to be tested is that using the least toxic cryoprotectant solution and the most efficient protocol, embryos will survive vitrification and reach stage 25.

Since there is no major study on vitrification of amphibian eggs and embryos, we used the vitrification protocol for fish as we know the physical characteristics of embryos of these two groups of animals at early stages are more similar than the other groups. Therefore, the vitrification study was based on a study on zebra fish - the main species of fish under study so far [29,31].

Vitrification was performed in two steps after applying cryoprotectants. The first step was to equilibrate the embryo and in this step embryos (n = 360) were exposed to 1M concentration of MeOH, Me2SO or PG (n = 120 for each of cryoprotectants) for