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ScienceDirect

Theriogenology 75 (2011) 220–232

Theriogenology

www.theriojournal.com

Cryopreservation of hormonally induced sperm for the conservation of threatened amphibians with *Rana temporaria* as a model research species

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Received 24 May 2010; received in revised form 15 August 2010; accepted 18 August 2010

Abstract

The survival of hundreds of threatened amphibian species is increasingly dependent on conservation breeding programs (CBPs). However, there is an ongoing loss of genetic variation in CBPs for most amphibians, reptiles, birds, and mammals. Low genetic variation results in the failure of CBPs to provide genetically competent individuals for release in supplementation or rehabitation programs. In contrast, in the aquaculture of fish the perpetuation of genetic variation and the production of large numbers of genetically competent individuals for release is accomplished through the cryopreservation of sperm. Successful protocols for the cryopreservation of amphibian sperm from excised testes, and the use of motile frozen then thawed sperm for fertilisation, have been adapted from those used with fish. However, there have been no protocols published for the cryopreservation of amphibian hormonally induced sperm (HIS) that have achieved fertility. We investigated protocols for the cryopreservation of amphibian HIS with the European common frog (*Rana temporaria*) as a model research species. We induced spermiation in *R. temporaria* through the intraperitoneal administration of 50 μg LHRHa and sampled HIS through expression in spermic urine. Highly motile HIS at a concentration of 200×10^6/mL was then mixed 1:1 with cryodiluents to form cryosuspensions. Initial studies showed that; 1) concentrations of $\sim 15 \times 10^6$/mL of HIS achieve maximum fertilisation, 2) TRIS buffer in cryodiluents did not improve the recovery of sperm after cryopreservation, and 3) high concentrations of DMSO (dimethylsulphoxide) cryoprotectant reduce egg and larval survival. We then compared four optimised cryopreservation protocols for HIS with the final concentrations of cryodiluents in cryosuspensions of; 1) DMSO, (½ Ringer Solution (RS), 10% sucrose, 12% DMSO); 2) DMSO/egg yolk, (½ RS, 10% sucrose, 12% DMSO, 10% egg yolk), 3) DMFA, (½ RS, 10% sucrose, 12% dimethylformamide (DMFA)), and 4) MIS/glycerol, (Motility Inhibiting Saline (MIS), 5% glycerol, 2.5% sucrose, 5% egg yolk). Cryosuspensions were frozen in LN_2 vapour, stored in LN_2, thawed in 40° C water bath, and activated by slow equilibration with 1:3 dilutions of cryosuspensions with 20 mM/L NaCl. Protocol efficacies were assessed through the post-thaw percentage of; 1) sperm motility, 2) sperm membrane integrity, 3) fertilisation, 4) fertilised eggs hatching, and 5) larval survival from fertilised eggs to 7 d. The DMFA cryodiluent proved superior to the DMSO based cryodiluents in recovery of sperm motility and fertility after cryopreservation. MIS/glycerol cryodiluent provided low sperm viability and no fertility. Considering the ease of obtaining HIS from many *Rana* species, the success of our protocols offer the potential for the perpetuation of the genetic variation of the 42 threatened *Rana* species and the 193 threatened Ranid species in total.
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Keywords: Cryopreservation; Amphibian; Sperm; Genetic variation; Hormonal induction

1. Introduction

There are many hundreds of threatened amphibian species whose survival is dependent on conservation breeding programs (CBPs) [1]. A major challenge in

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doi:10.1016/j.theriogenology.2010.08.008

CBPs has been the perpetuation of natural genetic variation [2]. Loss of the natural genetic variation of a species leads to reduced survival in both nature and in captivity. Low genetic variation results in, poor reproduction, less ability to cope with environmental change, and lowered success of conservation breeding, restocking, or rehabitation programs [3,4,5]. Without sperm cryopreservation, the perpetuation of genetic variation in CBPs requires the keeping of up to hundreds of brood stock of each species and strict regulation of pairings through studbook programs [2]. Unfortunately, these requirements have not been achieved in a CBP for any amphibian species, and rarely achieved for mammals, reptiles or birds [2]. The alternative means to perpetuate genetic variation through the use of cryopreserved sperm greatly reduces the numbers of brood stock required in amphibian CBPs and is relatively efficient, secure, and reliable [3,4,5]. The most dependable method of sampling amphibian sperm for cryopreservation has been from testes excised from sacrificed individuals [6,7,8,9]. The alternative use of hormonally induced sperm (HIS) does not require the sacrifice of individuals, however, until the current study there have been no protocols published for the cryopreservation of amphibian HIS resulting in subsequent motility and fertility.

The perpetuation of amphibian genetic variation could also potentially be achieved through the cryopreservation of oocytes, eggs, embryos or larvae [3,5]. However, with fish their cryopreservation has so far been prevented by factors including their large size and the high lipid content of yolks. In both fish and amphibians, the perivitelline membrane surrounding the yolk is non penetrable to most cryoprotectants and has also proven a major impediment to successful cryopreservation [10]. Nevertheless, the recent development of commercial techniques for the cryopreservation of small marine fish embryos are expected to have a wide area of application in breeding companies, research and development companies, vaccine producers and public conservation programs [11]. Amphibian oocytes and larvae are generally much larger than those of most fish [12,13] and their successful cryopreservation has not been achieved [3]. Nevertheless, the sampling of somatic cells, their cryopreservation, and subsequent nuclear transfer into ooctyes may eventually offer a practical, method to perpetuate both female and male genetic lineages [4,5].

The need for the reliable reproduction of amphibians during research [14] and in aquaculture [15,16] provided incentives for the development of protocols for the induction of HIS using pituitary extracts or hormones. Once sampled, HIS has been used fresh or through unfrozen storage for days in a refrigerator at 4° C [7,17,18]. Even limited numbers of motile sperm may then be used for direct artificial fertilisation of oocytes, and either motile or immotile sperm for artificial fertilisation of oocytes for a limited numbers of oocytes using intra-cytoplasmic sperm injection (ICSI) [5,19]. These techniques alone are adequate to perpetuate the genetic variation of threatened amphibians. Nevertheless, the development of cryopreservation protocols that recover highly motile and fertile HIS will also enable the direct fertilisation of the several hundreds to thousands of ooctyes spawned by many anuran species [5,12].

Cryopreserved sperm is a widely used reproduction technology in fish aquaculture to perpetuate genetic strains to produce large numbers of genetically competent individuals for conservation, restocking and consumption [20,21]. Fish and amphibians have a close phylogenetic relationship and many externally fertilising species have, similar fertilisation environments and strategies [3]. Consequently, the development of reproduction technologies for amphibians including protocols for the cryopreservation of amphibian sperm have largely been modeled on those for fish [3,6,22]. During cryopreservation and the subsequent fertilisation of ooctyes these technologies include similar types and concentrations of cryoprotectants, freezing and thawing rates, methods for osmotic equilibration of sperm and activation of motility, and fertilisation environments [3,6,21].

The sperm of saltwater fish are generally easier to cryopreserve that those of freshwater fish [21]. These differences are partly due to the sperm of freshwater fish retaining plesiomorphic (basic ancestral) morphologies and physiologies originating from the marine fertilisation environment [23,24,25]. The subsequent complex history of the fertilisation environment of anurans then included at least one episode of internal fertilisation, followed by external fertilisation, then reversion to internal fertilisation in some species [23,24]. This may explain anuran sperms' relatively long average period of motility of 60 min [Unpublished results], which is 7× longer than that of saltwater fish and 16× that of freshwater fish [25,26]. This extended period of motility in hypo-osmotic environments also shows the relatively high osmotic tolerance of anuran sperm when compared with fish sperm [Unpublished results]. Amphibian sperm also appears to be generally resistant to

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