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Cryosurvival of isolated testicular cells and testicular tissue of tench *Tinca tinca* and goldfish *Carassius auratus* following slow-rate freezing

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ABSTRACT

Experiments were carried out to test the efficiency of cryopreservation of whole testicular tissue in tench *Tinca tinca* and goldfish *Carassius auratus* and compare it to cryopreservation of isolated testicular cells. Additionally, effects of three cryoprotectants (dimethyl sulphoxyde – Me_2SO, methanol – MeOH and ethylene glycol – EG) at three concentrations (1 M, 2 M and 3 M) on post-thaw cell viability were assessed. Tissue pieces/isolated testicular cells were diluted in cryomedia and cryopreserved by slow-rate freezing (1 °C/min to −80 °C followed by a plunge into the liquid nitrogen). In both species Me_2SO and EG generally yielded higher cryosurvival of early-stage germ cells than MeOH, while spermatozoa of neither species displayed such a pattern. In most cases a 3 M > 2 M > 1 M viability pattern emerged in both species for both sample types regardless of the cryoprotectant used. Sample type (dissociated testicular cells vs testicular tissue) did not seem to affect viability rates of tench early-stage germ cells and goldfish spermatozoa, while the opposite was observed for tench spermatozoa and goldfish early-stage germ cells. Additionally, through histological analysis we displayed that tissue structure mainly remained unaltered after thawing in goldfish. These results indicate that cryopreservation of whole testicular tissue is indeed a valid alternative method to cryopreservation of dissociated testicular cells. Early-stage germ cells obtained from cryopreserved testis can be further used in different purposes such as transplantation into suitable donors while viable sperm might be used for fertilization when feasible.

1. Introduction

Current trends and developments in science and biotechnology lead to significant advances in industry, but also in understanding basic scientific questions. These advances lead to novel needs, primarily in the area of management and preservation of genetic resources. For instance, since the development of molecular genetics techniques thousands of mutant strains of mice, *Drosophila* and zebrafish have been created by transgenic techniques (Mazur et al., 2008). Consequently, safe storage of these genetic resources became necessary beyond the common practices of keeping breeding colonies. Additionally, many rare breeds and strains of different animals are at the verge extinction and need adequate management and conservation strategies.

Cryopreservation has quickly risen as a favorable method in conservation and preservation of genetic resources. Currently, many protocols have been developed for the cryopreservation of germplasm (gametes and/or embryos) in many species (Mazur et al., 2008), however, there are still major obstacles at some key points. One of them is the cryopreservation of fish oocytes and embryos due to their complex structure and large volume of yolk material. A novel practice has recently evolved which can overcome this barrier through cryopreservation of primordial germ cells (PGCs) and/or spermatogonial stem cells (SSCs), which can later develop into functional sperm and eggs following transplantation (Yoshizaki et al., 2011). One downside of this technique is that SSCs are firmly incorporated into the testicular tissue and need specialized techniques for successful isolation which may not be available when needed (due to technical or time restrictions). Therefore a new alternative concept arose in cryopreservation of whole testicular tissue.

Through this method both sperm and early-stage germ cells such as spermatogonial stem cells (SSCs) can be preserved. Although cryopreservation of testicular tissue has been achieved to a certain level in some mammalian (Ehmcke and Schlatt, 2008; Milazzo et al., 2008) and bird species (Liu et al., 2013), according to Mazur et al. (2008) there were no reports on successful cryopreservation of testicular tissue in ornamental fish species. Since then, this method has been tested in zebrafish (Bono-Mestre

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et al., 2009) and rainbow trout (Lee et al., 2015, 2013), however there is still a large gap in our knowledge whether cryopreservation of whole tissue is plausible in other species as well. Therefore in this study we aimed to assess the efficiency of cryopreservation of whole testicular tissue of two cyprinid fish species (tench and goldfish) by using different cryoprotective media and compare it to cryopreservation of isolated testicular cells.

2. Material and methods

2.1. Sampling procedure

A total of eight male tench *Tinca tinca* (TL: 172 ± 22 mm; W: 45 ± 18 g) and eight male calico goldfish *Carassius auratus* (TL: 109 ± 23 mm; W: 25 ± 14 g) individuals were captured from the wild and a local fish farm, respectively, and were brought to the Department of Aquaculture, Szent István University, Gödöllő, Hungary. Fish were held in a 1.5 m^3 tank supplied with aerated water at a constant temperature of 24 ± 1 °C until sacrificing. Fish were sacrificed by an overdose of 2-phenoxyethanol (Acros Organics 130240010).

2.2. Experimental design

Immediately following sacrificing, testes were excised; part of the testis of each individual was fixed in 8% formalin for histological and stereological analysis (counting of different testicular cell types), while the remainder was pooled and washed in phosphate-buffered saline (PBS; Sigma-Aldrich P-4417) on ice for further analyses (Fig. 1). Large blood vessels and fatty tissue were removed from the testes under a stereomicroscope and testes were cut into small pieces (~20–30 mg). One part of the obtained pieces was cryopreserved immediately in order to freeze the whole testicular tissue (three pieces per experimental group; in case of tench we mixed two immature testes pieces and one mature) while the remainder was digested in order to prepare for freezing of isolated testicular cells. Upon thawing of frozen tissues, pieces were digested and viability of cells was assessed. In case of goldfish, one thawed piece per experimental group was fixed in 8% formalin for histological and stereological analysis (analysis of testicular structure and spermatozoa nucleus volume).

2.3. Histological procedure

Fixed samples were dehydrated in ethanol series, cleared in xylene and embedded into paraffin blocks. One block per individual/experimental group was made. Three-micron thin sections were cut on a sliding microtome, mounted and stained by the standard hematoxylin and eosin (H&E) staining technique. Sections were observed by Nikon Eclipse 600 microscope and photographed by QImaging MicroPublisher 3.0 digital camera.

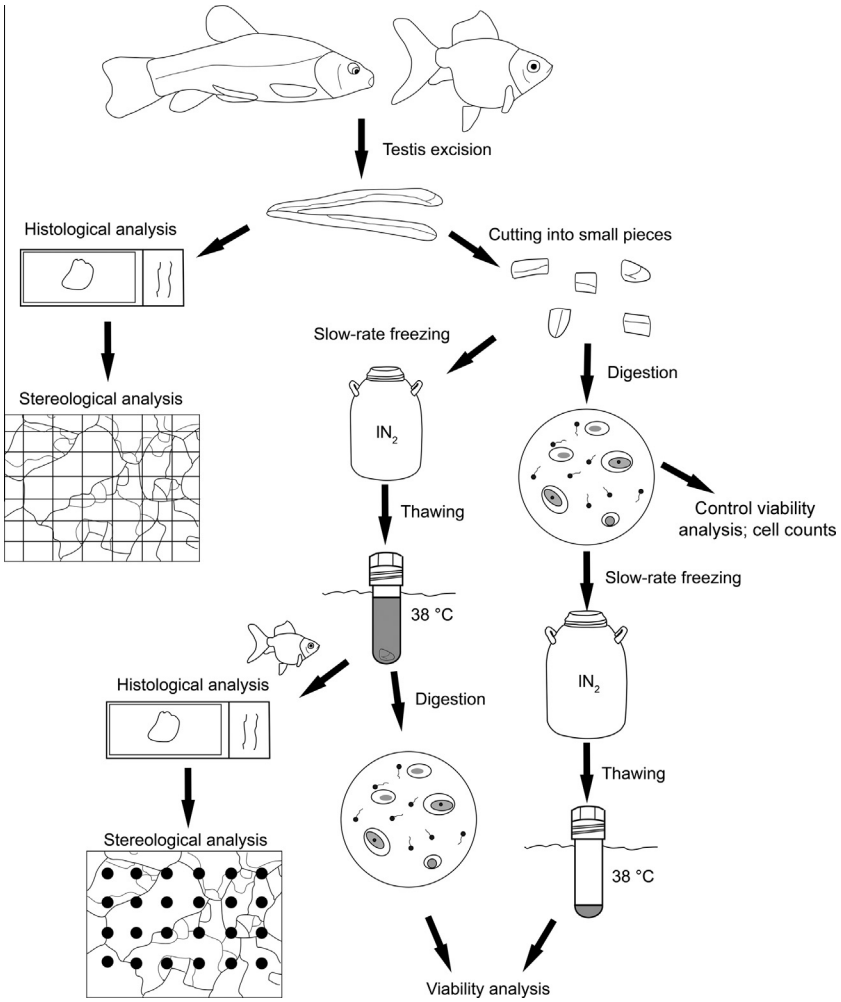

Fig. 1. Schematic representation of the experimental design.

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