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Progress and challenges of fish sperm vitrification: A mini review

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ABSTRACT

To survive low temperature is required for a long-term storage (cryopreservation), cells should be vitrified to a state in which intracellular water is solidified without ice crystal formation. Two different approaches are described for fish sperm cryopreservation: 1) sperm conventional cryopreservation, in which extracellular water is partially crystallized and 2) sperm vitrification, in which both intra- and extra-cellular liquids are vitrified. Sperm vitrification has been applied to some fish species with limited success. Traditional vitrification requires rapid cooling/warming rates, small sample carriers, and using high permeable cryoprotectant concentrations. The latter cause cytotoxic effects which must be well managed and will require continuous effort to match an appropriate cryoprotectant with suitable apparatus and warming methods. Novel cryoprotectant-free sperm vitrification approach has been applied to several fishes. This review summarizes development of basic procedures and discusses advantages and disadvantages of vitrification when applied it to fish sperm.

1. Introduction

Vitrification has become an increasingly popular method of preserving the cells and tissues by using permeable cryoprotectant concentrations which will develop an amorphous glassy state, while preventing intracellular and extracellular ice crystallization [1,2]. First reported in 1937, there has been recent renewal of interest after a long latent period [3]. During the last decade, scientists keep their attention on human sperm vitrification without any permeable cryoprotectants [4]. Researchers feel that vitrification might offer the capability to cryopreserve cells using simple and fast procedures needing no specialized equipment [4,5].

Successful vitrification of fish gametes requires high concentrations of permeable cryoprotectants, and a rapid temperature change. Vitrification has been applied for fish primordial germ cells [6], oocytes [7], eggs [8], testicular tissues [9] and embryos [10]. Spermatozoa were the first mammalian cell to be cryopreserved by slow-cooling using glycerol as a cryoprotectant [11]. The use of vitrification on fish spermatozoa is relatively a new application. Several scientists have tested vitrification on fish sperm; they have mainly focused on permeable cryoprotectant toxicity at various concentrations, exposure times, and temperatures [12,13]. Traditional vitrification on spermatozoa has been tested with limited success on: Russian sturgeon *Acipenser gueldenstaedtii* [14], Persian sturgeon *A. persicus* [15], rainbow trout *Oncorhynchus mykiss* [16], channel catfish *Ictalurus punctatus* [17], green swordtail *Xiphophorus hellerii* [18], spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus*, red drum *Sciaenops ocellatus* [19], Atlantic salmon *Salmo salar* [20], Tambaqui *Colossoma macropomum* [21], Eruasian perch *Perca fluviatilis*, and European eel *Anguilla anguilla* [22], while only few studies carried out on fish sperm by cryoprotectant-free vitrification [23,24].

The aim of this review is to (1) summarize the basic procedures of vitrification of fish sperm (2) discuss the current progresses in vitrification application for fish spermatozoa (3) compare the advantages and disadvantages of vitrification, and (4) to provide

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recommendations for the future research.

2. Vitrification approaches

The basic sperm vitrification procedures are to suspend the spermatozoa in a vitrification solution and then plunge the sample into liquid nitrogen to obtain a vitreous transparent state [25]. Success for fish spermatozoa by traditional vitrification depends on several factors, including high quality sperm, suitable cryoprotectants, equilibration time, and cooling and warming rates [26]. To prevent intracellular ice crystal formation, high concentrations of permeable cryoprotectants and rapid cooling rates are important [12]. Also the temperature and equilibration time need to be carefully controlled to reduce chemical toxicity, this is particularly critical because high concentrations of cryoprotectants are used [27]. For cryoprotectant-free vitrification, osmotic stress and chemical toxicity should be avoided and extremely high cooling rate is necessary to reduce ice crystal formation.

2.1. Development of traditional vitrification solutions

Most gametes will not survive cryopreservation without cryoprotectants, which reduce cryo-damage and protect them from ice crystallization [28]. Two forms of cryoprotectants are classified according to their capacity to penetrate the plasma membrane. Dimethyl sulfoxide (DMSO), glycerol (Gly), ethylene glycol (EG), methanol (MeOH), and propylene glycol (PG) are permeating cryoprotectants. They increase viscosity within the cell, thereby preventing water molecules to form ice crystals [29]. Non-permeating cryoprotectants include sucrose, albumins, dextran, egg yolk, hydroxyethyl, and polyethylene glycols. These cryoprotectants prevent cellular damage caused by freeze-thaw events, like crystallization and recrystallization [30]. Vitrification solutions should be considered relative to toxicity of the permeable cryoprotectants, the role or effect of temperature, the interactions between various intracellular biomolecules and membranes, inclusion of serum proteins, osmolytes, and buffers as supplements to the cryoprotectant solution that promote vitrification and protect the cells against cryoinjury [30]. Salinity of an extender can play an important role in sperm protection [26]. Multi-component salt media are effective for cryopreservation of salmonid spermatozoa [31]. Additionally, the extender composition with certain osmolality and pH can affect the sperm activation and motility in several fish species [32].

High molecular weight additives, disaccharides, can significantly reduce the required concentration of permeable cryoprotectant without passing through the cell membrane, as well as the reduction of toxicity of permeable cryoprotectants [33,34]. Sucrose benefiting plasma membrane of cells during cryopreservation [35], while trehalose can enhance glass formation [34]. Good post-thaw fertility was obtained by adding 0.6 M sucrose to semen of Asp *Aspius aspius* [36]. Similarity, high fertilization rates (82%) were acquired with thawed sperm of orange-spotted grouper *Epinephelus coioides* by adding trehalose into cryopreservation solution [37]. However, in contrast, sucrose was ineffective when added to the vitrification solution for rainbow trout spermatozoa [23]. Isachenko et al. [38] showed that putting sucrose and 1% human serum albumin into the vitrification medium of human sperm had a visible cryoprotective effect on mitochondrial membrane integrity.

Permeating cryoprotectants with low molecular weight, are widely used in the cryopreservation of fish spermatozoa [13,39]. Dimethyl sulfoxide is the most common cryoprotectant used for sperm cryopreservation of marine fishes because of the fast cell permeability which is little affected by temperature [40,41]. The basic requirement of vitrification is to determine the effective concentration of permeable cryoprotectants that is needed to form glass (Table 1). Glass formation can be distinguished by the appearance after plunging samples into liquid nitrogen as the sample remains transparent while the sample becomes milky white if crystallization occurs [30]. A high concentrations of permeable cryoprotectants is required to reach vitrification, consequently, toxicity is an obvious consideration in the selection of permeable cryoprotectants. Most permeable cryoprotectants are toxic and have hypertonic effects at concentrations needed for vitrification [42]. Also the reaction to a permeable cryoprotectant differs from one species to another (Table 2). An experiment performed in a marine fish demonstrated that ethylene glycol has the lowest toxicity followed by dimethyl sulfoxide [19], but Wayman et al. [43] found that dimethyl sulfoxide was the least toxic cryoprotectant and glycerol was among the most toxic.

Mixtures of several permeable cryoprotectants and stepwise addition have been used to reduce toxicity but attain the necessary concentration [44,45]. In general, reduced toxicity of permeable cryoprotectant mixtures has been explained by vitrification that occurs at lower concentrations and an aggregate effect of the combined properties of each permeable cryoprotectant [30]. One of the most commonly used cryoprotectant mixture for vitrification in mammalian gametes is dimethyl sulfoxide and ethylene glycol [46]. Dimethyl sulfoxide is a better glass former, while ethylene glycol is less toxic and permeates faster than dimethyl sulfoxide [46,47]. Average post-thaw motilities of 44% for spotted seatrout, 43% for red snapper and 20% for red drum were reported with a combination of dimethyl sulfoxide and ethylene glycol, along with trehalose [19]. Similar results were also achieved on Atlantic salmon spermatozoa by using a combination of a standard buffer (Cortland medium), 10% dimethyl sulfoxide, 2% bovine serum albumin (BSA), 0.13-M sucrose and 30%, 40%, and 50% of seminal plasma [20]. Employing acetamide in the vitrification of fish embryos and sperm achieved little success [19,48]. In addition, after evaluating three different addition methods, Cuevas-Uribe et al. [17] suggested that there is no advantage in adding the

Table 1
Appearance of cryoloop and droplets containing different concentrations of cryoprotectants after direct plunging into liquid nitrogen.

Cryoprotectant		Cryoloops (mm)			Droplet
		2	3	4	
methanol (MeOH)	10% MeOH	M	M	M	M
	20% MeOH	M	M	M	M
	30% MeOH	M	M	M	M
	40% MeOH	T	T	T	M
ethylene glycol (EG)	10% EG	M	M	M	M
	20% EG	M	M	M	M
	30% EG	M	M	M	M
	40% EG	T	T	T	I
dimethyl sulfoxide (DMSO)	10% DMSO	M	M	M	M
	20% DMSO	M	M	M	M
	30% DMSO	M	M	M	M
	40% DMSO	T	T	T	T
propylene glycol (PG)	10% PG	M	M	M	M
	20% PG	M	M	M	M
	30% PG	M	M	M	M
	40% PG	T	T	T	T
20% (MeOH)+ 20% (EG)		T	T	F	T
20% (MeOH)+10% (EG)+ 10% (PD)		T	T	F	T
5% (MeOH)+10% (EG)+ 20% (PD)		T	T	T	T

Note: M, milky; T, transparent; I, intermediate; F, fail to handle. 1, 2, 3 represent different size of cryoloops with diameter of 2 mm, 3 mm, 4 mm, individually; while droplets performed with 7 μL.