Vitrification of Buffalo Oocytes: Current Status and Perspectives

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ABSTRACT

During the past decade vitrification has been acknowledged as a viable alternative to traditional slow-rate freezing in both human and animal embryology. The buffalo is the major milk and meat producing farm animal in many developing countries. Buffalo oocytes obtained from slaughterhouse ovaries and matured \textit{in vitro} are useful sources. Cryopreservation of buffalo oocytes is very important in preserving this endangered species for future use. This review presents the recent buffalo oocytes vitrification procedures, the principle of vitrification, protocols for buffalo oocytes vitrification and the future of buffalo oocytes vitrification.

Keywords: buffalo, oocyte, vitrification

INTRODUCTION

Water buffalo are divided into two main types: river buffalo and swamp buffalo, which have 50 and 48 chromosomes respectively. Nowadays, the buffalo is the major milk and meat producing farm animal in many developing countries. Buffalo oocytes obtained from slaughterhouse ovaries and matured \textit{in vitro} are useful sources for reproductive procedures such as somatic cell nuclear transfer, IVF and intracytoplasmic sperm injection (ICSI), in which mainly cryopreserved spermatozoa are used. Yet the limited number of buffalo oocytes made it difficult to assess the success rate of these reproduction technologies. From this problem, the topic of “How to store the oocytes for further use?” has been raised several years ago.

Although, the biotechnologies of reproduction have been used in this special, most of them are not as efficient as in bovine. Hence, it is imperative to study the factors necessary to improve the success rate of the application for reproductive biotechnologies in this species.

Cryopreservation of oocytes is very important in preserving female gametes for future use. Efficient oocyte cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species. After cryopreservation, the oocytes presents compromised developmental competence. Optimal cryopreservation protocols should be adapted to individual

Accepted April 10, 2013; Online November 11, 2013.
species requirements, which present large variation in gamete size, permeability and sensitivity to cryoprotectant (CPA).

In assisted reproductive technology (ART), cryopreservation of embryos has become important for the best use of supernumerary embryos. During the cryopreservation of embryos, various types of injury may occur. Among the most damaging is the formation of intracellular ice. The first strategy to prevent intracellular ice from forming was to use a lower concentration of CPA and a long slow-cooling stage. This slow-freezing method has proven effective for embryos of a wide range of mammalian species. Unlike embryos of laboratory animals and domestic animals, in which dimethylsulphoxide (DMSO), glycerol or ethylene glycol (EG) are commonly used as the CPA. With slow freezing, however, it is difficult to eliminate injuries occurring from ice formation completely. Furthermore, the slow-freezing method requires a long period of time before embryos are stored in liquid nitrogen (LN2).

An alternative form of cryopreservation is vitrification. Vitrification is defined as “the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling” (Fahy et al., 1984). The rapid cooling process can minimise chilling injury and osmotic shock to the embryos. With recent improvements in past decades, vitrification has become the most reliable strategy because it is technically simple, and can lead to high survival and implantation rates.

Several advances have taken place in vitrification technologies over the past decade. It is proposed that vitrification will definitely become the most suitable method for cryopreservation of any cells and tissues in the near future. Therefore, vitrification technologies applied on buffalo oocytes and embryos has become more successful as an alternative to slow cooling methods.

**PRINCIPLES OF VITRIFICATION**

Vitrification is the alternative method of cryopreservation which uses an ultra rapid cooling rate, eliminating the need for programmable freezing equipment. Furthermore, the vitrification technique uses high concentrations of CPA which avoids water precipitation, preventing intracellular ice crystal formation.

The basic procedure for vitrification is simple. Embryos or oocytes are suspended in a vitrification solution and then plunged in LN2, or super-cooled air. Embryos are warmed rapidly and diluted quickly with a sucrose solution. The most important stage is the exposure of embryos to the vitrification solution before rapid cooling. In order to prevent intracellular ice from forming, a longer period of exposure is desirable. If the exposure is too long, however, embryos suffer from the toxicity of the CPA solution. Therefore, the optimal exposure time for successful vitrification must be a compromise between preventing the formation of intracellular ice and preventing toxic injury. Ironically, embryos may be injured by the toxicity of the cryoprotectant before enough cryoprotectant can permeate inside the embryos. To prevent this, a two-step procedure is commonly used, in which embryos are first equilibrated in a dilute (e.g. 10%) CPA solution, followed by a brief (30–60 s) exposure to a vitrification solution before embryos are cooled with LN2. The optimal exposure time in the vitrification solution depends on the CPA solution and the
temperature, as both the permeability of embryos and the toxicity of the CPA are largely influenced by the temperature (Rall and Fahy, 1985; Menezo et al., 1992). In vitrification, the selection of CPA requires extreme care because their concentration can be as high as 6 M, which can make the toxicity of these compounds a key limiting factor in cryobiology. The most appropriate characteristics of a penetrating CPA are low toxicity and high permeability. As a less toxic CPA, EG is commonly and widely used (Kasai, 2002).

PROTOCOLS FOR OOCYTES VITRIFICATION

Several protocols have been applied on buffalo oocytes vitrification. In those protocols, however, the basic concept is similar, and the differences between the protocols are related to the vitrification container, the type and concentration of CPA and duration of exposure of CPA. Protocols and outcomes are presented in Table 1.

Because of the high intracytoplasmic lipid content, buffalo oocytes are supposed to be particularly sensitive to chilling injuries (Boni et al., 1992). Slow freezing results of immature and matured buffalo oocytes demonstrated that slow freezing is not suitable for immature buffalo oocytes, as proven by both poor maturation rates and development to morulae (Gautam et al., 2008a), and that vitrification is more effective than slow freezing for the cryopreservation of in vitro-matured buffalo oocytes (Gautam et al., 2008b).

A two-step protocol for vitrification with French straw as a container using EG and DMSO as base-CPA for GV stage oocytes have been described at the beginning of the buffalo oocytes vitrification research (Dhali et al., 1999; Dhali et al., 2000). In 2004, the first successful production of a buffalo blastocyst derived from IVM and IVF of vitrified-warmed oocytes was reported (Wani et al., 2004), although the efficiency remained low. Insufficient cooling rates of oocytes were considered one of the principal obstacles in vitrification technology. In order to overcome this problem, several methods have been proposed using very small amounts of solution. Some improved vitrification methods have been successfully used for buffalo oocyte cryopreservation, including solid surface vitrification (SSV, Gasparini et al., 2007; Boonkusol et al., 2007; Liang et al., 2012), cryoloop (Gasparini et al., 2007), cryotop (Muenthaisong et al., 2007), open pulled straw (Mahmou et al., 2008), microdrop (Liang et al., 2011).

In 2007, Sharma and Loganathasamy proved that the meiotic stage affects survival rates of buffalo cumulus-oocyte complexes (COC) submitted to vitrification/warming, with higher values for those with 24 h maturation compared to those with a shorter one. One of the most successful ultra-rapid vitrification techniques is the Cryotop method that has resulted in excellent survival and developmental rates with human and bovine oocytes (Kuwayama et al., 2005). Cryotop (Muenthaisong et al., 2007; Attanasio et al., 2010; Liang et al., 2012) has been successfully utilized to cryopreserve buffalo oocytes, which retain the capability to develop into blastocyst following parthenogenetic activation, nuclear transfer, and IVF.

CPA mixtures may have some advantages over solutions containing only 1 permeable CPA (Vajta et al., 1998; Chian et al., 2004), and a mixture of EG and DMSO has been widely used for oocyte cryopreservation (Muenthaisong et al.,
THE FUTURE OF BUFFALO OOCYTES VITRIFICATION

With the aim of improving survival rate and quality of oocytes after vitrification, different approaches have been developed pointing to the use of different vitrification container, type and concentration of CPA.

The major damaging factors, which occur during cryopreservation, are associated with chilling injury, osmotic stress, CPA toxicity, and ice crystallization (Mazur et al., 1972). In general, we are trying to reduce these damages by increasing cooling and warming rates using vitrification. In the past, vitrification was based on the combination of a high cooling rate and high concentration of CPA, which caused breakthrough in the field of vitrification came when sample volume was reduced to a level that permitted lowering the CPA concentration. Success has been reported in a handful of mammalian species, but differences between species make cryopreservation techniques’ dissemination difficult.

Some attempts to improve cryopreservation outcome through manipulations to germplasm have been reported, but more studies are needed to identify the more promising ones, which will be incorporated into routine oocytes vitrification protocols.

ACKNOWLEDGEMENTS

This study was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Suranaree University of Technology. Y.Y. Liang was supported by SUT postgraduate research fellowships. A part of this research was supported by WCU (World Class University) program (R31-10056) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

REFERENCES


Muenthaisong, S., C. Laowtammathron, M. Ketudat-Cairns, R. Parnpai and S.


Table 1. Protocols, containers, CPA concentration, time and properties of the buffalo oocytes vitrification.

<table>
<thead>
<tr>
<th>Author and reference</th>
<th>Type of container</th>
<th>Oocytes stage</th>
<th>Equilibration step</th>
<th>Vitification step</th>
<th>Warming step</th>
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</thead>
<tbody>
<tr>
<td>Dhali et al., 1999</td>
<td>straw</td>
<td>GV</td>
<td>2.25M EG + 1.7M DMSO 1 or 3 min</td>
<td>4.5M EG + 3.4M DMSO 2 min</td>
<td>0.5M S. 5min</td>
</tr>
<tr>
<td>Dhali et al., 2000</td>
<td>straw</td>
<td>GV</td>
<td>I: 2.25M EG + 1.7M DMSO 1 or 3 min II: 1.75 M EG 1 or 3 min</td>
<td>I: 4.5M EG + 3.4M DMSO 2 min II: 3.5 M EG 2 min</td>
<td>0.5M S. 5min</td>
</tr>
<tr>
<td>Wani et al., 2004a,b</td>
<td>straw</td>
<td>GV</td>
<td>1.5 M of DMSO, EG, PROH and glycerol, respectively, 5 min</td>
<td>3.5, 4, 5, 6, and 7 M of DMSO, EG, PROH and glycerol, respectively, 5 min</td>
<td>0.5M S. 5min+ 0.25M S. 5min+ 0.1M S. 5min</td>
</tr>
<tr>
<td>Gasparrini et al., 2007</td>
<td>SSV, CLV</td>
<td>MII</td>
<td>SSV: 4%EG 12-15min CLV: 7.5%EG + 7.5% DMSO 3min</td>
<td>SSV: 35%EG + 5%PVP + 0.4M trehalose 25-30s CLV: 16.5%EG + 16.5% DMSO 25s</td>
<td>SSV: 0.3M trehalose 3min CLV: 1.25M S. 1min + 0.62M S. 30s + 0.42 M S. 30s + 0.31 M S. 30s</td>
</tr>
<tr>
<td>Sharma et al., 2007</td>
<td>straw</td>
<td>MII, GV, 6h IVM, 12h IVM 18h IVM</td>
<td>40%PROH + 0.2M trehalose 3 min</td>
<td>1 M S. 15min</td>
<td></td>
</tr>
<tr>
<td>Boonkusol et al., 2007</td>
<td>SSV, straw</td>
<td>MII</td>
<td>SSV: 4%EG 5-10min Straw: 4%EG 5-10min</td>
<td>SSV: 35%EG + 5%PVP + 0.4M trehalose 25-30 s Straw: 40%EG + 5% PVP + 0.4M trehalose 1 min + LN2 vapor 3min</td>
<td>SSV: 0.3M trehalose 1min + 0.15M trehalose 2min + 0.075M trehalose 2min Straw: 0.3M trehalose 1min</td>
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<tr>
<th>Author and reference</th>
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<th>Warming step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muenthaisong et al., 2007</td>
<td>cryotop</td>
<td>MII, enucleated MII</td>
<td>7.5% EG+7.5%DMSO 4, 7 or 10 min</td>
<td>15%EG+15%DMSO+S. 1 min</td>
<td>0.5M S. 5min</td>
</tr>
<tr>
<td>Mahmoud et al., 2008</td>
<td>Straw, open pull straw</td>
<td>GV</td>
<td>1: 3M EG 2: 1.5M EG+1.5M DMSO 3: 1.5M EG+1.5M glycerol 4: 1.5M DMSO+1.5M glycerol 45 s each</td>
<td>1: 6M EG 2: 3M EG+3M DMSO 3: 3M EG+3M glycerol 25 s each</td>
<td>0.5M galactose 5 min</td>
</tr>
<tr>
<td>Gautam et al., 2008b</td>
<td>straw</td>
<td>MII</td>
<td>1: 10%, 25%, 40% EG each 1 min 2: 10%, 25%, 40% DMSO each 1 min 3: 10%EG+10%DMSO 1min 4: 10%EG+10%PROH 1min</td>
<td>1: 40%EG 1min 2: 40%DMSO 1min 3: 20%EG+20%DMSO 1min 4: 20%EG+20%PROH 1min</td>
<td>0.5M S. 1min+ 0.33M S. 1min+ 0.17M S. 1min</td>
</tr>
<tr>
<td>Attanasio et al., 2010</td>
<td>cryotop</td>
<td>MII</td>
<td>10%EG+10%DMSO 3 min</td>
<td>20%EG+20%DMSO 20-25 s</td>
<td>1.25M S. 1min+ (0.62, 0.42, 0.31) M S. 30s each</td>
</tr>
<tr>
<td>Liang et al., 2011</td>
<td>microdrop</td>
<td>MII</td>
<td>10%EG+10%DMSO 1 min</td>
<td>20%EG+20%DMSO 30 s or 45 s</td>
<td>0.5 M S. 5 min</td>
</tr>
<tr>
<td>Liang et al., 2012</td>
<td>cryotop, SSV</td>
<td>GV</td>
<td>1: 7.5 μg/mL CB 15 min +10%EG+10% DMSO 1 min 2: 10%EG+10% DMSO 1 min</td>
<td>20%EG+20%DMSO 30 s</td>
<td>0.5 M S. 5 min</td>
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