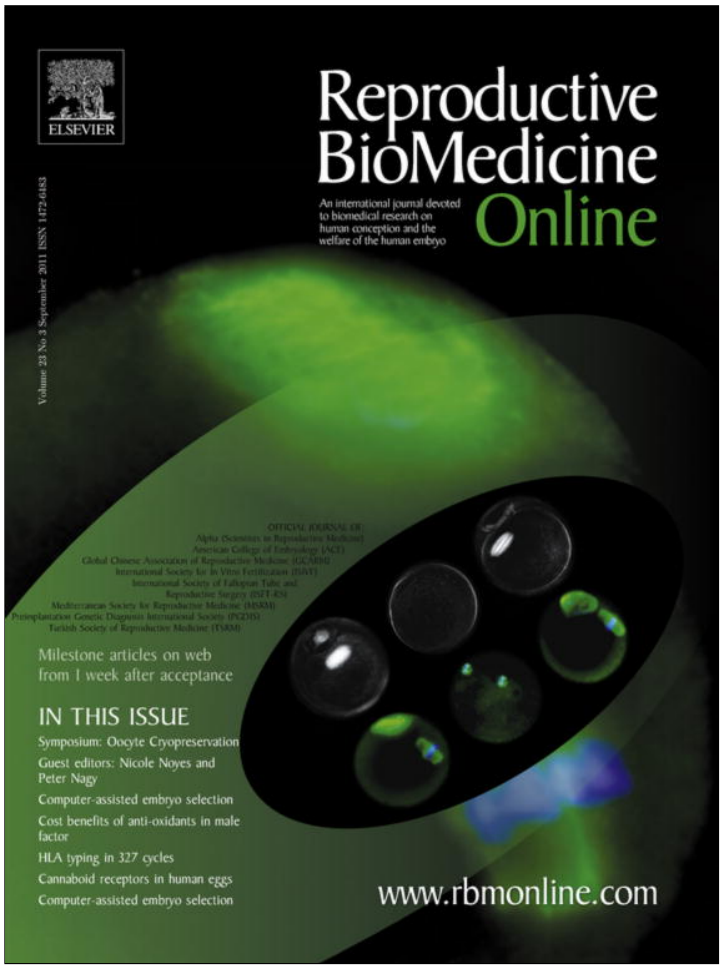

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SYMPOSIUM: OOCYTE CRYOPRESERVATION
REVIEW

Cryopreservation of oocytes in experimental models

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Abstract Until recently, success in oocyte cryopreservation has been very limited mainly due to poor understanding of the complex physiological processes that lead to cell damage during cryopreservation. In the past three decades, however, a wealth of information has been collected using various different animal models, which has led to development of new technologies and optimization of existing ones. The use of these models has provided the opportunity for research that may not have been possible with human material. Today, results of these studies still continue to form the basis of oocyte cryobiology. This review discusses these studies, especially the physiological impacts of cryopreservation on oocyte biology. It will also focus on the role that animal models have played in improvement strategies, validation before translating new techniques into the human model and the advances made in the human in IVF because of these animal models. Finally, existing investigations and their potential impact in other areas of research will be discussed.

KEYWORDS: animal models, oocyte cryopreservation

Introduction

Since the birth of the first 'test tube' baby in 1978 (Steptoe and Edwards, 1978), IVF has grown into a prominent field of the human healthcare system. In 2008, in the USA alone, the number of total in-vitro cycles (autologous plus donor oocyte cycles) from clinics registered with the Society for Assisted Reproductive Technologies was just short of 101,000 (http://www.sart.com/). Of note, almost 10% of these cycles used donor oocytes. The European Society of Human Reproduction and Embryology (ESHRE) reported for 2006 almost 440,000 cycles from 32 countries, which included about 3500 cycles from frozen–thawed oocytes (de Mouzon et al., 2010). It can be only assumed that, since then, these numbers on oocyte cryopreservation have continued and will continue to increase exponentially as the technology becomes more widely available and reliable in the public eye. Since patients typically produce more oocytes than the number of embryos that are preferred for replacement, oocyte cryopreservation is becoming increasingly desirable as an alternative for embryo cryopreservation for IVF patients. Its value is amplified even more if one considers

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the ethical concerns and limiting legislation in some countries regarding embryo freezing. Furthermore, oocyte cryopreservation allows a means to obtain donated oocytes without having to rely on a fresh donation.

The first live human birth from a cryopreserved oocyte was reported in 1986, following a slow cooling protocol using dimethylsulphoxide (DMSO) as a cryoprotectant (Chen, 1986). This news came almost 10 years after the first birth in a mammalian animal model, the mouse (Whittingham, 1977). The success of Wittingham was a direct result of more than 30 years of extensive experimentation with cryopreservation in various animal species (Whittingham, 1971; Willadsen, 1977; Wilmut and Rowson, 1973). This research laid the foundation upon which sperm, embryo and oocyte freezing still rests today. Since the first successful human oocyte cryopreservation was performed, the techniques have been improving consistently by dedicated workers, greatly impacting clinical infertility treatment. Animal models played a critical role in these studies and it can be said without a doubt that, had it not been for the accessibility of mammalian animal oocytes, the procedure for human oocyte cryopreservation would not have been nearly as advanced as it is today, and perhaps would not have occurred yet.

Today, animal models still continuously provide imperative data that facilitates further advancements in oocyte cryobiology. This review will focus on the physiological impacts, current improvement strategies and future applications of oocyte cryopreservation using animal models as they benefit not only human oocyte cryopreservation procedures, but also the human species through their usefulness in agriculture, medicine and conservation.

The effect of cryopreservation on oocyte physiology in experimental models

Ca^{2+} ion increases after oocyte cryopreservation

A current potential risk with oocyte cryopreservation is the induction of an oocyte activation event. Mammalian sperm and oocyte fusion induces the elevation of the concentration of the intracellular calcium ions (Ca^{2+}) (Lawrence et al., 1997). Fertilization ultimately causes a rise of Ca^{2+} that starts from the point of sperm entry and spreads in a wave-like manner across the entire egg (Eisen et al., 1984; Gilkey et al., 1978; Sardet et al., 2002). Although the Ca^{2+} rise is largely due to release of calcium ions from internal cellular reserves, particularly from stores within the endoplasmic reticulum. This elevated Ca^{2+} may also originate from the external environment or from combination of the two (Shiina et al., 1993). The rise of Ca^{2+} could also trigger cortical granule exocytosis (Kline and Kline, 1992). The cortical granules then biochemically and structurally modify the zona pellucida and plasma membrane so that spermatozoa can no longer bind or remain bound to the egg. However, if the Ca^{2+} rise was prematurely triggered in the mature oocyte by environmental factors, it could cause parthenogenetic activation, cellular degeneration and render the oocyte non-fertilizable.

Elegant investigations in the mouse model (Larman et al., 2007a) demonstrated that the cryoprotectant 1,2-propanediol (PROH) could cause a significant rise in intracellular calcium that is capable of inducing cellular degeneration and oocyte activation. The study also showed that zona hardening occurred. These irreversible effects were observed after oocyte cryopreservation using PROH with slow-freezing procedures (Chang et al., 2010; Shaw and Trounson, 1989; Van der Elst et al., 1992). Interestingly, fetal development rates of mouse oocytes cryopreserved in a choline-based slow-freezing medium with PROH were comparable to the fresh oocytes (Stachecki et al., 2002). It has been shown that PROH can support the organization of the metaphase-II spindle to resist the subphysiological temperature; however, an anaphase/telophase spindle or an early stage pronucleus were observed in approximately 12.5% of mouse oocytes after the subsequent slow freezing and thawing process (Chang et al., 2010), which suggested that oocyte activation may have been triggered in a portion of oocytes. However, it is still not clear whether the oocyte activation and zona hardening are simply because of the cryoprotectant exposure, the process of slow freezing and thawing (i.e. inter- and intra-cellular ice crystal formation) or both, since parthenogenetic activation of unfertilized mouse oocytes by exposure to PROH was also influenced by temperature, oocyte age (hours post human chorionic gonadotrophin), and cumulus removal (Shaw and Trounson, 1989). Likewise, other cryoprotectants commonly used in the vitrification, DMSO and ethylene glycol (EG), could cause a large increase in intracellular calcium concentration in mouse metaphase-II oocytes (Larman et al., 2006; Takahashi et al., 2004). The amplitude of this rise in Ca^{2+} due to cryoprotectants is equivalent to the initial increase triggered at fertilization (Saunders et al., 2002), but lasts about 50% longer than the natural Ca^{2+} spike (Larman et al., 2006). This calcium influx is thought to be responsible for the zona hardening caused by exposure to the cryoprotectants. It is, however, worthy to note that zona hardening can be prevented by adding serum albumin to the freezing medium (George et al., 1992) and keeping the exposure temperature low, as was indicated in the mouse model (Carroll et al., 1990; dela Pena et al., 2001; Hotamisligil et al., 1996). In contrast, although the significant increase of Ca^{2+} could possibly be induced by DMSO and EG, oocyte activation was not noticed after 4 h of warming in sequential spindle observations when mouse oocytes were vitrified with a mixture of DMSO and EG (Chang et al., 2011). The use of animal models in oocyte cryopreservation provides the opportunity for further investigations, which could lead to understanding the basis of oocyte cryobiology.

Cytoskeletal change after oocyte cryopreservation

The meiotic spindle is extremely sensitive to low temperatures, as it may result in significant meiotic spindle disruption (Magistrini and Szollosi, 1980). Disassembly of the meiotic spindle is highly associated with dispersion of metaphase chromosomes and subsequent chromosomal anomalies (Boiso et al., 2002; Bouquet et al., 1992, 1995; Glenister et al., 1987; Kola et al., 1988; Nasmyth, 1999; Sathananthan et al., 1988). During oocyte cryopreservation, intracellular ice formation, cryoprotectant toxicity, osmotic stress, nonphysiological temperatures, phase transition and pH instability are factors believed to affect the structure of the meiotic spindle. The influence of cryopres-

ervation on metaphase-II oocyte spindle dynamics has received a lot of attention in recent years (Chen et al., 2004; Ciotti et al., 2009; Gomes et al., 2008; Larman et al., 2007b; Stachecki et al., 2004). However, studies involving the structure of the oocyte spindle during cryopreservation have been complicated by contradictive observations among different laboratories and protocols. Thus far, understanding of the structural change of the spindle during cryopreservation remains vague.

Introduction of the Polscope has allowed real-time investigation of the meiotic spindle in living oocytes. It enables noninvasive detection and assessment of the meiotic spindle as a means to evaluate oocyte quality without injuring the oocytes. Therefore, in recent human oocyte cryopreservation studies, most of the spindle observations were performed using this device (Chen et al., 2004; Ciotti et al., 2009; Gomes et al., 2008; Larman et al., 2007b). However, these Polscope observations of oocyte spindle structure during cryopreservation were inconsistent between investigators and protocols. Details of structural changes of the spindle during cryopreservation also remain unclear, because of technical limitations of polarized light microscopy. Chromosome positioning cannot be detected in Polscope observations. In some cases, morphometric evaluation of the spindle through Polscope was not consistent with confocal analysis (Coticchio et al., 2010). Although the oocytes have to be fixed, it is believed that immunocytochemistry with confocal microscopy still provides more sensitive and detailed information about the spindle structural changes and chromosome arrangement (Bromfield et al., 2009; Huang et al., 2008). Accordingly, an animal experimental model using immunocytochemistry with confocal microscopy could provide a distinctive method to clarify the effects of oocyte cryopreservation on the spindle without the interference of potential artefacts.

Strategies to improve oocyte cryopreservation using experimental models

The evaluation of subsequent embryo development is one of the most important ways to assess the safety and efficiency of a novel assisted reproductive technology. Therefore, an animal experimental model should always be at the forefront of such research to validate a new technology or a new treatment, since very limited experimentation is possible on human embryos or oocytes. Parameters such as fertilization rate, cleavage rate, blastocyst rate, embryo implantation rate and live birth rate are often chosen as indicators of overall effectiveness. Therefore, based on those experimental models, some strategies have been developed to improve the efficiency of oocyte cryopreservation.

The use of cryoprotectants

All successful oocyte cryopreservation methods have one common objective: to avoid ice crystal formation. Since the first successful mammalian oocyte cryopreservation was performed using DMSO as a cryoprotectant (Whittingham, 1977), several other agents such as EG and PROH have been widely applied to oocyte cryopreservation. These consist of small molecules that penetrate cell membranes to form hydrogen bonds with water molecules, thereby preventing formation of ice crystals. Among these permeating cryoprotectants, PROH is most commonly used in oocyte slow freezing, and its experimental model was originally established in mouse oocytes (Gook et al., 1993). Exposure of oocytes to high concentrations of cryoprotectants can be detrimental to oocytes because of osmotic and toxic effects (Joly et al., 1992; Oda et al., 1992; Vincent et al., 1989). The potential genotoxicity has been indicated when mammalian cells were exposed to PROH without cryopreservation (Aye et al., 2010); however, PROH has been and is still being used as a major cryoprotectant for oocyte slow freezing. Ethylene glycol is an effective cryoprotectant for mouse oocyte vitrification protocols without any known compromise to morphology and development (Hotamisligil et al., 1996). Thus, EG became a universal cryoprotectant for human oocyte vitrification (Kuleshova et al., 1999; Kuwayama et al., 2005; Yoon et al., 2003). In bovine oocyte vitrification studies, the mixture of permeable cryoprotectants (EG and DMSO) provided advantages over solutions containing one permeable cryoprotectant (Chian et al., 2004; Vajta et al., 1998), since the mixture allowed the use of lower concentrations of individual cryoprotectants to achieve the same level of protection. Thus, the permeable cryoprotectant mixtures (i.e. EG + DMSO or EG + PROH) are often chosen as a vitrification formula for vitrifying human oocytes (Chang et al., 2008; Chian et al., 2009; Cobo et al., 2008; Kuwayama et al., 2005; Lucena et al., 2006). In this example, a bovine model helped to develop a human cryopreservation protocol with reduced cytotoxic effects when compared with previous methods (Chian et al., 2004; Hamano et al., 1992; Kuwayama et al., 1992).

Modifications of cryopreservation solution

The mouse model has been used extensively to modify cryopreservation solutions in an attempt to enhance cryopreservation outcomes. In oocyte slow freezing, high intracellular sodium concentrations that may result from freezing are undesirable for normal cell functions. Therefore, sodium toxicity has been specifically suggested to be a major factor in cryopreservation-related cell damage (Stachecki et al., 1998). Based on the evidence that the removal of sodium from the media used to cryopreserve mouse oocytes resulted in higher survival and pregnancy rates (Stachecki et al., 1998, 2002), the sodium-depleted system was applied to human oocyte cryopreservation (Boldt et al., 2003; Petracco et al., 2006; Quintans et al., 2002; Stachecki et al., 2006). In a mouse vitrification model, cryoprotectant EG causes an influx of calcium across the plasma membrane from the external medium. Removal of extracellular calcium from the medium could thus prevent the increase of Ca^{2+} after oocyte vitrification. Therefore, removal of calcium from the EG-based vitrification media used in these experiments facilitated IVF and development to the 2-cell stage (Larman et al., 2006).

Cytoskeleton stabilizers

Paclitaxel interferes with the normal function of microtubule breakdown and hyper-stabilizes microtubule

structure, rendering the microtubule complex unable to disassemble when oocytes are exposed to paclitaxel, inducing the formation of cytoplasmic asters and changing the shape of the spindle. The cytoskeleton stabilizer was first used to improve cryopreservation of porcine embryos (Dobrinsky et al., 2000), since then it was found that paclitaxel pretreatment can also improve developmental competence of vitrified mouse (Park et al., 2001), ovine (Zhang et al., 2009), porcine (Shi et al., 2006) and bovine (Morato et al., 2008) oocytes. Recently, paclitaxel was used in human oocyte vitrification (Fuchinoue et al., 2004). The results indicated that the pretreatment of oocytes with paclitaxel could potentially reduce the damaging effects of cryopreservation and improve the subsequent development of embryos derived from vitrified oocytes.

Dobrinsky et al. (2000) found that cytochalasin B has a positive effect on in-vitro and in-vivo survival of vitrified expanding and hatched pig blastocysts. Cytochalasin B is a microfilament inhibitor, which can be used to stabilize microfilaments during cryopreservation. In the porcine oocyte vitrification model, the pretreatment of cytochalasin B was beneficial for developmental competence of oocytes (Fujihira et al., 2004). However, in the bovine model, this advantage of cytochalasin B pretreatment was not observed during oocyte vitrification (Silvestre et al., 2006).

Intra- and extracellular sugars

A wide variety of organisms in nature, including arctic frogs, tardigrades, insects, bacteria and fungi, are able to survive extreme temperatures by accumulating large amounts of intracellular sugars such as sucrose, trehalose and glucose (Crowe et al., 1992; Potts, 1994). Therefore, the use of intracellular sugar has been proposed as an effective cryoprotectant for oocyte cryopreservation (Eroglu et al., 2000, 2002, 2003). Eroglu et al. (2000, 2003) described such a technique for the first time by injecting trehalose into mouse oocytes before cryopreservation. His experiment showed that trehalose can protect mouse oocytes against freezing-associated stresses when present both intracellularly and extracellularly (Eroglu et al., 2009). The same intracellular trehalose microinjection technique also provided beneficial effects on the cryosurvival of human oocytes (Eroglu et al., 2002). However, the disadvantage is that intracellular sugar could not be removed once it was injected into the oocytes.

The extracellular sugar draws free water from within the cell, since mammalian cell membranes are not permeable to sugars. Therefore, in combination with permeating cryoprotectants, extracellular sugars could further prevent ice crystal formation. The most commonly used extracellular sugar is sucrose. However, other types of sugar such as trehalose have also been used for oocyte cryopreservation (Eroglu et al., 2009). Using the mouse model, Eroglu et al. (2009) demonstrated that extracellular trehalose allowed lowering the concentration of permeating cryoprotectants which reduces the toxicity of the cryomedium during oocyte cryopreservation (Eroglu et al., 2009).

Macromolecules

Solutions used for vitrification of embryos and oocytes usually contain high concentrations of permeable cryoprotectants. At these concentrations, embryos or oocytes could only tolerate the toxicity for short periods of time. Therefore, macromolecule polymers such as polyvinylpyrrolidone, Ficoll and dextran have been used in the vitrification solutions in order to reduce the concentrations of permeable cryoprotectant, thus alleviating the negative impact. The macromolecules could not only modify the vitrification tendencies when they interact with EG-based vitrification solutions (Shaw et al., 1997), but they also provide the advantage of using lower concentrations of permeable cryoprotectant to vitrify embryos (Kuleshova et al., 2001).

Future applications of animal models in oocyte cryopreservation

Experimental animal models have always been at the research forefront for validating a new technology or a new treatment. Additionally, it can provide a model for exploring the prospective applications of human disease treatments. For example, therapeutic cloning, whereby somatic cell nuclear transfer (SCNT) is used to derive cloned embryonic stem cells, has been proposed as a very promising approach for the treatment of many human diseases, such as diabetes and Parkinson's disease (Barberi et al., 2003). The consistent and efficient generation of both heterologous and autologous stem cells using SCNT has the potential to revolutionize understanding and development of treatments for degenerative diseases. However, there are many practical constraints for therapeutic cloning, including an insufficient supply of human oocytes. Based on preliminary results of oocyte cryopreservation for SCNT in mice (Chang et al., 2009; Sung et al., 2010), oocyte cryopreservation could be considered one method to overcome this particular obstacle. It is still not clear whether cryopreserved human oocytes function like their fresh counterparts and whether cryopreserved oocytes retain their nuclear reprogramming activity in the cytoplasm. Therefore, before application of therapeutic cloning in humans, it is important to test the validity and efficiency of embryonic stem cell derivation through SCNT by using cryopreserved oocytes in an animal model. Other applications, such as assessment of genotoxicity of the cryoprotectants or epigenetic modifications after oocyte cryopreservation, will likely be developed in the future as a result of the significant biomedical data that can be obtained through animal models.

Conclusions

The use of animal models in oocyte cryopreservation provides the opportunity for further investigations of the principles of oocyte cryobiology. As in the past, it will most likely continue to help to improve and optimize current technology. Based on the foundation of invaluable research and intensive validation in the animal models thus far, the ability to cryopreserve human oocytes has already provided

tremendous advantages to the field of human IVF. It enables preservation of female fertility, delay of childbearing and avoidance of embryo cryopreservation with its associated moral and ethical concerns, and facilitates upgraded oocyte donation programmes for immediate donor—recipient matching. The successful development of animal models in oocyte cryopreservation will advance not only human oocyte cryopreservation technologies, but will be of enormous benefit to shape the future of its applications in the biomedical field.

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