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Minireview

Cryopreservation of bovine oocytes: Current status and recent developments

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Abstract — The cryopreservation of oocytes of most animal species remains a challenge due to their complex structure. Equilibrium freezing is not satisfactory because oocytes seem to be damaged by exposure for several minutes to temperatures near 0 °C. Therefore, cryopreservation of oocytes by vitrification – especially the use of very high cooling rates of oocytes suspended in extremely small volumes of various cryoprotective additive modifications – seems the most appropriate method.

oocyte / bovine / cryopreservation

The cryopreservation of bovine oocytes remains a challenge despite significant recently reported progress [1, 2]. Conventional as well as vitrification methods have been applied to immature and mature oocytes and very few calves have been born following the transfer of blastocysts derived from frozen or vitrified oocytes [2–7]. The factors affecting the cryopreservation of mammalian oocytes have been reviewed [2–9]. Since there is a growing interest in the possibility of obtaining blastocysts from cryopreserved oocytes for the application to new technologies, research on cryopreservation of oocytes has become a priority.

In this paper, I examined the reasons for this poor success rate at different levels and how it might be improved. Reviews on the cryobiology of mammalian oocytes have been published [8, 10, 11].

1. PROBLEMS CURRENTLY ASSOCIATED WITH CRYOPRESERVATION OF BOVINE OOCYTES

1.1. Source of oocytes

Experiments on cloning and transgenesis need a lot of oocytes and if they could be stored frozen this would be very interesting from a logistical point of view [12]. The main source of oocytes is from abattoir ovaries then ovum pick up. Abattoir material is very heterogenous because oocytes

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are punctured from follicles of different sizes and we know that the ability of oocytes to complete all stages of development and probably also to support freezing is related to follicle size [13] and consequently to the size of the oocyte [14]. So, not only the mixed origin but also the fact that the subsequent maturation is performed in vitro means a compromised situation compared with in vivo maturation.

1.2. Stage of maturation

The oocyte is a unique and large cell with a low surface area volume ratio, surrounded by a zona pellucida (ZP) and several layers of granulosa cells forming the cumulus oocyte complex (COC). The cells immediately adjacent to the oocyte, the corona radiata cells, have long cytoplasmic extensions which penetrate the ZP and terminate in bulbous swellings closely associated with the oocyte membrane. The presence of these processes and of gap junctions play an important role in the metabolic cooperation between oocyte and cumulus cells during the growth phase of the oocyte [15].

When oocytes are aspirated from follicles on ovaries derived from an abattoir, they are at the GV (Germinal Vesicle) stage of maturation. An important characteristic of the GV stage oocyte is that the genetic material is contained at early prophase within the contours of a nucleus. Since no spindle is present, this type of oocyte is assumed to be less prone to microtubular and chromosomal damage. However, progress in the cryopreservation of GV bovine oocytes has been limited and only two papers report the production of offspring derived from them [5, 6].

The other stage that is traditionally cryopreserved is the mature oocyte at the metaphase II stage of maturation. The cumulus surrounding the oocyte is expanded and cell cohesion is maintained by hyaluronic acid. A series of cytoskeletal elements play an active role during the maturation process of the oocyte. For example microfilaments of actin are involved in cell shape modifications and movements and microtubules form the spindle apparatus [8].

Exposing oocytes to cryoprotective additives (CPAs) and/or cooling them from about 37 ºC to 20 ºC or below causes various cytoskeletal and chromosomal alterations. Nevertheless, calves have been obtained from mature oocytes that have been frozen slowly [3, 5, 16, 17] as well as from vitrified ones [4, 7]. Therefore, whatever the cryoprotectant or the method of cryopreservation, the developmental potential of oocytes frozen at the GV or the MII stage appears to be quite similar [17].

Although mature and immature oocytes have been cryopreserved, cooling GV oocytes to 4 ºC or 0 ºC reduces the formation of normal meiotic spindles [18] and of fertilization. Cryoprotectants and exposure of mature oocytes to low temperatures can cause chromosome dispersal, microtubule depolymerisation [19, 20]. There are also changes in the composition and permeability of the plasma membrane during maturation. The permeability of bovine oocytes to water and to cryoprotectants changes after in vitro maturation [21]. Furthermore, the permeability of bovine oocytes to DMSO is higher than that to ethylene glycol. The maturation stage has a significant influence on the developmental competence of oocytes after cryopreservation. This was observed [22–25] when oocytes were cryopreserved at different times of the maturation process; various meiotic stages exhibit different sensitivities to cooling. Maturation protocols also affect an oocyte's ability to tolerate cryopreservation [25, 26]. It should be noted that DNA may be damaged by cryopreservation whatever the method used [27].

1.3. Cumulus cell investment

Cumulus and granulosa cells are necessary to provide the oocyte with nutritive

substances and may have a supportive role at fertilization in vitro. The multiple roles of the cumulus oophorus have been reviewed [15, 28]. These cells may be an obstacle to the penetration of cryoprotectants [29] or the gap junctions can be altered by cryopreservation.

1.4. Osmotic stress

It has been shown that exposure to anisotonic conditions has a significant effect on the developmental competence of bovine MII COC at both the GV and MII stages. Oocytes at the GV stage are more sensitive to anisotonic stress than MII oocytes [30]. High concentrations of sodium are detrimental to mouse embryos during freezing [31].

1.5. Chilling sensitivity

In bovine oocytes, low temperature sensitivity is attributed to two different factors. Besides damage of the numerous lipid droplets, the meiotic spindle of the metaphase II oocyte suffers serious injury when cooled to 4 or even 25 °C [1, 19]. Unlike the lipid alteration, damage to the spindle after short-time low-temperature exposure is reversible, but even so may disturb the subsequent fertilization process.

2. HOW TO IMPROVE OOCYTE CRYOPRESERVATION?

The following possibilities should be considered to achieve higher blastocyst rates and healthy offspring after fertilization of cryopreserved bovine oocytes:

(1) Cryopreserve only competent oocytes, preferably those that have been ovulated. To my knowledge, nobody has tried to freeze oocytes matured in vivo in the cow. Oocytes that are competent to develop to term are probably also more resistant to freezing. It has been demonstrated that the blastocyst yield is significantly higher when oocytes are matured in vivo compared with in vitro [32]. Another study [33] comparing in vitro with in vivo maturation made it clear that the developmental potential of in vitro matured oocytes is generally lower than that of in vivo matured oocytes.

(2) Cryopreserve oocytes from large follicles that have reached the diameter at which they are considered competent. There is evidence that oocytes collected in the presence of a dominant follicle are of lesser quality than those collected during follicular growth [34, 35].

(3) Culture oocytes during arrested meiosis to improve their developmental competence after fertilization [36, 37]. Multiple attempts have been made with various inhibitors, the aim being to allow the oocyte to accumulate sufficient material to support development. Recently, two compounds, butyrolactone and roscovitine were tested separately or together [38, 39]. Calves have been born after such treatments (our unpublished observations). Apparently, cryopreservation of such premature oocytes has not been attempted.

(4) Partially remove the cumulus cell investments several hours after the start of the in vitro maturation. This step may facilitate cryoprotectant penetration while maintaining the supportive role of these cell layers in the subsequent final maturation, fertilization and also further development [40].

(5) The permeating component(s) of the cryoprotectant mixture should penetrate bovine oocytes rapidly so as to minimise osmotic shock and also to ensure appropriate protection for the whole cell. Ethylene glycol, propylene glycol and dimethylsulphoxide seem to be the best candidates.

(6) Cryopreservation of oocytes after centrifugation to eliminate cytoplasmic lipid droplets partially responsible for chilling sensitivity. Attempts undertaken were not conclusive [41]. The polarization of cytoplasmic lipid droplets had a negative effect on the survival of frozen thawed

oocytes. Thus this treatment did not improve the frequency of normal fertilization and development to blastocysts, compared with that of frozen control oocytes. However the frequency of polyspermy of those surviving freezing-thawing was less than those of surviving control oocytes.

(7) Another and probably more efficient approach to avoid chilling injury is to increase the cooling rate through the dangerous temperature zone (between +15 and –15). There are several methods; one of them is the solid-surface vitrification where oocytes are vitrified in microdrops on a precooled metal surface [12]. Cryopreservation of oocytes in 0.25 mL straws immersed directly into liquid nitrogen versus those placed on top of very small microscope grids to cool them very rapidly showed that 15% of the latter developed into blastocysts after fertilization and culture [42]. This was interpreted as being due to small size and the very high cooling rate achieved with the grids. The open-pulled straw (OPS) method consisting in pulling a conventional 0.25 mL straw to half of its diameter and wall thickness so that a very small volume of vitrification medium is aspirated by capillarity with oocytes [40]. Another method is to create a thin film of cryoprotectant solution in a small nylon loop [43]. Cryoloop vitrification was compared to the OPS method and with in vitro matured oocytes, 33% of those fertilized continued development to the morula/blastocyst stage ($n = 42$). These new high speed vitrification methods dramatically decrease the chilling injury, permit the use of less concentrated and less toxic solutions and shorten the time of exposure with the final cryoprotectant both before cooling and after warming. High rates of survival have been obtained using a new cryoloop vitrification technique followed by ICSI [44]. Additionally, the small volume of the solution prevents heterogeneous ice formation [45]. Finally, the use of liposomes may alter chilling sensitivity of bovine oocytes [46].

Consequently, a considerable advance in the cryopreservation of certain mammalian ova and embryos has been achieved. These results have generated a search for new methods for further increasing the cooling rates [43, 44].

3. CONCLUSION

The cryopreservation of mammalian oocytes remains a challenge in most animal species due to its complex structure. Equilibrium freezing is not satisfactory and it seems that in this special case vitrification is the most appropriate method.

The ways indicated in this brief review to cryopreserve this special cell, the oocyte, are the existing ways but perhaps there are other possibilities by exploring for example the reasons why it can be arrested for several decades before resuming meiosis. Cryopreservation of primordial follicles is also a new although a complicated way [47].

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