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Morphologic, viability and ultrastructural analysis of vitrified sheep preantral follicles enclosed in ovarian tissue

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ABSTRACT

The main objective was to compare the efficiency of vitrification techniques and solutions on the preservation of morphology, ultrastructure and viability of sheep preantral follicles enclosed in ovarian tissue fragments. The fragments were cryopreserved by using macrotube vitrification (MTV), solid-surface vitrification (SSV) or conventional vitrification (CV). These techniques were combined with one of the six solutions containing 6 M ethylene glycol (EG) and with or without sucrose (SUC) (0.25 or 0.50 M) and with or without fetal calf serum (FCS) (10%). After one week, samples were warmed and histological analysis was performed, showing that the percentage of normal follicles after CV (66.20 ± 8.87%) using a solution containing 6 M EG, 0.25 M SUC and 10% FCS (vitrification solution 4 – VS4) was similar to fresh control (79.40 ± 7.83%), MTV (53.40 ± 10.60%) and SSV (56.75 ± 15.33%), all of them with the same vitrification solution ($P < 0.05$). For follicular viability evaluation, ovarian fragments were vitrified as described above. After warming, follicles were assessed by trypan blue dye. Controversially, the highest percentage of viable follicles was observed in MTV (97.06%) and was similar to fresh control (92.62%) ($P < 0.05$), but was significantly different from SSV (81.08%) and CV (83.81%) ($P < 0.05$). These results were validated by transmission electron microscopy that showed normal follicles observed in MTV and in fresh control. In addition, to verify the MTV with VS4 (a combination of the best technique plus the best solution), follicle viability was evaluated after 48 h *in vitro* culture. The viability assay was performed by fluorescence microscopy (calcein-AM and ethidium homodimer-1) analysis as follows: follicles isolated from fresh tissue were forthwith analyzed or underwent 48 h *in vitro* culture before analysis, whereas others fragments were vitrified/warmed and immediately analyzed or underwent 48 h *in vitro* culture before analysis. These results showed that, although follicular viability after MTV/VS4 (65%) was reduced when compared to the non-vitrified follicles at day 0 (100%), follicular viability after MTV/VS4 at day 2 (36.5%) was similar to follicles vitrified at day 0 (65%) and similar to non-vitrified follicles at day 2 (62.5%) ($P > 0.05$). As the decrease of viability in non-vitrified follicles at day 2 was similar to the decrease of MTV/VS4 in the same time, follicle viability at day 2 is not affected by MTV/VS4. In conclusion, using the experimental conditions of the present study, an efficient solution (VS4: 6 M EG, 0.25 M SUC and 10% FCS) and technique (MTV) were successfully used to vitrify ovine ovarian tissue.

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1. Introduction

Advances in cryopreservation techniques and protocols for germinal tissue over the past decades have contributed greatly to the establishment of germplasm banks. These genetic banks are crucial for the preservation of genetic material with potentially high economic value or for use with endangered species populations (Liu et al., 2008; Santos et al., 2010). In addition, the association between cryotechnology and assisted reproduction techniques (ART) has important clinical relevance, as it permits the development of alternative strategies for restoring fertility in women at risk of premature ovarian failure, especially those undergoing cancer therapies. Admittedly, high dose chemotherapy and radiotherapy destroy a significant portion of ovarian follicular population, often times leading to permanent infertility in women (Meirow and Nugent, 2001; Chemaitilly et al., 2006).

The main alternatives for fertility preservation in routine clinical use are limited to the protection of the ovaries (oophoropexy) against radiation, or emergency *in vitro* fertilization (IVF) (Sonmezer and Oktay, 2004). Although oophoropexy may offer some protection to germ cells, this technique can greatly reduce the success of future pregnancies (Wallace et al., 2005). There are also serious limitations in the emergency use of IVF in patients with cancer, as hormonal stimulation is required to obtain mature oocytes. The possibility of utilizing these hormones in patients with hormone-sensitive cancers, as well as in prepubertal patients (Sonmezer and Oktay, 2004), is immensely restricted. Currently, cryopreservation of ovarian tissue is a possible fertility preservation alternative for patients in need of treatment for malignant diseases and is recommended by the American Society of Clinical Oncology (ASCO) (Lee et al., 2006).

In veterinarian medicine, embryo cryobiology has been emphasized when regarding conservation of endangered species or pets. However, this practice is not feasible in cases of accidental or sudden loss of valuable females and, therefore, cryopreservation of ovarian tissue is indicated as a better alternative in these situations (Takahashi et al., 2001). With regard to the ovarian tissue cryopreservation of livestock animals, such as sheep, several studies have reported the feasibility of applying both slow freezing (Gosden et al., 1994; Salle et al., 2002, 2003; Imhof et al., 2006) and vitrification (Bordes et al., 2005; Lornage et al., 2006) methods through the birth of healthy offspring after transplantation of ovarian tissue.

Vitrification is a fairly recent alternative method of cryopreservation and, when compared to slow freezing, is quicker and cheaper. However, the vitrification method is characterized by using high concentrations of cryoprotectants (Vajta et al., 1998), which can increase the toxic effect caused by these substances on preantral follicles. Moreover, it is known that factors such as high concentrations of cryoprotectant agents, osmotic stress and the techniques used for vitrification loading may contribute to the reduction of normal preantral follicles after warming (Huang et al., 2008).

In the last decade, studies have been completed using ethylene glycol (EG) with vitrified ovarian tissue or isolated preantral follicles in rat (Sugimoto et al., 2000), mouse (Kagabu and Umezu, 2000; Kim et al., 2010), goat (Santos et al., 2007; Carvalho et al., 2011), cow (Gandolfi et al., 2006; Kagawa et al., 2009), pig (Moniruzzaman et al., 2009) and human (Isachenko et al., 2003; Silber et al., 2010). However, very few investigators have tested EG in vitrification solution with ovine preantral follicles enclosed in fragments of ovarian tissue (Amorim et al., 2003; Melo et al., 2011). Developments in sheep ovarian vitrification may have relevance as ewe ovaries are similar to the human ovary in its anatomy and physiology (Gosden et al., 1994; Oktay et al., 2000; Salle et al., 2002). While positive results have recently been obtained with the vitrification of mouse ovaries (Wang et al., 2011), these methods cannot be easily transferred to human tissue. This is, in part, due to the vast morphological and physiological differences between mouse and human ovaries. Despite having larger ovaries, neither bovine nor porcine can be considered a relevant model for human tissue vitrification (Gandolfi et al., 2006). In addition, researchers have published promising results regarding ovarian tissue cryopreservation in the presence of an extra-cellular cryoprotectant, like sucrose (SUC) (Santos et al., 2006a) or fetal calf serum (FCS) (Chen et al., 2006). Information detailing whether the addition of sucrose at concentrations of 0.25 or 0.5 M with or without 10% FCS may be essential for ovarian tissue vitrification, despite being important, is limited in sheep.

The current study aimed (1) to compare different vitrification techniques in ovine ovarian tissue and (2) to test the effects of varying concentrations of SUC, FCS or both combined with 6 M EG as a vitrification solution (VS). Morphology, by classical histology and transmission electron microscopy, and viability, by trypan blue stain and fluorescent markers, were assessed in fresh ovarian fragments, vitrified/thawed fragments, and vitrified/thawed samples after *in vitro* culture (IVC).

2. Materials and methods

2.1. Source and preparation of ovarian tissue

Ovaries ($n = 30$) were collected at a local abattoir from 15 adult non-pregnant mixed-breed ewes. Immediately after postmortem, under aseptic conditions, the ovaries were washed in 70% alcohol for 10 s, followed by two washes in HEPES buffered minimum essential medium (MEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 100 g/mL penicillin and 100 g/mL streptomycin. The ovaries were transported into tubes containing 20 mL of MEM within thermos flasks maintained at 20 °C to the laboratory within 1 h after they were recovered.

2.2. Experiment I: morphology, viability and ultrastructure of preantral follicles in vitrified ovarian cortex

2.2.1. Ovarian tissue vitrification: solution composition and technique

At the laboratory, ovarian pairs ($n = 5$) were stripped of adhering tissue and fat, and cut with a scalpel into approximately 3 mm × 3 mm × 1 mm (9 mm^3) or 1 mm × 1 mm × 1 mm (1 mm^3) fragments, according to the vitrification technique used, macrotube vitrification (MTV), solid-surface vitrification (SSV) or conventional vitrification (CV) and were randomly assigned to each treatment. One fragment (9 mm^3) from each pair of ovaries was immediately fixed in Carnoy's solution for 12 h for histological analysis (fresh control). Twelve 9 mm^3 fragments (for MTV or for SSV) and six 1 mm^3 fragments (for CV) were exposed to one of the six vitrification solutions (VS): (VS1–6, description to follow) for 5 min at 20 °C. After this duration, the fragments underwent MTV, SSV or CV. The base medium (BM), composed of 6 M ethylene glycol (EG) in MEM, was supplemented

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