VITRIFICATION OF METAPHASE II PLATE IN SHEEP OOCYTES: PRELIMINARY STUDY

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Summary
The objective of this work was to develop a technique for cryopreservation of the metaphase II plate (MPII) of sheep oocytes. For this, MPII were isolated from in vitro matured and vitrified oocytes. After warming, the recovered MPII were chemically activated and cultured for 24 h in SOF, then they were stained with Hoechst and the extruded polar bodies counted under a fluorescent microscope. The results indicated that it is possible to vitrify MPII of sheep oocytes.

Introduction
Advances in reproduction technologies, such as in vitro maturation (IVM), in vitro fertilization (IVF), in vitro culture (IVC) and nuclear transfer, have stimulated research for efficient cryopreservation techniques for mammalian oocytes. Different cryoprotectants and types of freezers have been tried (Martino et al., 1996; Arav et al., 2000; Vajta et al.,1998) however, for species like cattle, usually less than 15% of all cryopreserved, in vitro fertilized oocytes develop to the blastocyst stage with only a few offspring resulting after transfer (Vajta et al.,1998). Martino et al. (1996) placed oocytes on electron microscope grids and obtained a 15% blastocyst rate following in vitro fertilization and culture. Vajta et al. (1998) vitrified bovine oocytes in 0.25 ml pulled plastic straws (OPS) and obtained a 25% rate of blastocyst formation. Further improvement in increasing the cooling rate during vitrification was achieved using a small nylon cryoloop (Lane et al.,1999), later the blastocyst yield rate was improved to 33% by dropping oocytes directly onto a metal surface chilled with liquid nitrogen (Dinnyes et al., 2000). However, none of these methods could overcome the difficulties of survival of oocytes after cryopreservation in a reliable fashion. It is well known that the oocyte is the largest cell of an animal's body, and as such is full of water and, in many species, of fat making it difficult to cryopreserve. The aim of this paper was to reduce the volume of the oocyte by vitrifying only the MPII plates obtained by enucleation of the in vitro matured oocytes.

Materials and Methods
Oocytes were obtained by aspiration of follicles from ovaries of slaughtered animals. Oocytes covered by at least 2 layers of granulosa cells and with an evenly granulated cytoplasm were selected for IVM. The maturation medium was TCM-199, containing 1 mM of glutamine, 10% FBS, 5 μg/ml follicle stimulating hormone (FSH), 5 μg/ml luteinizing hormone (LH), 1 μg/ml estradiol, 0.3 mM sodium pyruvate and 100 mM cysteamine. The oocytes were incubated in 400 μl of medium in 4-well dishes covered with mineral oil. IVM conditions were 5% $C0_2$ in humidified air at 39°C for 24 h. Following maturation, the oocytes were denuded of granulosa in a medium with 300 UI/ml of hyaluronidase. Then they were placed

for 10 min in a media with Hoechst 33342 (3µg/ml) and cytochalasin B (5µg/ml) to facilitate the enucleation of the MPII with a minimum volume of ooplasm. The MPII plates were divided in three groups: the vitrification group (n 71) was exposed to vitrification under the follow conditions: 40% ethylene glycol (ET) + 40% DMSO + 1M trealose for 20 sec, and plunged into liquid nitrogen (LN_2). It was warmed (37°C) in TCM-199 at 20% FBS. After 15 min of incubation the oocytes were activated for extrusion of the second polar body for 5 min in ionomicyn (5µM), washed for 5 min followed by 4 h of culture in 6-DMAP (0.12mM) + cycloheximide (0.6µl/ml). After activation the MPII were washed and cultured for 20 h. The control group (n 63 fresh oocytes) received the same treatment, but they were not vitrified. The toxicity test group (n 44 fresh oocytes) was loaded for 20 sec in the vitrification solution, washed and shortly thereafter activated like the others groups**.** Differences between the experimental groups were tested using Chi-square test ($p<0.05$ was considered significant). The MPII plates were considered viable when integrity of cytoplasmic membrane and extrusion of the second polar body was seen under the light microscope (fig 1-2).

Results

As shown in table 1 the membrane integrity was related to the treatment and more than 56% of the vitrified MPII were not disrupted after warming ($p<0.001$). The expulsion of the second polar body after activation was observed in more than 61% and 57% of the MPII that were not vitrified (control and toxicity test groups), while 40% of vitrified plates had expulsion of polar bodies.

Discussion

These preliminary studies showed that it is possible to vitrify MPII plates. In our opinion it is possible that different vitrification protocols (times of exposure, cryoprotectants, devices) for the MPII can improve the results. On the other hand, the drastic reduction of the volume of the oocytes might make cryopreservation possible with greater efficiency. More work is necessary to confirm these data. In our future studies the insertion of the MPII soon after warming into the cytoplasm of a mature oocyte and its subsequent development after culture will be tested.

Table 1. Integrity of cytoplasmic membrane and expulsion of II polar body after vitrification of MPII in vitro matured sheep oocytes.

MPII Treatment	MPII n	Membrane Integrity (%)	Expulsion II polar body (%)
Vitrified	71	40/71 (56.3)^{a}	16/40 (40)^{x}
Fresh	63	63/63 (100)^{b}	39/63 (61.9)^{y}
Toxicity Test	44	40/44 (90.9)^{c}	23/40 (57.5)^{xy}

Values with no common superscripts are significantly different; ^{a,b} $P<0.001$; ^{b,c} $P<0.05$; ^{x,y} $P<0.05$.

MPII membrane integrity after warming

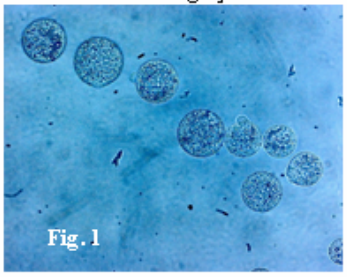

Fig. 1

Expulsion second polar body

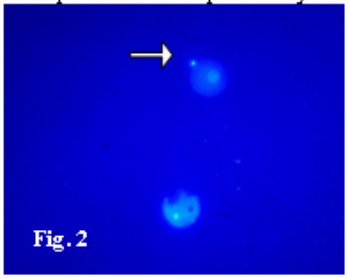

Fig. 2

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