Developmental Capacity of Bovine Follicular Oocytes after Ultra-Rapid Freezing by Electron Microscope Grid
Ⅰ. Cryopreservation of Bovine Immature Oocytes

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Electron Microscopic Grid를 이용한 초급속동결이 소 난포란의 발달능에 미치는 영향
I. 소 미성숙난자의 동결에 관한 연구

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본 연구는 소 미성숙난자를 electron microscope grid와 동해제인 EFS30을 이용하여 초급속 동결하였을 때 정상적인 배 발달의 유도가능성 여부를 조사하고자 실시하였다. 동해제는 30% ethylene glycol, 18% ficoll, 0.5 M sucrose와 10% FBS등이 PBS에 첨가되어 제작된 EFS30을 사용하였다. 난자생존의 평가기준으로는 성숙, 수정 및 배발달을 조사하였다. 본 연구에서 얻어진 결과는 다음과 같다. 초급속동결-융해 후, 소 미성숙란의 생존율은 43.2%을 나타내었다. 동결-융해군의 체외성숙 (84.1%) 과 정상 자웅전핵 형성율 (57.5%) 은 대조군의 결과 (92.5, 65.0%) 와 비교하여볼 때 유의한 차이는 없었다. 또한, 동결군의 체외수정 이후의 ≥2-세포기 형성 (65.0%) 과 배반포형성율 (30.8%) 도 대조군 (73.7, 35.7%) 의 결과와 유의한 차이를 나타내지 않았다. 따라서 소 미성숙난자는 EM grid와 EFS30 동결액을 이용한 초급속 동결방법에 의해 정상적인 배발달이 유도될 수 있다는 것을 알 수 있었다.

INTRODUCTION

Cryopreservation of bovine oocytes at germinal vesicle (GV) stage or metaphase II stage was impeded by poor survival rates according to more increased chilling sensitivity than later stage such as morula or blastocyst (Fahning and Garcia, 1992). Also, it has known that developmental capacity of frozen immature bovine oocytes was lower than that of frozen mature oocytes (Fuku *et al*., 1992). In major reason of that, tolerance of immature oocytes to the cryoprotectant is very poor than that of mature oocytes (Fuku *et al*., 1995). So far, the study on cryopreservation of bovine oocytes was more concentrated at slow freezing using a programmable freezer than rapid-freezing or vitrification. Recently, a new ultra-rapid freezing method using eletron microscope (EM) grids was introduced into bovine oocyte cryopreservation, which adopted from vitrification of exceedingly chilling sensitive *Drosophila*. By this technique, Martino *et al*., (1996) reported that higher developmental capacity of frozen bovine mature oocytes was obtained. Therefore, this study was carried out to examine whether the developmental capacity of bovine immature oocytes frozen ultra-rapidly using EM grids and EFS30 can be obtained.

MATERIALS AND METHODS

1. Oocyte collection

GV, immature bovine oocytes were collected from visible follicles (2 6 mm) of ovaries which were obtained at local slaughterhouse. Oocytes with a complete dense cumulus oophorus and dark evenly granulated cytoplasm were only used. Recovered immature oocytes were washed with TALP-HEPES (Parrish *et al*., 1988) and then they were suspended in Dulbecco's phosphate-buffered saline (D-PBS) containing 10% fetal bovine serum (FBS) at room temperature (25℃).

2. Freezing and thawing

As freezing solution, EFS30 which containing of 30% ethylene glycol, 18% ficoll, 0.5 M sucrose and 10% FBS added D-PBS was used. In addition, for the ultra-rapid freezing, 400 mesh copper EM grids (1GC 400; Pelco international, USA) were used as a physical support to achieve very high cooling rates when plunged into liquid nitrogen (LN_2). A mean number of oocytes loaded on one grid were 10 15. The total time that elapsed from the immersion of oocytes to cryoprotectants to the plunge of oocytes-grid into LN_2 was about 30 sec. After thawing, cryoprotectants were removed by 3-step procedures at 37℃. At thawing, grids with oocytes storaged in LN_2 were transferred as soon as possible rapidly into 0.5 M sucrose (S). And then they were transferred to 0.25 MS and 0.125 MS. Each step needs for 1 min.

3. *In vitro* maturation (IVM)/*in vitro* fertilization (IVF)/*in vitro* culture

The culture procedures employed in the production of preimplantation embryos from frozen-thawed bovine immature oocytes were as outlined by Park *et al.* (1995). For IVM, oocytes recovered from warming were cultured in maturation medium composed of TCM-199 (Gibco) + 10% (v/v) fetal bovine serum (FBS) supplemented with sodium pyruvate (0.2 mM), follicle-stimulating hormone (1 μg/ml), estradiol-17β (1 μg/ml), and gentamycin (25 μg /ml) at 39℃, 5% CO_2 incubator. After 22 - 24 h incubation, oocytes were inseminated with highly motile frozen-thawed sperm recovered on a discontinuous percoll column and heparin (2 μg/ml) and PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) were also added in fertilization drop. From day 2 after IVF, cleaved embryos were co-cultured in cumulus monolayer cell drop added CR1 medium supplemented with 10% FBS. Cumulus cell drop was prepared with the recovered cumulus cells from matured bovine oocytes. Final assessment of developmental capacity in this study was determined with blastocyst formation at day 8 after IVF.

4. Evaluation of oocyte survival

To analyse the abnormality of maturation and fertilization after ultra-rapid

freezing of bovine immature oocytes, oocytes and eggs were examined by hoechst staining at 24 h after IVM (Fig. 1E) and 18 h after IVF, respectively. In addition, to assess the developmental capacity of frozen-thawed immature oocytes, the rates of survival, ferilization and blastocyst formation were examined (Fig. 1). Survival was assessed with oocytes showed no difference of cytoplasmic appearance and membrane integrity at day 1 after IVF. Fertilization and blastocyst formation were determined at day 2 and day 8 after IVF, respectively.

5. Hoechst staining

For the comparison of rates of maturation and fertilization between control and frozen-thawed oocytes, the matured oocytes and fertilized eggs recovered at appropriate treatment time were fixed with 2% formalin solution for 2 3 min and stained with bisbenzimide solution (No. 33342, 2.5 μg/ml, Sigma). Observation was carried out under ultra violet filter incorporated fluorescent microscope on 1 day after making sample.

6. Statistical analysis

Difference in the rates of maturation, fertilization and developmental capacity among treatment group was compared using the Chi-square test.

RESULTS

To determine the effect of ultra-rapid freezing to the developmental capacity of bovine immature oocytes, the rates of maturation, fertilization and embryonic development were examined after thawing. After ultra-rapid freezing and thawing, 43.2% of immature oocytes were survived. When maturation was identified at 24 h after thawing, the maturation rate in control and freezing group was 92.5% and 84.1%, respectively (Table 1). Also, fertilization rate at 18 h after insemination was evaluated as total penetration (90.9, 87.0%), normal 2 pronuclei formation (65.0, 57.5%) and mean number of sperm/oocyte (1.44, 1.40), respectively (Table 2). The rate of two-cell

formation in freezing group was 65.0%, and there was not significantly different when compared to control (73.7%). In addition, development to the blastocyst on day 8 after IVF of freezing group was 30.8% from the cleaved oocytes. As shown in Table 3, development to the blastocysts of frozen-thawed oocytes was not significantly different to that of the control (35.7%).

Table 1. Maturation of ultra-rapidly frozen bovine immature oocytes (r=2)

Treatment	No. of oocytes	No. of matured (%)*
Control	40	37 (92.5)
Freezing	44	37 (84.1)

* Matured oocytes were defined as Metaphase II stage by hoechst staining at 24 h after IVM.

Table 2. Fertilization of ultra-rapidly frozen bovine immature oocytes (r=2)

Treatment	No. of oocytes insemi.	No. of oocytes penetrated (%)*			Mean no. of sperm/oocyte
		Total	Polyspermy	2PN	
Control	44	40 (90.9)	9 (22.5)	26 (65.0)	1.44
Freezing	46	40 (87.0)	10 (25.0)	23 (57.5)	1.40

* Oocytes were examined by hoechst staining at 18 h post insemination.

Table 3. Developmental capacity of ultra-rapidly frozen bovine immature oocytes (r=3)

Treatment	No. of oocytes	No. of oocytes survived (%)	No. of ≥2-cell (%)	No. of blastocyst on day 8 (%)
Control	114	-	84 (73.7)	30 (35.7)
Freezing	139	60 (43.2)	39 (65.0)	12 (30.8)

DISCUSSION

These data demonstrate that immature bovine oocytes can be successfully cryopreserved with ultra-rapid freezing method using electron microscope grid. Until now, cryopreservation of bovine oocytes was mainly carried out at metaphase II stage. Many researchers reported that developmental capacity of frozen immature oocytes was very poor than that of frozen oocytes at mature stage. Through the two papers (1992, 1995), Fuku *et al.* concentrated that immature bovine oocytes are more sensitive to cryoprocessing and poor tolerant to cryoprotectants than mature stage. Also, bovine oocytes are chilling sensitive. A limiting factor for achieving cryopreservation of oocytes is direct chilling injury (DCI) which occurs during cooling. The primary target of DCI is the plasma membrane. Arav *et al*, (1996) reported that the phase transition of the membrane lipids of immature oocytes occured broadly between 13 and 20 ℃ and thus holding immature oocytes at the phase transition temperature is more damaging to their membranes. Also, Rebecca and Parks (1994) demonstrated that microtubules of the meiotic spindle of bovine oocytes are sensitive to cooling, start to depolymerize even at room temperature and not resilient as mouse with respect to spindle recovery following cooling. Thus, it is important to maintain temperatures as close to 39℃ as possible for the survival of frozen-thawed bovine oocytes. In the viewpoint, all procedures in this study were performed at 37℃. Considering with those characteristics of

bovine oocytes, Martino *et al.* (1996) introduced a new ultra-rapid freezing method which adopted from cryopreservation of exceedingly chilling sensitive *Drosophila*. In the study, they reported that higher developmental capacity was obtained in frozen mature oocytes. However, by this technique, we obtained the developmental capacity of frozen immature oocytes. Although the survival rates of frozen-thawed immature oocytes were low (43.2%), fertilization (65.0%) and embryonic development (30.8%) of them were no significant differences when compared to those of control (73.7% and 35.7%, respectively). On the other hand, we used EFS30 which is modified from EFS40 used for vitrification of mouse and bovine blastocyst as freezing solution (Zhu *et al.*, 1993; Tachikawa *et al.*, 1993). EFS30 containing non-permeable ficoll and sucrose indicated the better survival rates than EG5.5 M which used by Martino *et al.* (1996)(data not shown). Therefore, these results demonstrate that developmental capacity of frozen-thawed bovine immature oocytes can be successfully obtained by ultra-rapid freezing method using EM grid and EFS30.

SUMMARY

This study was carried out to examine whether the developmental capacity of bovine immature oocytes frozen ultra-rapidly using electron microscopic (EM) grids and EFS30 can be obtained. As freezing solution, we used EFS30 which consisted of 30% ethylene glycol, 0.5 M sucrose, 18% ficoll and 10% FBS added in D-PBS. As criterior of oocyte viability, the rates of maturation, fertilization and embryonic development were determined. The results obtained in this experiment were summarized as follows: When ultra-rapidly frozen immature oocytes were thawed, 43.2% of them were survived. The rates of maturation (84.1%) and normal 2 pronuclei formation (57.5%) of frozen immature oocytes were not significantly different when compared to those of control (92.5, 65.0%). In addition, the rates of $\geq$2-cell (65.0%) and blastocyst formation (30.8%) of freezing group were not significantly different when

compared to those of control (73.7, 35.7%). These results demonstrate that developmental capacity of frozen-thawed bovine immature oocytes can be successfully obtained when survived from the ultra-rapid freezing method using EM grid and EFS30.

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Fig. 1. Treatment procedures and developmental morphology of ultra-rapid freezing and thawed immature bovine oocytes. A) Immature bovine oocytes before being exposed to cryoprotectants. B) Immature oocytes loaded on electron microscope grid immediately before being plunged in LN_2. C) Oocytes after warming. Arrows indicate damaged ooplasm after freezing-thawing. D) In vitro matured oocytes after freezing and thawing. E) Hoechst stained oocytes at 24 h after IVM. F) Hatched blastocysts at day 9 after IVF.