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Developmental Capacity of Bovine Follicular Oocytes after Ultra-Rapid Freezing by Electron Microscope Grid
Ⅱ. Cryopreservation of *In Vitro* Matured Bovine Oocytes

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Electron Microscopic Grid를 이용한 초급속 동결이 소 난포란의 발달능에 미치는 영향
II. 체외 성숙된 소 미수정란의 동결에 관한 연구

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요 약

본 연구는 체외에서 성숙된 소 미수정란을 electron microscope grid와 동해제인 EFS30을 이용하여 초급속 동결하였을 때 정상적인 배 발달의 유도 가능성 여부를 조사하고 동해제 및 동결방법의 유해성 여부를 indirect immunocytochemistry방법으로 확인하고자 실시하였다. 동해제는 30% ethylene glycol, 0.5 M sucrose, 18% ficoll과 10% FBS가 들어 있는 PBS로 된 EFS30을 사용하였다. 본 연구에서 얻어진 결과는 다음과 같다. 동해제와 동결과정이 난자의 microtubule, microfilament 및 chromatin의 형태에 미치는 영향을 indirect immunocytochemistry방법으로 조사하였던 바, 동해제 노출뿐 아니라 동결에 의해서도 대조군과 차이를 나타내지 않았다. 초급속 동결이 소 미수정란의 체외수정에 미치는 영향을 검토했을 때, 총 정자침투율(96.7%, 90.0%), 정상 자웅전핵 형성율(74.6%, 68.9%)과 난자당 정자수(1.50, 1.44)가 동결군과 대조군에 있어서 차이는 볼 수 없었다. 또한, 초급속 동결-융해 후의 체외발달능을 조사했던 경우, 85.5%의 높은 난자 생존율과 74.5%의 난할율, 그리고 31.4%의 배반포 형성율을 얻었다. 이러한 결과는 난자의 생존율을 제외한 수정율과 배반포 형성율에 있어서 대조군(76.0%, 34.6%)과 노출군(77.9%, 33.0%)의 결과와 매우 유사한 것이었다. 이와 더불어, 각 처리군에서 얻어진 배반포기배를 Hoechst 염색방법으로 총세포수를 조사하였을 때도 그 차이는 확인할 수 없었다. 따라서 체외에서 성숙된 소 미수정란은 EM grid와 EFS30 동결액을 이용한 초급속 동결방법으로 동결하였을 때 정상적인 배발달을 유도할 수 있다는 것을 알 수 있었다.

(Key words : Mature bovine oocyte, Ultra-rapid freezing, EFS30 Electron microscope grid, Developmental capacity)

Ⅰ. INTRODUCTION

It will be possible to preserve genetic resources of oocytes for establishing of gamete banks, if the cryopreservation of bovine oocytes can be

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obtained successfully(Hamano et al., 1992). Until now, only a few limited number of full term development was reported using frozen-thawed mature oocytes in cryopreservation of bovine oocytes. The viability and developmental capacity of cryopreserved bovine oocytes are very poor because they are chilling sensitive and not resilient as mouse oocytes with respect to spindle recovery following cooling(Martino et al., 1996). Thus, it is important to maintain temperatures as close to 39℃ as possible for the survival of frozen-thawed bovine oocytes(Rebecca and Parks, 1994). Considering with this chracteristics, a new freezing-thawing method of bovine oocytes was introduced by Martino et al.(1996). This study was carried out to investigate 1) the effect of the cryoprotectants and freezing method on the microtubule, microfilament and chromatin morphology of bovine oocytes using indirect immunocytochemistry 2) whether the developmental capacity of bovine mature oocytes frozen ultra-rapidly using electron microscopic (EM) grids and EFS30 can be obtained.

Ⅱ. MATERIALS AND METHODS

1. Oocytes collection and *in vitro* maturation(IVM)

The culture procedures employed in the production of preimplantation embryos from bovine follicular oocytes were as outlined by Park et al. (1995). Cumulus oocyte complexes(COCs) were collected from visible follicles(2~6mm) of ovaries, washed with TALP-HEPES(Parrish et al., 1988) and cultured into 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.

In this study, COCs were divided into three groups according to experimental purpose; control, exposure to cryoprotectant and freezing.

2. Freezing and thawing

After incubation for 22~24 h in IVM medium, the oocytes were treated with 0.05% hyaluronidase solution to remove the cumulus cell, washed sufficiently with TALP-HEPES and then they were suspended in Dulbecco's phosphate-buffered saline(D-PBS) at 39℃ before the each treatment. Briefly, major factors of freezing method used in this study were electron microscope grids(EM grids, Pelco International) and EFS30. EM grids were used as a physical support to achieve very high cooling rates when it was plunged into liquid nitrogen(LN_2). The cryoprotectant is consisting of 30% ethylene glycol, 0.5 M sucrose, 18% ficoll and 10% FBS added D-PBS. 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. Especially, in exposure group, all treatment including before-freezing and thawing procedures was done as the same method as freezing group.

3. *In vitro* fertilization(IVF) and *in vitro* culture(IVC)

After recovered from the exposure and thawing, the oocytes were incubated in fertilization drop for 30min, and then they were subjected to IVF. For IVF, highly motile sperm recovered from frozen-thawed semen separated 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 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 before each treatment. Final assessment of developmental capacity in this study was determined with blastocyst formation at day 8 after IVF. Also, total cell numbers of blastocysts in each group were examined by Hoechst staining to compare the embryo quality among treatment group.

4. Evaluation of oocyte survival

To evaluate the effects of cryopretectant and freezing procedure on the microtubule, microfilament and chromatin morphology of oocytes, recovered oocytes were stained with indirect immunocytochemistry at 1 h after each treatment(Fig. 1). Also, to analyse the fertilization abnormality after ultra-rapid freezing of bovine mature oocytes, sperm penetration was examined by Hoechst staining 18 h after IVF. In addition, to assess the developmental capacity after each treatment, the rates of survival, fertilization and blastocyst formation were examined(Fig. 2). Survival in each treatment 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. Immunocytochemistry

Microtubules, microfilaments and DNA of recovered oocytes from the each treatment were detected by indirect immunocytochemical techniques described by Kim et al.(1996). Brifly, surrounding cumulus cells were removed by 0.05% hyaluronidase solution and repeated pipetting, the oocytes were permeabilized in a modified Buffer M(Simerly and Shatten, 1993) for 10 min at 39℃, fixed in methanol at −20℃ for 10 min and stored in PBS 0.02% sodium azide and 0.1% bovine serum albumin at 4℃ until before the staining. All staining procedures were carried out at 39℃. Microtubule localization was performed using 1:300 of α-tubulin monoclonal antibody(Sigma) and 1:100 of FITC labeled goat anti-mouse antibody(Sigma) for 90 min, respectively. DNA was observed by exposure to 1 μg/ml propidium iodide(Sigma) for 90 min. Also, distribution of microfilaments of oocytes was detected from treatment of 10 μg/ml FITC-labeled phalloidin(Sigma) for 60 min. Stained oocytes were then mounted under a coverslip with antifade mounting medium(Universal Mount, Fisher Scientific Co, Huntsville, AL, USA) and examined using laser-scanning confocal microscope(BIO-RAD MRC 1024).

6. Hoechst staining

At 18 h after IVF, eggs were fixed with 2% formalin solution for 2~3 min. and stained with bisbenzimide solution(No. 33342, 2.5μg/ml, Sigma). Observation was carried out under ultra violet filter incorporated fluorescent microscope on 1 day after making sample.

7. Statistical analysis

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

Ⅲ. RESULTS

When the effects of cryoprotectant and freezing procedure on the microtubule, microfilament and chromatin morphology of oocytes were evaluated with indirect immunocytochemistry at 1 h

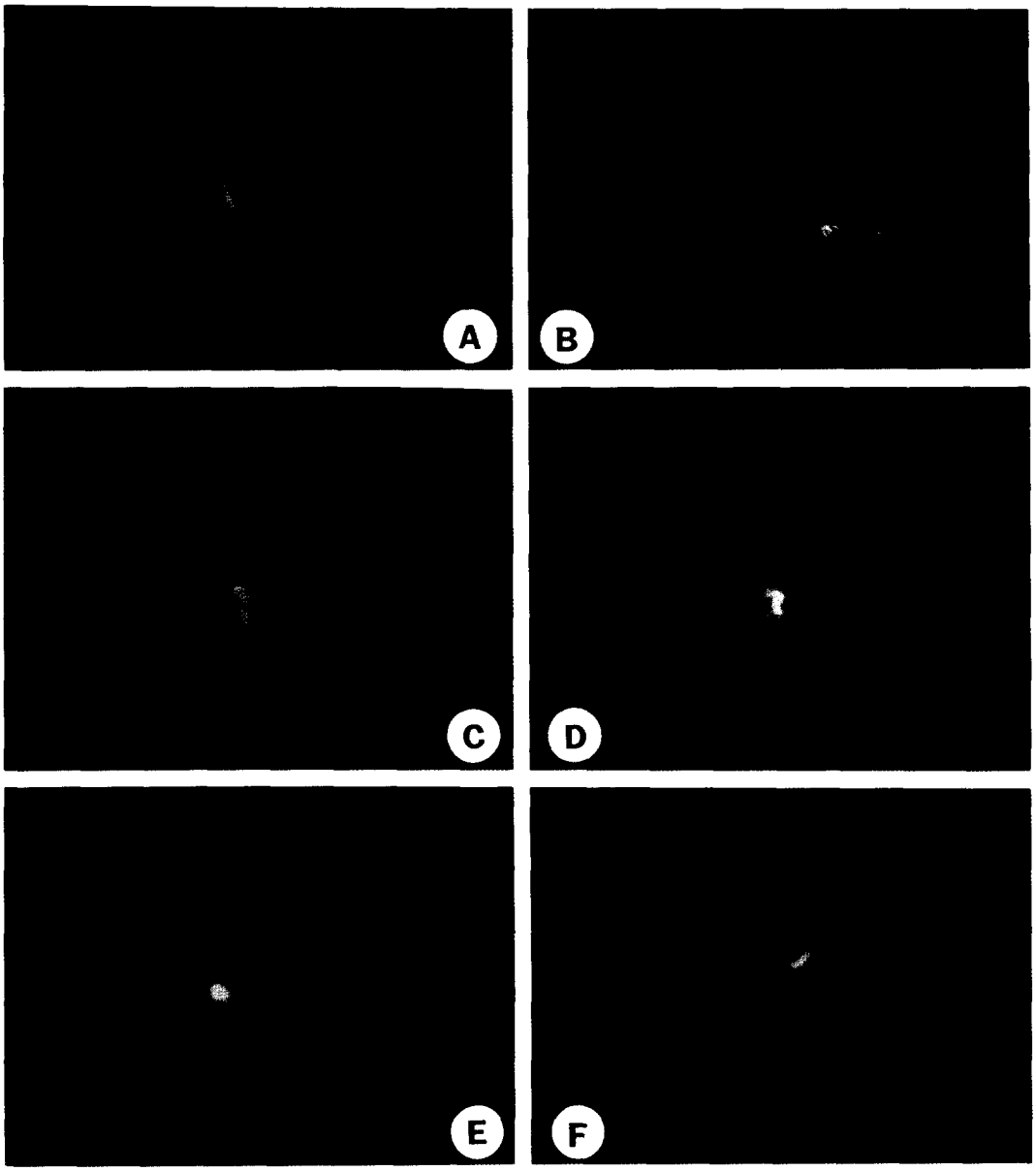

Fig. 1. Immunofluorescence localization of microtubules(A~D) and microfilaments(E~F) in frozenthawed bovine oocytes. (A) Normal (B, C and D) abnormal. Green, microtubules and microfilaments; red, chromatin; yellow, area of microtubules and DNA overlapping. A. Metaphase plate and polar body at metaphase II stage. Microtubules were detected in the spindle and some in around the polar body. B. Metaphase chromatin was not located in the cortex of oocyte. C. Microtubules were found in the midbody. D. Separated chromatin particles. (E and F) Normal microfilaments E. Control oocyte. F. Frozen-thawed bovine oocyte. Microfilaments were dispersed evenly through the ooplasm

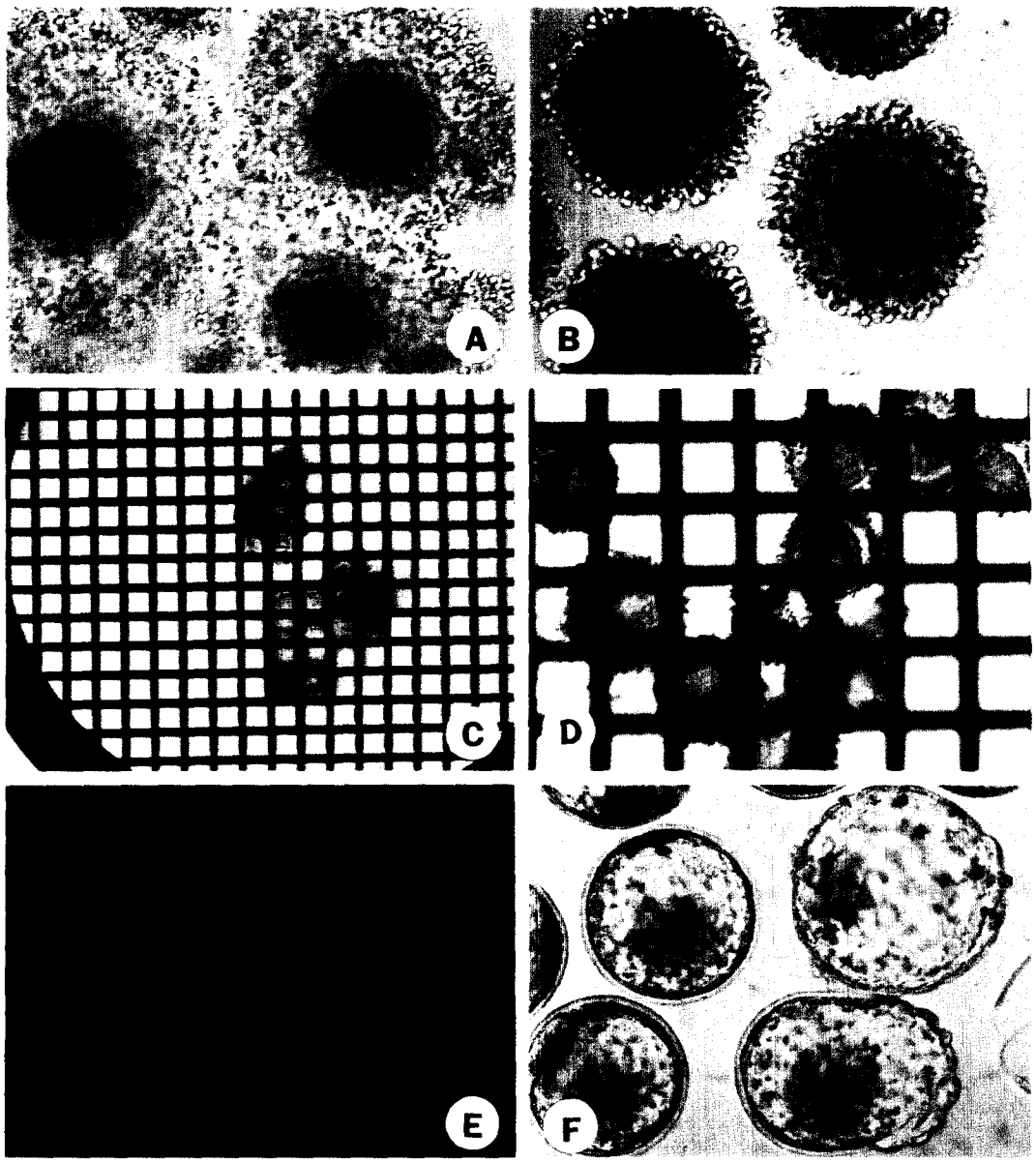

Fig. 2. Treatment procedures and developmental morphology of ultra-rapid freezing and thawed bovine mature oocytes. A) *In vitro* matured bovine oocytes B) Cumulus cell removed oocytes before being exposed to cryoprotectants. C) Mature oocytes loaded on electron microscope grid immediately before being plunged in LN_2. D) Oocytes after warming. E) Hoechst stained oocytes to confirm the sperm penetration at 18 h after IVF. F) Blastocysts produced from the coculture environment at day 8 after IVF

after each treatment, the results of freezing as well as exposure group were not different with that in the control oocytes. However, as shown in Fig. 1, there is a little abnormal microtubule pattern in all groups. When the fertilization abnormality after ultra-rapid freezing of bovine mature oocytes was examined by Hoechst staining 18 h after IVF, the rates of total penetration(96.7, 90.0%), normal two pronuclei formation(74.6, 68.9%) and mean number of sperm/oocyte(1.50, 1.44) were not significantly different between control and freezing group(Table 1). In addition, when the developmental capacity of frozen-thawed oocytes was assessed, a high mean percentage of oocytes(85.5%) was survived, 74.5% of them were cleaved and 31.4% of cleaved embryos were developed to blastocyst(Table 2). These data were similar to those of the control(76.0%, 34.6%) and exposure(74.5%, 33.0%) except survival rates(Table 2). Furthermore, when the total cell number of blastocysts produced from the each treatment at day 8 after IVF was examined by Hoechst staining, there were not different in among groups(Table 3).

Ⅳ. DISCUSSION

This study presents that developmental capacity of ultra-rapidly frozen bovine mature oocytes can be obtained successfully as described by Martino et al.(1996). If bovine oocytes could

Table 1. Fertilization of ultra-rapidly frozen bovine mature oocytes

Treatment	No. of oocytes	No. of oocytes penetrated(%)*			Mean no. of
(r=2)	insemination	Total	Polyspermy	2PN	sperm/oocyte
Control	61	59(96.7)	14(23.7)	44(74.6)	1.50
Freezing	50	45(90.0)	13(28.9)	31(68.9)	1.44

*Oocytes were examined by Hoechst staining at 18 hr post insemination.

Table 2. Developmental capacity of bovine mature oocytes after exposure to EFS30 and ultra-rapidly freezing(r=5)

Treatment	No. (%) of					
	Oocytes	Exposed	Freezed	Survived	≥ 2-cell	Blastocyst
Control	171	–	–	171(100.0)	130(76.0)	45(34.6)
Exposed		280	–	280(100.0)	218(77.9)	72(33.0)
Freezing			275	235(85.5)	175(74.5)	55(31.4)

Table 3. Blastocyst development and their cell number(r=5)

Treatment	No. (%) of blastocyst					Cell no.
	Total	≤ MB*	EdB	HgB	HdB	(≥ MB)
Control	45	28(62.3)	8(17.8)	7(15.6)	2(4.4)	125.0 ± 7.1
Exposed	72	40(55.6)	18(25.0)	14(19.4)	–	122.4 ± 5.6
Freezing	55	29(52.7)	13(23.6)	12(21.8)	1(1.8)	122.4 ± 8.0

*≤ MB: Early to middle blastocyst, EdB: Expanded blastocyst, HgB: Hatching blastocyst, HdB: Hatched blastocyst.

be successfully cryopreserved, an embryo bank could be established, and the techniques for advancing of genetic improvement could be promoted(Otoi et al., 1992). However, the pregnancy and live calves have been derived from cryopreserved bovine oocytes(Fuku et al., 1992; Hamano et al., 1992; Otoi et al., 1992), although the survival of oocytes as judged by development into blastocysts has been low, usually amounting to < 3% of all oocytes treated(Lim et al., 1991; Otoi et al., 1994; Schellander et al., 1994). In major reason of that, the most important factor in cryopreservation of bovine oocytes is mitigating of chilling sensitivity. Martino et al,(1996) reported that cooling of bovine mature oocytes to 0℃ for as little as 5 sec significantly decreased their capability to cleave and develop further after IVF compared to control(21.3% vs. 40.4% in blastocyst development). In addition, it has been known that normal spindle formation after thawing is very important to obtain of normal fertilization and development and is temperature-dependent. By Rebecca and Parks(1994), meiotic spindles in bovine oocytes did not fully reform after rewarming, while mouse oocytes are resilient against cooling and thus they appeared normal morphology after rewarming. Thus, they demonstrated that it is important to maintain bovine oocytes at temperatures as close to 39℃ as possible during *in vitro* procedures in order to maintain normal spindle morphology. In this study, we confirmed that there are not detrimental effects on the microtubules and microfilaments of ultra-rapidly frozen bovine oocytes, when all of the procedures in this study were carried out at 39℃. On the other hand, the advantage of rapid cooling methods over vitrification procedures lies in the use of lower, and hence less toxic, concentrations of penetrating cryoprotectants(Abbeel et al., 1997). In this study, we used EFS30 as freezing solution which consisted of 30% ethylene glycol, 18% ficoll, 0.5 M sucrose and 10% FBS in D-PBS. EFS30 was modified from EFS40 which generally used in vitrification of bovine and mouse blastocysts. When the toxicity of EFS30 solution to the bovine oocytes was examined, there was not detrimental effect on fertilization and embryonic development of exposure group(Table 2). However, by ultra-rapid freezing using EM grids and EFS30, a high developmental capacity of frozen bovine matured oocytes was 85.5% in survival, 74.5% in cleavage and 31.4% in blastocyst formation. These data were higher than that of Martino group(30.0% cleaved and 15% blastocyst from the total number of oocytes). Thus, it will be possible to obtain the higher developmental capacity of frozen bovine oocytes, if the more improved culture environment were introduced.

Therefore, these results demonstrate that developmental capacity of frozen-thawed bovine mature oocytes can be successfully obtained by ultra-rapid freezing method using EM grid and EFS30, thus it means that the establishment of bovine ovum bank has been actually possible.

V. SUMMARY

This study was carried out to confirm whether the developmental capacity of bovine mature oocytes frozen ultra-rapidly using electron microscopic(EM) grids and EFS30 can be obtained, and whether the cryoprotectants and the freezing method used in this study effect detrimentally to the bovine oocytes by indirect immunocytochemistry. 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. The results obtained in this experiment were summarized as follows: When the effects of cryoprotectant and freezing pro-

cedure on the microtubule, microfilament and chromatin morphology of oocytes were evaluated using indirect immunocytochemistry, the results of freezing as well as exposure group were not different with that of the control oocytes. When the fertilization abnormality after ultra-rapid freezing of bovine mature oocytes was examined by Hoechst staining, the rates of total penetration(96.7, 90.0%), normal two pronuclei formation(74.6, 68.9%) and mean number of sperm /oocyte(1.50, 1.44) were not different between control and freezing group. In addition, when the developmental capacity of frozen-thawed oocytes was assessed, a high mean percentage of oocytes(85.5%) was survived, 74.5% of them were cleaved and 31.4% of cleaved embryos were developed to blastocyst. These data were similar to those of the control(76.0%, 34.6%) and exposure(74.5%, 33.0%) except survival rates. Also, when the total cell number of blastocysts produced from the each treatment at day 8 after IVF was examined by hoechst staining, there were not different among groups. These results demonstrate that developmental capacity of frozen-thawed bovine mature oocytes can be successfully obtained by ultra-rapid freezing method using EM grid and EFS30 solution.

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