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소 수정란의 동결처리에 의한 난자성숙과 발육능 획득에 관한 연구
1. 미성숙 소난자의 시험관내 성숙과 수정시 Propanediols의 독성 효과

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Studies on Bovine Oocytes Maturation and Developmental Acquisition after Treatment with Different Cryoprotectants
1. Toxic Effects of Propanediols on *In Vitro* Maturation and Fertilization of Bovine Oocytes

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초 록

소 난자의 시험관내 수정시 이성체인 1, 2 propanediol과 1, 3 propanediol과의 동결보호제의 이용에 대한 독성효과를 조사하였다.

프랑스에 있는 도축장의 난소에서 채취한 미성숙 난자 212개를 시험관내의 성숙과 heparin(10 μg/ml)첨가에 의한 수정 및 배양을 실시하였다.

소 난자의 시험관내 성숙과 수정시 propanediol의 독성효과를 시험한 결과 1,3 propanediol은 80%의 정상 난할분할율과 5.7%의 낮은 다핵분할구율을 나타내었다. 이것은 1,2 propanediol의 73.9%, 10.3%와 미처리대조구의 73.9%, 7.7%와 비교되었으나 처리간의 유의성은 없었다.

Heparin 처리구는 1,2 propanediol의 첨가 유무에 관계없이 미처리구에 대하여 고도의 유의성 차이가 인정되었다. ($P<0.05$)

INTRODUCTION

The entire process of *in vitro* maturation (IVM), *in vitro* fertilization(IVF) and *in vitro* culture(IVC) is tedious and consuming. So efforts are made to find freezing procedures adapted to each step from oocyte to blastocyst in order to create gamete and embryo banks and gain more flexibility in their use.

Freezing of oocytes offers many advantages (Mermillod *et al.*, 1992). It will greatly facilitate IVF studies relating to the process of fertilization; provide a germ-plasm bank to evaluate the fertility of future sires by IVF; allow greater flexibility in genetic management of a breed in combination with semen bank; economically provide fertilized eggs in the pronuclear stage for gene insertion; provide for the preservation of oocytes of individual cows beyond their normal limits of fertility and allow for IVF with the sperm of future sires.

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So far, procedures for the cryopreservation of oocytes have not been developed to the same extent, particularly in the case of farm animals, due to factors such as the generally lower yield of viable embryos resulting from IVM, IVF and IVC as well as a lack of economic incentives. However the recent rapid improvement of the techniques in the bovine has generated new interest in the preservation of oocytes at low temperatures(Fuku *et al.*, 1992).

In 1949, Polge *et al.* reported the protective effect of 1,2 propanediol. The wholly amorphous state of its aqueous solution has great stability at subzero temperatures and has a higher glass formation tendency than does glycerol or DMSO(Boutron and Kaufmann, 1979). Recently the data(Fuku *et al.*, 1992; Fahning and Garcia, 1992; Niemann, 1990) reported that the use of propanediol instead of glycerol or DMSO significantly improved survival and development of cryopreserved bovine oocytes to the morphological criterion for maturation and fertilization. The toxic data of 1,3 propanediol against bovine oocytes are scarcely introduced.

In the first paper we investigated the toxic effects of 1,2 propanediol and 1,3 propanediol in order to select the cryoprotectants in the non-freezing atmosphere previous to freezing oocytes and this two different isomeric propanediols, applied to immature and *in vitro* matured bovine oocytes, were compared.

MATERIAL AND METHODS

1. Oocytes recovery

Cow ovaries were obtained from a slaughter house and transported to the laboratory within 2 hr. In our experiments after repeated washing of ovaries in a large volume of saline, follicle with an apparent diameter ranging from 2 to 4 mm are aspirated through a 28G 1/2 needle connected to a controlled depression of 4cm Hg (Boccart *et al.*, 1991). A total number of 212 oocytes were studied. From those, 74 were used to assess cryoprotectant treated groups, the 65 oocytes were only fixed to the non-treated control group. And the 73 remaining oocytes were examined for evaluation with or without heparin treatment.

2. *In vitro* maturation

The harvested oocytes are examined under a stereomicroscope and only those surrounded by more than three compact layers of cumulus cells are selected. They are washed in Tyrode modified medium(low bicarbonate TALP, Parrish *et al.*, 1986) and cultured in groups of about 100 in 4-cell tissue culture plates with 500 μl maturation medium. Maturation medium is TCM 199 containing 10% heat-treated fetal calf serum, 1 mg/ml 17 β-estradiol, 5 μg/ml pLH and 0.5 μg/ml pFHS. They are incubated for 24hr at 39℃ under a 5% CO_2 in air, humidified atmosphere.

3. Treatment of cryoprotectants

For the comparative examination of the toxic effects between 1,2 propanediol or 1,3 propanediol and non treated control, matured cumulus oocytes were washed four times with a maturation medium and placed into the each well of three levels of propanediol concentrations (1.5M, 1.0M and 0.5M) for 10 minutes per each step and then washed by the medium. After removal of the cryoprotectant, oocytes were separately transferred to each fertilization test well including non-treated oocytes group.

4. *In vitro* fertilization

The medium used for sperm/oocyte co-cul-

ture is TALP supplemented with the appropriate heparin concentration, in 50㎕ droplets under mineral oil and containing 10 oocytes and 1×10^6sperm/ml(Parrish *et al.*, 1986). Finally we use Percoll sperm selection(Guerin *et al.*, 1989) and heparin(10㎍/ml) sperm capacitation(Parrish *et al.*, 1986). Fertilization is practically done in 4-well tissue culture plates with about 25~40 oocytes(100 oocytes per well and 10^6sperm in 500㎕/well of heparin supplemented TALP). Oocytes and sperm are incubated for 18hr at 39℃ under a 5% CO_2 in air, water saturated atmosphere. Fertilization quality is assessed by determining after fixation and the staining with fluorochrome bisbenzimide(Hoechst 33342).

The morphological criterion for fertilization is determined on the presence of polar bodies and pronuclei for examination under a fluorescent microscope at ×200 and ×400.

5. Statistical analysis

Data from four replicate experiments were pooled and the treatment effects were compared by χ^2 analysis.

RESULTS AND DISCUSSION

Development capacity after treatments with 1,2 propanediol and 1,3 propanediol on *in vitro* maturation and fertilization of bovine oocytes are presented in Table 1.

It was observed that the normal cleaved oocytes were majority but others development were minority. The developmental category was classifies to oocytes of not fertilized(NF), two prenuclei(2PN), one prenucleus(1PN) and more than two prenuclei(>2PN), respectively. Epifluorescence micrographs of bovine oocytes stained with the Hoechst 33342 fluorescent dye is represented in Fig. 1A, 1B, 1C and 1D.

We have investigated the toxicity of propanediols on immature bovine oocytes by exposing them to increasing concentrations. They show that two isomeric propanediol are not toxic and could be interesting candidates which is similar to non-treated control group. The rates of morphologically normal cleavage formation for them were showed in exceed 74%(Table 2).

The effect of propanediol on *in vitro* maturation and fertilization of bovine oocytes was indicated in Table 2.

Generally speaking, the best results comes out by which the higher percent of normal cleaved and the lower percent of polyspermy formation. 1,3 Propanediol was shown 80% in normal cleaved and 5.7% in polyspermy. This figures compared with 76.9% and 10.3% of 1,2 propanediol, and 73.9% and 7.7% of control group.

Table 1. Developmental capacity of propanediol treatments on *in vitro* maturation and fertilization of bovine oocytes

Treatment	NF	2PN	1PN	>2PN	Total
1,2 propanediol	–	30	5	4	39
1,3 propanediol	4	28	1	2	35
non-treated (control group)	6	48	6	5	65
Total	10	106	12	11	139

NF:not fertilized oocytes 2PN:two prenuclei oocytes(normal cleaved, dispermy)

1PN:one prenuclus oocyte(monospermy)

>2PN:more than two prenuclei oocytes(polyspermy)

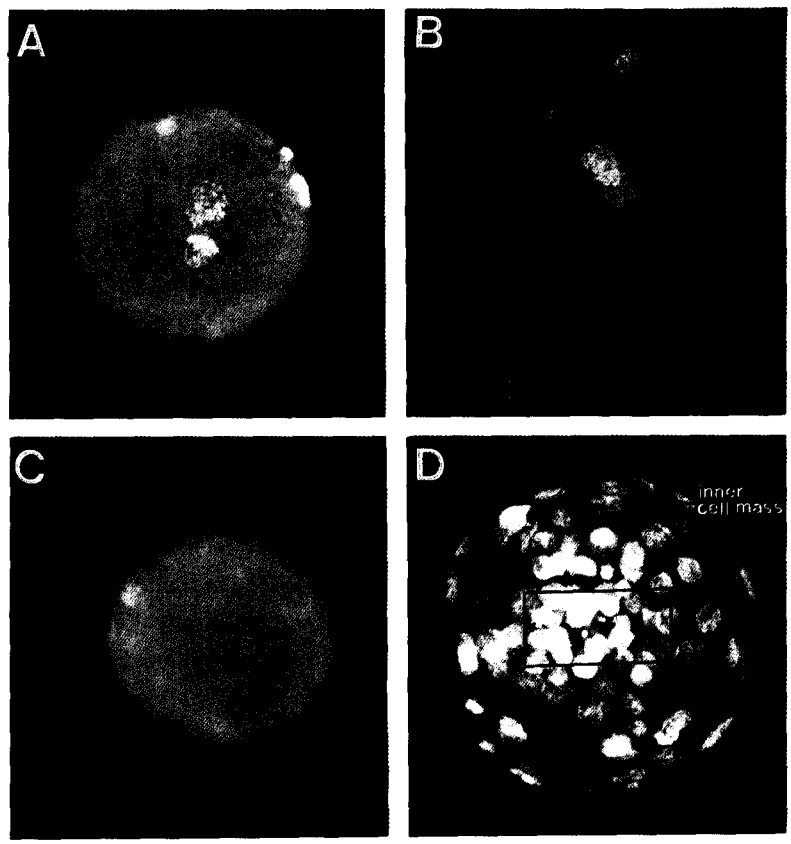

Fig. 1. Epifluorescence micrographs(magnification x400) of bovine oocytes stained with the Hoechst 33342 fluorescent dye.
(A)*in vitro* fertilized zygote with the two polar bodies(PB) and the two pronuclei(2PN). (B)*in vitro* fertilized polyspermic zygote and one prenucleus oocyte. (C)non-fertilized oocyte.
(D)*in vitro* cultured blastocyst showing inner cell mass.

Table 2. Effect of propanediol on *in vitro* maturation and fertilization of bovine oocytes

Treatment	n	Normal cleaved	%	No of polyspermy	%
1,2 propanediol	39	30	76.9	4	10.3
1,3 propanediol	35	28	80.0	2	5.7
non-treated (control)	65	48	73.9	5	7.7
Total	139	106	76.3	11	7.9

There was a few distinction in the formation of normal cleaved and polyspermy among propanediol treated groups and control non-treated group. However, there were no significant differences among any of the treatments for any response.

Table 3. Toxic effects of 1,2 propanediol *in vitro*-derived bovine embryos after maturation of oocytes in TCM-199 supplemented with or without heparin

Treatments		n	Normal cleaved (2PN)	%
1,2 propanediol (118μg/ml)	heparin (10μg/ml)			
+	−	40	10	25.0[a]
−	+	29	22	76.0[b]
+	+	39	30	77.0[b]
−	−	33	3	9.0[a]
Total		141	65	46.1

a, b (within columns): $P<0.05$

Toxic effects of 1,2 propanediol *in vitro*-derved bovine embryos after maturation of oocytes in TCM-199 supplemented with or without heparin were represented in Table 3.

In TCM-199 supplemented with heparin treatment was shown highly significant differences than that without heparin having nothing to do with or without 1,2 propanediol($P<0.05$).

The most common method to undergo a sperm capacitation used in bovine IVF involves heparin(Parrish *et al.*, 1986). Heparin, as well as other glycosaminoglycans present in the female genital tract(Lenz *et al.*, 1982) is able to capacitate bovine fresh and frozen sperm(Parrish *et al.*, 1986, 1988). The optimal heparin concentration ranges from 0.05(Marquant-Le Guienne *et al.*, 1990) to 100μg/ml(Fukui *et al.*, 1990). We use percoll sperm selection and heparin(10μg/ml) sperm capacitation and the rate of zygotes presenting both male and female pronuclei is about 76~77% with the Holstein sire. The results showed in lower than 80% of Mermillod *et al.*, but higher than 53% of Fuku *et al.*

Depiesse *et al.*(1991) have investigated the toxicity of four cryoprotectants on immature bovine oocytes by exposing them to increasing concentrations of each one. Survival was evaluated by determining IVM rate after cryoprotectant treatment. They show that DMSO is toxic and that glycerol, 1,2 propanediol and ethylene glycol could be interesting candidates although maturation rates do not exceed 50%.

Our results were shown in higher maturation rates of exceed 74% in only 1.5M isomeric propanediols. 1,3 propanediol that is an isomeric type of 1,2 propanediol are not well-known cryoprotectants, but no toxic, good results of normal cleaved formation and lower polyspermy. Accordingly, the conclusion is 1,3 propanediol seems to be used to freezing bovine oocytes as an useful cryoprotectants as well as 1,2 propanediol.

SUMMARY

In vitro fertilization assays were performed to investigate their toxic validity in evaluting cryoprotectants; two isomeric types of 1,2 propanediol and 1,3 propanediol. A total of 212 immatured bovine oocyte collected from ovaries at the abattoir, were matured by *in vitro* maturation, *in vitro* fertilization used in heparin (10μg/ml) for sperm capacitation and subsequently by *in vitro* culture.

The effects of the propanediol on *in vitro* maturation and fertilization of bovine oocytes were studied. 1,3 Propanediol was shown 80% in normal cleaved and 5.7% in polyspermy. This figures compared with 76.9% and 10.3% of 1,2

propanediol, and 73.9% and 7.7% of control group. There were no significant differences among of the treatments.

The treatment of heparin was shown highly significant differences than that without heparin having nothing to do with or without 1,2 propanediol($P<0.05$).

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