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Studies on the *In vitro* Culture of Early Bovine Embryos

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Abstract

In vitro maturation and fertilization of oocytes collected from slaughtered bovine ovaries were investigated. Immature bovine extrafollicular oocytes were cultured for 24 hrs in TCM 199 supplemented with fetal calf serum in a humidified CO_2 incubator. Fertilization *in vitro* was performed using frozen-thawed bull semen which was treated by Ca Ionophore A23187.

Fourty percentage of oocytes cultured had matured to the metaphase II. There were no effects of the concentration of fetal calf serum and of the addition of HEPES on the maturation rate. The mean proportions of *in vitro* fertilized eggs and of cleaved eggs were 23.1% and 14.4%, respectively.

Introduction

Economics of embryo availability and production largely govern the extent of practical utilization of embryo transfer. The supply of ova available by superovulation is limited. The ability to mature *in vitro* from follicles of the bovine ovary would greatly increase the yield of oocytes available for fertilization.

The successful development of *in vitro* fertilized bovine eggs to morula or blastocyst stage has been reported(Sirard *et al.*, 1985; Lambert *et al.*, 1986; Lu *et al.*, 1988; Fukui *et al*1989). Following *in vitro* fertilization of oocytes collected form slaughtered bovine ovaries, pregnamcy and newborn calves were obtained by Hanada *et al*(1986).

However, successful rate of the development to morula stage was very low and a small number of laboratories performed successfully *in vitro* maturation and fertilization.

The purpose of the present study was to investigate *in vitro* maturation and fertilization of oocytes collected from slaughtered bovine ovaries.

Materials and Methods

Oocyte Maturation: Ovaries were obtained from Holstein and korean native cattle killed at a slaughterhouse. They were transported to the laboratory within 2 hrs, held at 35℃ to 37℃ in physiological saline(0.9%) supplemented with 100I.U. of penicillin and 100 μg/mℓ of streptomycin. The cumulus-oocyte complexes were collected from follicles of 2~5mm in diameter using a 10mℓ syringe with an 18 gauge needle. The collection of oocytes was carried out in Dulbecco's phosphate

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saline (PBS) supplemented with 10% calf serum. After collection, the oocytes were classified under a stereomicroscope(15~30x). The medium used for the culture of extrafollicular oocytes was TCM 199(with Earle's salts, L-glutamine, 2,200mg/L sodium bicarbonate ; GIBCO) supplemented with 10% heat-inactivated (at 56℃ for 30min) fetal calf serum, 100 μg/mℓ streptomycin, 100I.U. of penicillin, 1 μg/mℓ of estradiol benzoate and 25mM HEPES. Only apparently normal oocytes with a dense layer of cumulus cells were placed in a 30mm petri dish(Nunc, 8~10 oocytes per drop) covered with paraffin oil and cultured for 24 hrs in a humidified CO_2 incubator(5% CO_2 and 95% air at 38℃).

Treatment of Spermatozoa : The standard medium used for capacitation of spermatozoa and for *in vitro* fertilization was essentially the same as that used by Brackett and Oliphant(1975). Two 0.5mℓ-frozen straws of extended semen from a Holstein bull were thawed at 35℃ for 1 min and concentration was determined by counting using a hematocytometer. Thawed semen was mixed with 8mℓ of BO medium with caffeine in test tube and washed twice by centrifugation for 5 min at 1500~1600rpm. The final pellet was resuspended in BO medium with caffeine to yield a concentration to 5×10^6 spermatozoa/mℓ and then spermatozoa was treated with 0.1μM Ca Ionophore A23187 free acid. The equal volume of BO medium with BSA was added to result in a stop of Ionophore reaction. The 80~100 μℓ of sperm droplets were incubated under paraffin oil for 2 to 3 hrs in a CO_2 incubator.

***In vitro* Fertilization** : Five to ten oocytes which had been cultured for maturation were added per drop of spermatozoa. Spermatozoa and oocytes were incubated together for 5 hrs in a humidified CO_2 incubator. Oocytes were transferred to TCM 199 containing 10% fetal calf serum after *in vitro* insemination. The examination of fertilization and cleavage was carried out at 18hrs and 48hrs after transfer, respectively. After cultrure, the oocytes were freed from follicular cells by treatment with 5% hyaluronidase and by repeated passage through a fine pipette and were fixed in acetic acid and ethyl alcohol(3 : 1) and stained with 1% aceto-orcein, and examined by phase-contrast microscope.

Results and Discussion

The result of *in vitro* culture of bovine follicular

Table 1. Effects of FCS and HEPES on Maturation of Bovine Extrafollicular Oocytes

Concentration of serum(%)	HEPES	No. oocytes examined	Stage of maturation			maturation rate(%)
			Deg*	PI-TI**	MII***	
5	+	80	7	42	31	38.8
	−	67	6	38	23	34.3
10	+	83	5	36	42	50.6
	−	75	7	35	33	44.0
20	+	64	3	33	28	43.8
	−	71	5	37	29	40.8

* : Degeneration, ** : prophase I-Telophase I, *** : Metaphasse II

Table 2. Result of *In vitro* Fertilization after Insemination of Spermatozoa Pretreated with 0.1μM Ca Ionophore

Inseminated hours after insemination	No. oocytes inseminated	No. oocytes fertilized(%)	No. oocytes cleaved(%)
44	87	20(22.9)	12(13.8)
50	60	14(23.3)	9(15.0)

oocytes is shown in Table 1.

A total of 440 oocytes were examined; Overall, 42% of the follicular oocytes had matured to the second metaphase. The Maturation rate of the present study was lower than that of other researchers (Hunter *et al.*, 1975 : 91%; Thibault *et al.*, 1975 : 97%; Shea *et al.*, 1976 : 73%). Although pH and temperature variation in the medium were reported to affect on the maturation of oocyte, the exact cause which lowered maturation rate could not be found in the present study There was no significant difference in maturation rates between the groups.

The result of *in vitro* fertilization by frozen-thawed bull spermatozoa treated with caffeine and Ca Ionophore A23187 is summarized in Table 2.

Capacitation of mammalian spermatozoa *in vitro* has been reported following a variety of treatments, including exposure to BSA. bovine follicular fluid(Fukui *et al.*, 1985), heparin(Parrish *et al.*, 1985), caffeine(Goto, 1988) and Ca Ionophore (Byrd., 1981). Chung and Im(1988) reported 22.0% of cleavage rate when albumin was used for capacitation. In the present study most of spermatozoa treated with Ca Ionophore A23187 free acid (SIGMA) for 30 sec had shown hyperactivation and resulted in a mean 23% of fertilization and 14.4% of cleavage.

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소 초기배의 체외수정에 관한 연구

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초 록

소 난소로부터 난자를 채취하여 체외성숙과 체외수정을 실시하였다. 난의 성숙배양을 위한 배양액은 소 태아혈청을 첨가한 TCM 199으로 CO_2배양기에서 24시간 배양하였다. 체외수정에는 동결융해정액을 사용하였으며 Caffeine과 Ca Ionophore 처리후 매정에 공하였다. 배양난자중 40%가 Metaphase II까지 성숙하였다. 성숙율에 대한 소 태아혈청의 농도변화와 HEPES 첨가여부의 효과는 없었다. 체외수정율과 분할율의 성적은 각각 23.1%와 14.4%였다.