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Calf Production by In Vitro Fertilization of Follicular Oocytes Matured In Vitro

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Abstract

The procedure for bovine *in vitro* fertilization (IVF) involving the collection of ovaries at the slaughterhouse, maturation of oocytes *in vitro* (IVM), capacitation and insemination *in vitro* and development to blastocysts *in vitro* is well established. Transportation of ovaries in physiological saline at 23 and 38°C afforded a large number of oocytes capable to develop to blastocysts after in vitro maturation and fertilization. The follicular contents were scrubbed out with a small spoon after incision of the follicle to collect a large number of oocytes (13.4 per pair of ovaries) rather than aspiration with an 18-G needle (7.2 per pair of ovaries). Cleavage ability after maturation and *in vitro* fertilization depended on the characteristics of the cumulus cells surrounding the oocytes. A period of 20 hr for the culture to achieve maturation and for the time of the insemination was preferable to 24 or 28 he for the development to blastocyst. For *in vitro* capacitation of sperm, heparin was effective and the oocytes fertilized by heparin-treated sperm developed well to full term after transfer to recipient cattle.

Bovine IVM/IVF can be used for commercial beef calf production and for the supply of embryos with known stages of development for research purposes and for the development of new technologies.

Discipline: Animal Industry/Biotechnology
Additional key words: blastocyst, bovine, capacitation, embryo, spontaneous maturation

Introduction

The first ruminant offspring produced after *in vitro* fertilization (IVF) was a bull calf born in 1981[2]. Oocytes from mammalian species are able to resume meiosis from the dictyate stage to metaphase II when liberated from follicles and cultured in a suitable medium. This phenomenon, called spontaneous maturation *in vitro*, was reported by Pincus and Enzmann in 1935[19]. The bovine oocytes undergoing *in vitro* maturation (IVM) could develop to the fetal stage when they were transferred to an inseminated heifer and re-transferred to a recipient heifer after development to blastocysts[16], although it was reported that the IVM oocytes were not able to elicit the formation of male pronucleus after IVF and did not develop further[26].

The first calves from oocytes which became mature and were fertilized *in vitro* were born in 1985[6]. This was a remarkable achievement because the method of capacitation was induced and large numbers of embryos were capable to develop to calves at a low cost[14].

To obtain non-surgically transferable blastocysts, the rabbit oviduct was necessary as a temporary incubator. Now it is well known that embryos derived from IVM/IVF oocytes can develop to blastocysts when cultured in TCM 199 supplemented with bovine serum[3,10].

The main purpose of this article is to review the results of IVM/IVF of bovine oocytes for the production of embryos in the experiments conducted at National Institute of Animal Industry (NIAI).

General procedures

Cattle ovaries collected at a local slaughterhouse

were placed into warm physiological saline supplemented with 100 U of penicillin (crystalline penicillin G potassium, Meiji Conf. Co., Japan) and 100 μg of streptomycin/m*l* (streptomycin sulfate, Meiji Conf. Co.) and transported within 2 hr to the laboratory in NIAI.

Oocytes were then aspirated from small (below 5 mm) vesicular follicles using a 5 m*l* syringe with an 18-G needle. The aspirates were mixed with modified Dulbecco's phosphate buffered saline supplemented with 3 mg bovine serum albumin/m*l* (Fraction V, Miles, Naperville, USA) and antibiotics.

The oocytes were selected based on the thickness and compactness of the cumulus cell layers for maturation and cultured in 100 μ*l* of 25 mmol HEPES-buffered TCM 199 (Gibco, Grand Island, USA) supplemented with 10% bovine serum and 0.002 AU of FSH (Antrin, Denka Pharmac. Co., Japan) and 1 μg of estradiol/m*l* (17ß-estradiol, Sigma Chem. Co., USA) under mineral oil (Squibb and Sons Inc., USA) for 20 to 22 hr. Atmospheric conditions of the culture were 5% carbon dioxide and 95% moisture air at 39°C.

For the capacitation, frozen bull semen was thawed and poured into a 10 m*l* test-tube and mixed well with 10 m*l* of Caff-BO[25] (modified DM of Brackett and Oliphant, 1975[1]), without bovine serum albumin and with 10 mmol sodium caffeine benzoate).

Then the sperm suspension was centrifuged at 500 × g for 5 min, the supernatant was aspirated and resuspended in the Caff-BO, again centrifuged and the supernatant was removed. The sperm pellet was resuspended in 0.5 to 1.0 m*l* with Caff-BO and the sperm concentration was measured and adjusted between 1×10^7 and 2×10^7 spermatozoa/m*l*. The adjusted sperm suspension was diluted to 1:1 with BSA-BO-Hep (modified DM of Brackett and Oliphant, 1975[1]), with 20 mg of crystallized BSA (Sigma Chem. Co., USA) and 5 to 10 μ*l* of heparin/m*l* (Novo Heparin, Novo Industry A/S, Denmark)). Aliquots of sperm (0.1 m*l*) were placed under mineral oil and incubated for 15 min at 39°C in an atmosphere of 5% carbon dioxide and 95% moisture air.

The cultured oocytes were transferred into sperm droplets for insemination. After a sperm-oocyte incubation period of 4 to 6 hr, the oocytes were transferred to the culture medium for cleavage and development. The culture medium consisted of 25 mmol HEPES buffered TCM 199 supplemented with 10% bovine serum and antibiotics.

Several oocytes were picked up, and the cumulus cells were removed, mounted on a 4-wax-spot glass slide and fixed in alcohol-acetic acid for the examination of sperm penetration and pronuclear formation 10 to 18 hr after insemination.

The remaining oocytes were cultured and examined for cleavage on day 3 or 4 (day 1 = the day of the insemination). For the examination, the cumulus cell layers of the oocytes were removed by gentle pipetting, the cleaved embryos were cultured in the same dish and the blastocyst development was observed during 7 to 10 days.

Results

1) Collection of ovaries at slaughterhouse

Ovaries were transported at 4°, 23° and 38°C, within 2 to 4 hr after the death of the animals. The oocytes from the ovaries transported at 4°C showed a marked decrease of the developmental capability to blastocysts after IVM/IVF. The transportation at 23°C had an advantage over that at 38°C, resulting in a development rate of the blastocysts ranging from 15.5% (37/239) to 20.5% (39/190) (Tables 1 and 2)[27].

Table 1. Effect of temperature of saline used for ovary transport on the development rare to blastocysts after IVM/IVF

Bull	Temperature of saline (°C)	Oocytes used	Cleavage rate (%)	Blastocyst (%)
Toch	38	177	77.4	23(13.0)
	23	190	71.6	39(20.5)
688	38	293	53.2	24(8.2)[a]
	23	239	51.5	37(15.5)[b]

Figures with superscripts a and b are significantly different ($P<0.01$) by the χ^2-test.

Table 2. Effect of low temperature of saline used for ovary transport on the development rate to blastocysts after IVM/IVF

Temperature of saline (°C)	Oocytes used	Cleavage rate (%)	Blastocyst (%)
23	470	71.3[a]	102(21.7)[a]
4	506	34.0[b]	33(6.5)[b]

Figures within the same column with different superscripts are significanty different ($P<0.01$).

The possibility of transporting ovaries for a long period of time was confirmed because 16.0% (109/683) of the oocytes obtained from ovaries kept at 20°C for 8 hr were able to develop to the blastocyst stage (Abe & Shioya, 1991, unpublished).

2) Collection and selection of oocytes for maturation

(1) Collection of oocytes

Although about 40 small follicles were counted on the surface of a pair of ovaries, approximately 20 oocytes were obtained by aspiration with a syringe and a needle. Only one third of the oocytes obtained was surrounded with compact cumulus cells and suitable for IVM/IVF[11].

It was reported that the stage of estrous cycle did not affect the number of oocytes collected and the oocytes from various stages of the estrous cycle developed to blastocysts after IVM/IVF[11].

Several methods were used for the collection of follicular oocytes including mincing and slicing[9]. Recently a new method, whereby follicular contents are scrubbed out into modified PBS supplemented with 1 μl of heparin/m*l* with a small spoon after incision of the follicle with a surgical blade has been used to collect a larger number of oocytes (Table 3). More research is necessary to collect and utilize all the oocytes in the ovary.

(2) Selection of the oocytes for maturation

Oocytes were classified according to the characteristics of the cumulus cells under a stereomicroscope as follows: Class A oocytes had compact and dense cumulus cell layers; Class B oocytes showed compact but dense cumulus cell layers, some were partially naked oocytes with compact cumulus cells; Other B class oocytes (B″) were partially naked oocytes, with thin cumulus cell layers or with small remnants of cumulus cells; Class C consisted of

Table 3. Methods of collection of follicular oocytes

	Aspiration[a]		Scrubbing[b]	
	No. of cows	Oocytes for IVF	No. of cows	Oocytes for IVF
	11	11.4	10	19.1
	28	6.5	15	10.8
	25	6.9	9	9.7
	14	7.1	4	17.3
	8	7.1	11	13.6
Total	86	7.2	49	13.4

a): 18-G needle & 5 m*l* syringe.
b): Incision on the follicle with a scalpel and scrubbing out of the follicular contents with a small spoon.

Table 4. Cleavage capability of IVF oocytes classified on the basis of the characteristics of the cumulus cells

Class	Oocytes cleaved/oocytes cultured (%)
A	232/364(63.7)[a]
B″	36/122(29.5)[b]
C	28/158(17.7)[b,c]

Figures with superscripts a, b ($P<0.01$) and b, c ($P<0.05$) are significantly different.

cumulus-free oocytes. Oocytes classified as A, B″ and C were cultured for maturation, inseminated *in vitro* and cultured for cleavage. It was clearly demonstrated that the characteristics of the cumulus cells influenced the cleavage rate after IVM/IVF (Table 4)[22]. Oocytes classified as A and B were able to develop to blastocysts while those with only a small remnant of cumulus cells and without them were not suitable for IVM/IVF experiments due to the lack of blastocyst development.

3) Culture of oocytes for maturation

Oocytes cultured were inseminated *in vitro* at 16 to 28 hr after the beginning of the maturation culture to determine when the oocytes should be inseminated. It was shown that insemination should be performed not later than 24 hr after the culture (Table 5)[7].

It was reported that the maturation of sheep oocytes cultured within follicles *in vitro* was related to hormone supplementation in the culture medium[15]. Oocytes were cultured in TCM 199 supplemented with 10% calf serum (CS), FSH (0.002 AU/m*l*) and 17-ß estradiol (1 μg/m*l*) and in the medium with CS

and without hormones to assess the effect of hormone supplementation on IVM/IVF and development to blastocysts. It was demonstrated that hormones were not necessary for the acquisition of the developmental competence of the oocytes liberated from small follicles (Table 6)[21], although the cumulus cells surrounding the oocytes were stimulated and expanded by the hormones.

The same results were reported by Fukushima et al.[4] and calves were born from oocytes matured *in vitro* without hormones and fertilized *in vitro*. To avoid the effect of hormones in CS, oocytes were cultured for maturation in TCM 199 supplemented with BSA and lacking hormones[21]. The oocytes developed to blastocysts after IVM/IVF. All these data demonstrate that bovine oocytes cultured without hormones can develop to blastocysts after IVM/IVF.

4) Capacitation and insemination

Mammalian spermatozoa cannot penetrate the zona pellucida before capacitation, which normally occurs in the female reproductive tract. Several methods have been developed for the capacitation of ejaculated bovine spermatozoa *in vitro* to obtain high and reproducible fertilization frequencies, including Ca-ionophore treatment[5,25], preincubation in BSA-rich medium[12,20] and heparin treatment[17,18]. The ionophore treatment in which sperm washed with Caff-BO medium (DM, without BSA supplemented with 10 mmol caffeine sodium benzoate[1]) was treated with 0.1 μmol ionophore for 1 min, and then diluted two times with BSA-BO (modified DM by 20 mg BSA/m*l*) for insemination, showed a high fertilization rate in ejaculated bovine semen[5,25].

Heparin concentration was tested on ejaculated bovine sperm for the ability to increase *in vitro* fertilization frequencies. Zero to 50 μl heparin (Novo Heparin for injection, Novo Ind. A/S, Denmark) was added to 1 m*l* of BSA-BO medium. Sperm washed with Caff-BO was diluted to half by BSA-BO supplemented with heparin. The spermatozoa of one bull showed a high fertilization rate both "with" and "without" heparin. The spermatozoa of two other bulls showed a low fertilization rate ranging from 13 to 55% and the heparin treatment increased the fertilization rate above 90% (Table 7)[8]. Thus the heparin treatment was effective in inducing capacitation *in vitro*. Until now, spermatozoa of over 10 bulls have been tested and they showed adequate fertilization and blastocyst development rates.

Sperm concentrations in the fertilization medium are important to obtain adequate fertilization rates. The numbers of spermatozoa in the fertilization medium were adjusted to 5 to 100×10^5/m*l* and IVM/IVF was carried out. A lower sperm concentration, 5×10^5/m*l* resulted in lower fertilization and blastocyst development rates (Table 8). An amount of over 50×10^5 spermatozoa/m*l* was sufficient to ensure adequate development rates[23].

5) Development to blastocysts in vitro

After co-incubation with spermatozoa, the oocytes should be transferred to the medium for development to blastocysts. Co-incubation for 4 to 6 hr

Table 5. Effect of the duration of the culture period of the oocytes for maturation on the development rate to blastocyst

Culture period (hr)	Oocytes used	Cleavage rate (%)	Blastocyst (%)
16	430	65.1	59(13.7)[a]
20	410	65.4	62(15.1)[a]
24	477	65.0	37(7.8)[b]
28	403	64.5	16(4.0)[b]

Figures with superscripts a, b ($P<0.01$) and b ($P<0.05$) are significantly different.

Table 6. Effect of gonadotropin and estradiol-17ß in culture medium for maturation on the development rate to blastocysts after IVM/IVF

Bull	Estradiol (μg/m*l*)	FSH (AU/m*l*)	Fertilization rate (%)	Oocytes used	Cleavage rate (%)	Blastocyst (%)
Satu	0	0	76.5	52	40.4	6(11.5)
	1	0.002	79.4	129	53.5	10(7.8)
5321	0	0	88.1	133	56.4	15(11.3)
	1	0.002	86.0	139	63.3	23(16.5)

with sperm and oocytes is sufficient depending on the bull used. TCM 199 supplemented with bovine serum contributes to the cleavage to blastocysts. Although different sources of bovine serum including fetal calf, calf, steer and estrous cow serum could be used for the culture, it is important to select a suitable lot of serum.

In the experimental system of IVM/IVF, the oocytes are surrounded by cumulus cell layers which exert a favorable effect on blastocyst development. The cumulus cells fall down automatically at the bottom of the culture dish and make a cell sheet. To clarify the effect of cumulus cells on the blastocyst development, the oocytes after 8 hr of insemination were pipetted with a fine glass pipette to get rid of the cumulus cell layers and cultured in the medium only or with oviductal epithelium cells. The use of oviductal epithelium cells effectively increased the blastocyst development rate of cumulus-free oocytes, unlike that of oocytes with cumulus cell layers (Table 9)[13].

Table 7. Effect of heparin concentration of BSA-BO on *in vitro* fertilization rate of bovine oocytes undergoing maturation *in vitro*

Concentration of heparin (μl/m*l*)	Fertilization rate (%) (penetrated/examined)		
	Bull		
	Oo	Atsu	Satu
0	13.0(3/23)	100.0(11/11)	54.5(12/22)
5	100.0(21/21)	100.0(14/14)	94.4(17/18)
10	90.5(19/21)	100.0(13/13)	100.0(10/10)
20	90.9(20/22)	100.0(14/14)	95.2(20/21)
50	91.3(21/23)	100.0(14/14)	94.1(16/17)

The effect of the cumulus cells on the development of IVM/IVF oocytes is ascribed to the presence of diffusible factors since the oocytes cultured on a permeable filter placed between them and the cumulus cell monolayers were able to develop to blastocysts[24].

6) *Developmental competence of embryos produced in vitro*

Table 10 shows the results of transfer of embryos from IVM/IVF oocytes and their use. Blastocysts obtained after IVM/IVF were fertile after non-surgical transfer to recipient cattle.

Two to three blastocysts can be obtained from one pair of ovaries collected at a slaughterhouse at present (Table 11), and if transferred to recipient cattle, one or two calves are expected to be born. Therefore, it is necessary to develop a more efficient method for IVM/IVF for application to calf production on a commercial basis.

Table 9. Effect of cumulus and oviductal cells on the development rate to blastocysts after IVM/IVF

Culture conditions		Blastocysts developed /Oocytes used (%)
Cumulus cells[a]	Oviductal cells[b]	
Intact	+	28/139(20.1)
Intact	−	40/136(29.4)
Removed	+	30/131(22.9)
Removed	−	1/125(0.8)

a): Removed; The cumulus cell layers were removed from the oocytes after 8 hr of insemination to make them naked.

b): +: Oviduct cells from newly ovulated cows were added to the culture medium (100,000 cells/m*l*)

Table 8. Effect of sperm concentration in the insemination medium on the development rate of blastocysts after IVM/IVF

Bull	Sperm con. $\times 10^4$	Fertilization rate (%)	Poly-spermy rate (%)	Oocytes used	Cleavage rate (%)	Blastocyst (%)
Toch	25	43.6	0.0	153	8.5	1(0.7)
	250	94.3	18.0	159	42.8	22(13.8)
	500	98.9	34.7	271	48.3	29(10.7)
	1,000	98.6	45.2	296	60.5	32(10.8)
Fuji	25	49.4	9.8	226	10.2	4(1.8)
	125	78.6	9.1	307	53.7	45(14.7)
	250	94.8	44.0	278	62.9	47(16.9)
	500	96.7	56.2	317	73.2	65(20.5)
	1,000	95.0	54.4	173	71.7	24(13.9)

Table 10. Results of transfer of embryos derived from IVM oocytes fertilized *in vitro* by heparin-treated sperm

Reci.	Bull	Days after E.[a]	Embryos		Results[e]
			Age[c]	Trans.[d]	
344	Ara	7(AI)[b]	9	1	IVF-M, 40 kg & AI-calf
341	Ara	8(AI)	8	2	IVF-M, 37 kg & AI-calf
345	Toch	7(AI)	8	1	IVF-M, 52 kg & AI-calf
365	Fuji	7(AI)	8	1	—(estrus-84 days)
346	Toch	8	9	2	IVF-M, 38 kg & M, 27 kg
370	Toch	8	9	2	IVF-F, 25 kg & M, 25 kg
373	Toch	8	9	2	—(estrus-27 days)
372	Toch	7	9	2	—(estrus-47 days)
626	Toch	8	8	2	IVF-M, 22 kg & M, 29 kg
374	Toch	8	8	2	abortion (Twins, 196 days)
356	Toch	7	8	2	IVF-M, 45 kg
355	Fuji	7	8	1	Abortion (156 days)

a): Estrus day = 0-day.
b): AI; The recipients were artificially inseminated beforehand.
c): Insemination *in vitro* = 1-day.
d): Number of embryos transferred.
e): AI-calf; calf from artificial insemination.
IVF-M; male calf from transfer of *in vitro* fertilized oocyte. Figure indicates the birth weight.

Table 11. Collection of follicular oocytes with a small spoon and development rate to blastocysts after IVF

	No. of cows	No. of oocytes used for IVF (per cow)	No. of blastocysts (per cow)
	22	278(12.6)	44(2.0)
	19	328(17.3)	58(3.1)
	34	521(15.3)	92(2.7)
	12	204(17.0)	51(4.3)
	24	382(15.9)	43(1.8)
	30	513(17.1)	57(1.9)
Total	141	2,226(15.8)	345(2.4)

Source: Abe & Shioya (1991 unpublished).

Other important applications of IVM/IVF include the supply of mature oocytes and of embryos with definite stages of development for techniques relating to animal production. Research and uses of IVM/IVF will undoubtedly expand for the improvement of genetic performance involving new techniques.

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