The Effects of Dimethyl-Sulfoxide Added to the Fertilization Medium on the Motility and the Acrosome Reaction of Spermatozoa and the Subsequent Development of Oocytes in Bovine

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ABSTRACT : This experiment was conducted to evaluate the influence of dimethyl-sulfoxise (DMSO, 0, 5, 50, 100 and 500 μM) on the motility and acrosome reaction of the frozen-thawed spermatozoa from 3 different bulls (Bull A, B and C). Also we evaluated the developmental capacity of bovine embryos fertilized in a medium containing DMSO at various concentrations. DMSO had negligible effects on the sperm motility and acrosome reaction in all three bulls. However, the development rates from 2 to 16 cells stage on the 3rd day after insemination with 50, 100 and 500 μM DMSO in Bull-B, and up to the blastocyst stage fertilized with 5, 50, 100 and 500 μM in Bull-A were significantly higher ($p<0.05$) than those of control (0 μM DMSO) group from each bull. Furthermore, the rates of blastocysts per cleaved embryos of 5 to 500 μM DMSO group in Bull-A and of 5 to 100 μM DMSO in Bull-C were also significantly higher ($p<0.05$) than those for their 0 μM groups, respectively. These results indicate that DMSO at micromol level used for *in vitro* fertilization might stimulate the development of embryos for some bulls. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 6 : 739-747*)

Key Words : DMSO, *In Vitro* Fertilization, Bovine Oocytes

INTRODUCTION

The capacitation of mammalian spermatozoa is needed before the spermatozoa can penetrate the ovum (Austin, 1951; Chang, 1951). To induce sperm capacitation, chemical reagents such as with caffeine (Niwa et al., 1988; Tsuzuki and Fujihara, 1998), heparin (Parrish et al., 1988; Yang et al., 1993), caffeine and heparin (Niwa and Ohgoda, 1988; Larocca et al., 1997; Tsuzuki et al., 1998), a mixture of heparin, penicillamine, hypotaurine and adrenaline (Long et al., 1994) or penicillamine, hypotaurine and epinephrine with somatic cells (Miller et al., 1994) were used for the treatment of bovine sperm during *in vitro* fertilization. However, the percentage of transferable embryos at the blastocyst stage is still lower *in vitro* than *in vivo* (Holm and Callesen, 1998).

Differences among bulls in the ability of sperm to fertilize bovine oocytes *in vitro* have been reported in many papers (Iritani et al., 1986; Leibfried-Rutledge et al., 1989; Tsuzuki et al., 1991; Larocca et al., 1997). This seems to be one of the important factors which affect embryonic development after *in vitro* fertilization (Leibfried-Rutledge, 1999). With these reports, it is considered that the further improvement of capacitation methods of bovine spermatozoa for *in vitro* fertilization.

Dimethyl-sulfoxide (DMSO) is widely used in the cyropreservation of various kinds of cells including oocytes and embryos (Yu and Quiun, 1994). It can enhance the maturation and development of bovine oocytes fertilized *in vitro* (Tsuzuki et al., 1998). For humans, DMSO can stimulate the motility of immobilized sperm treated with reactive oxygen species (de Lamirande and Gagnon, 1992), which can be occur during the spermatozoal normal metabolic process (Aitken and Clarkson, 1987). Sperm motility is an important factor for fertilizing ability with oocytes in humans (Baker et al., 1993). Also, for bovines, motile spermatozoa separated from semen could stimulate embryonic development (Parrish et al., 1995).

It is suggested that acrosome reaction, which occurs before penetration to the ovum in mammals, is a sequel to the preceding changes covered under the sperm capacitation (Gordon, 1994). Therefore measurement of acrosome reaction would be one of the valuable strategies to estimate sperm capaci- tation (Baker et al., 1993). In addition, *in vitro* fertilization technique is also useful to provide further evidence on sperm capacitation in bovines (Cormier et al., 1997).

The present study was undertaken to evaluate the effect of adding DMSO to the insemination medium on the motility and the acrosome reaction from 3 different bulls. We also assessed the development capacity of bovine embryos fertilized in the medium containing DMSO at various concentrations.

MATERIALS AND METHODS

Embryo development assay

1) Oocyte maturation *in vitro*

Bovine ovaries were obtained from a local

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slaughter house and transferred to our laboratory within 7 h in physiological saline (0.9% NaCl) supplemented with 400 U/ml penicillin and 500 μg/ml streptomycin (Katayama Chemical Co., Osaka, Japan) at 23 to 33℃. Oocytes were aspirated from superficial small follicles (2 to 5 mm in diameter) with a 20 G needle (Terumo, Tokyo, Japan). Oocytes with attached cumulus cells were selected and placed in 2.5 ml of 25 mM Hepes buffered TCM-199 with earl's salts (Gibco BRL Products, Rockville, MD, USA) supplemented with 5% calf serum (CS, heat-inactivated at 56℃ for 30 min, Gibco BRL Products), 0.12 iu/ml follicle stimulating hormone from pig pituitary (F-8001, Sigma, St Louis, Mo, USA), 50 μM DMSO (134-07, Nacalai Tesque, Kyoto, Japan) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, Katayma Chemical Co. and 100 μg/ml dibekacin sulfate, Meiji Seika Co., Tokyo, Japan). Then, oocytes were incubated for 24 to 25 h at 39℃ in 5% CO_2, 95% air and saturated humidity.

2) Sperm penetration and fertilization *in vitro*

Frozen semen samples from 3 different Japanese black bulls (Bull A, B and C) were thawed in a water bath (37 to 39℃) for 1 min. Semen from each bull was diluted step-wise (2, 4, 8, 16 and 32 times) with BO solution (Brackett and Oliphant, 1975) at 2 min intervals in a 37 to 39℃ water bath to remove glycerol. Thereafter, the semen was centrifuged at 750 ×g for 8 min at room temperature and diluted with an equal volume of BO solution supplemented with 15 mg/ml bovine serum albumin (Fraction V, Katayama Chemical Co.) and 0, 10, 100, 200 and 1,000 μM of DMSO or 10 mM caffeine (caffeine sodium benzoate, 50% w/w mixture, C-4144, Sigma) plus 20 μg/ml heparin sodium salt (Acros Organics, NJ, USA). Thus, these solutions gave final concentrations of 0, 5, 50, 100 and 500 μM DMSO or 5 mM caffeine and 10 μg/ml heparin, and the final concentration of spermatozoa was adjusted to 1,800 to $2,000 \times 10^4$ cells/ml. Some aliquots of each semen were used for *in vitro* fertilization, and the remainder of each semen was used for mobility assessment and acrosome reaction test described below. For *in vitro* fertilization, the matured oocytes were transferred to a 100 μl insemination drop (20 to 40 oocytes/drop) and incubated at 39℃ for 6 h. After fertilization, oocytes were moved to 100 μl of 25 mM Hepes buffered TCM-199 supplemented with 1% CS, 50 μM DMSO, 5 mM sodium lactate and 0.4 mM sodium pyruvate. On the 3rd day after insemination, the oocytes were removed from cumulus cell layer with a mouth operated pipette and cultured for an additional 7 days.

Assessment of sperm motility

The semen concentration was adjusted to 600 to 700×10^4 spermatozoa/ml to record their motilities by videomicroscopy (magnification of the black and white monitor was approximately×600) at 39℃ on a thermostatically controlled warm plate. The measurements were made with 100 to 200 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of motility. The percentages of spermatozoa characterized by straight progressive motility was counted.

Acrosome reaction test

We used triple-stain method for analysis of the sperm acrosome reaction (Talbot and Chacon, 1981). The semen (600 to 700 spermatozoa/ml) was placed in an 1.5 ml Eppendorf tube, and was incubated in 0.75% trypan blue (Chroma-Gesellschaft Schmid GmbH & Co., Köngen, Germany) in BO solution for 15 to 20 min at 37 to 39℃ in water bath. The stained semen was mounted on the glass slides, dried in air and fixed with 3% gluteraldehyde (Nacalai Tesque) diluted with 0.1M cacodylate buffer (pH 7.4). Slides were washed with distilled water and soaked for 5 min in 0.4% Bismark Brown (Chroma-Gesellschaft Schunid GmbH & Co., at 40 to 41℃) in HCl solution (pH 1.8) for 5 min. Thereafter, slides were inmersed in 0.4% Rose Bengal in 0.1 M Tris buffer (pH 5.3) for 5 min (Chroma-Gesellshaft Schmid GmbH & Co., at 25 to 27℃). After staining, the slides were transferred to 99.5% ethanol to dehydrate, cleared in xylene and protected with bio-light and coverslip.

More than 400 spermatozaoa were observed at 400 × magnification and the criteria of Talbot and Chacon (1981) for identifying reacted and unreacted live sperm were adopted.

Statistical analysis

Statistical comparison of the cleavage and developmental rates up to the blastocyst stage was performed using chi-square test with Yate's correction (Snedecor and Cochran, 1980). The values of sperm motility and acrosome reaction test were analyzed by Student t-test.

RESULTS

Embryo development assay

The cleavage from 2 to 16 cells and development rates up to the blastrocyst stage in each bull are shown in tables 1 to 3. The cleavage rate of 2 to 16 cells stage of the oocytes cultured with Bull-A semen was found to be significantly higher ($p<0.05$) when supplemented with caffeine and heparin (CH) than in the other groups. Among DMSO treated groups, no significant difference was found in the cleavage rates. However, the percentage of embryos developed to the blastocyst stage in all of DMSO treated groups (5, 50,

Table 1. Embryo development rate of *in vitro* matured oocytes after *in vitro* fertilization with Bull-A semen in the medium containing DMSO

Treatments	N	% of embryos developed to 2 to 16 cells on the 3rd day after insemination	% of embryos developed up to the blastocyst stage	% of cleaved oocytes developed to blastocyst stage
Conc. of DMSO (μM)				
0	78	24.4[a]	3.8[a]	15.8[a]
5	79	29.1[a]	16.5[bc]	56.5[bc]
50	78	32.1[a]	25.6[bc]	80.0[c]
100	85	34.1[a]	25.9[c]	75.9[bc]
500	98	28.6[a]	15.3[bc]	53.6[b]
CH	82	78.0[b]	13.4[ab]	17.2[a]

Note: Data are combined from 3 experiments.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin.
[a,b,c] Within a column, values with different superscripts are significantly different ($p<0.05$).

Table 2. Embryo development rate of *in vitro* matured oocytes after *in vitro* fertilization with Bull-B semen in the medium containing DMSO

Treatments	N	% of embryos developed to 2 to 16 cells on the 3rd day after insemination	% of embryos developed up to the blastocyst stage	% of cleaved oocytes developed to blastocyst stage
Conc. of DMSO (μM)				
0	174	12.6[a]	4.0	31.8
5	169	10.7[a]	3.0	29.4
50	154	28.6[b]	6.5	22.7
100	149	24.2[b]	6.7	27.8
500	157	25.5[b]	5.1	20.0
CH	141	44.7[c]	8.5	19.0

Note: Data are combined from 4 experiments.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin.
[a,b,c] Within a column, values with different superscripts are significantly different ($p<0.05$).

Table 3. Embryo development rate of *in vitro* matured oocytes after *in vitro* fertilization with Bull-C semen in the medium containing DMSO

Treatments	N	% of embryos developed to 2 to 16 cells on the 3rd day after insemination	% of embryos developed up to the blastocyst stage	% of cleaved oocytes developed to blastocyst stage
Conc. of DMSO (μM)				
0	138	9.4[a]	0.7[a]	7.7[a]
5	131	8.4[a]	3.8[a]	45.5[ab]
50	117	6.0[a]	3.4[a]	57.1[ab]
100	137	3.6[a]	4.4[a]	125.0[b]*
500	130	3.8[a]	2.3[a]	60.0[ab]
CH	146	37.7[b]	12.3[b]	32.7[a]

Note: Data are combined from 4 experiments.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin.
[a,b] Within a column, values with different superscripts are significantly different ($p<0.05$).
* 100 percentage was substituted for this value when we analyzed with X^2 test.

100 and 500 μM DMSO) was significantly higher ($p<0.05$) than that in control (0 μM) group. The percentage development in the 100 μM DMSO group was the highest within DMSO treated all groups, and this value showed a significant difference ($p<0.05$) compared to the CH group. In addition, percentages of cleaved oocytes developed to the blastocyst stage in DMSO treated group were significantly higher ($p<0.05$) than the 0 μM and CH groups (table 1).

With Bull-B, the cleavage rate of 2 to 16 cells

Table 4. Motility rate of Bull-A spermatozoa treated with or without DMSO during *in vitro* fertilization

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	37±7.4	20±4.9[b]	22±1.6[b]	10±2.8[a]	4±2.4[a]	1±0.4
5	40±6.8	23±5.2[b]	25±2.2[b]	15±3.9[b]	3±0.8[a]	2±1.1
50	36±4.4	24±3.6[b]	19±3.4[b]	12±6.9[b]	4±1.7[a]	1±1.9
100	39±8.3	26±4.8[b]	24±2.7[b]	10±7.2[a]	5±0.4[a]	4±2.3
500	40±8.0	23±3.2[b]	28±4.7[b]	9±2.2[b]	7±0.3[b]	4±3.6
CH	36±7.4	9±2.9[a]	3±0.5[a]	3±0.3[a]	2±0.5[a]	1±0.6

[a,b] Within a column, values with different superscripts are significantly different ($p<0.05$).
Each figure represents mean±SE motility of frozen semen in 3 trials.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

Table 5. Motility rate of Bull-B spermatozoa treated with or without DMSO during *in vitro* fertilization

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	36±7.5	40± 1.2[b]	38±4.2[b]	42± 3.8[a]	28±4.2[b]	15±3.2[b]
5	48±8.2	47± 6.2[b]	44±8.8[b]	37±17.3[b]	17±5.6[a]	16±2.2[b]
50	38±7.6	44±12.0[b]	45±2.2[b]	39± 6.7[b]	23±0.9[b]	3±2.6[a]
100	40±2.6	45± 7.9[b]	40±8.1[b]	42± 7.9[a]	27±9.3[a]	4±3.0[a]
500	35±6.0	50± 9.2[b]	40±9.9[b]	40± 4.1[b]	26±4.4[b]	11±1.6[b]
CH	40±3.1	7± 2.7[a]	3±4.3[a]	2± 1.1[a]	5±0.4[a]	3±1.4[a]

[a,b] Within a column, values with different superscripts are significantly different ($p<0.05$).
Each figure represents mean±SE motility of frozen semen in 3 trials.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

stage in CH group was significantly higher ($p<0.05$) than the other group. Although the cleavage rate of groups with 50 to 500 μM DMSO were significantly higher ($p<0.05$) than those in 0 and 5 μM DMSO groups, the development rates up to blastocyst were almost the same in all DMSO treated groups, and these values were almost the same as that in CH group. No significant differences were a found between groups in the proportion of blastocyst stage embryos per cleaved oocytes (table 2).

The cleavage rate of embyos from 2 to 16 cells stage and the percentage of development up to the blastocyst stage in CH, inseminated by Bull-C, were significantly higher ($p<0.05$) than those of the other groups. No significant differences were found in percentage of development from 2 to 16 cells stage and up to the blastocyst stage among all DMSO treated groups. However, the percentages of cleaved oocytes developed to blastocyst stage in the 100 μM DMSO group was significantly higher ($p<0.05$) than those of 0 μM and CH groups (table 3).

Sperm motility assessment

The sperm motility in each bull is shown in tables 4 to 6. Our results showed that motility of CH was sharply decreased from 0.5 to 6 h in all bulls while the motility of all of DMSO treated groups or 0 μM group were maintained for 0 to 2 h and gradually declined from 2 to 6 h. In table 4, initial motilities for Bull-A in all treatment groups were almost the same, but after 0.5 h incubation, the motility for the CH group was significantly lower ($p<0.05$) compared with the other groups, and maintained at lower level. Five, 50 and 500 μM DMSO groups for 2 h incubation and 500 μM treated spermatozoa showed higher motility compared with the 0 μM group ($p<0.05$).

The sperm motility of CH was significantly decreased ($p<0.05$) from 0.5 to 6 h compared with 0 to 500 μM DMSO groups in Bull-B (table 5). The motility of Bull-B spermatozoa treated with each concentration of DMSO was not significantly higher than 0 μM DMSO groups for all incubation periods.
In the case of Bull-C, during the initial incubation for 0.5 h, sperm motility in CH significantly decreased ($p<0.05$) as compared with the other groups (table 6). DMSO treated semen maintained motility from 0 to 2 h and gradually declined; motilities of only the 5 and 100 μM DMSO group for 6 h incubation were significantly higher than that of the 0 μM DMSO group.

Acrosome reaction test

The percentages of acrosome unreacted and reacted

Table 6. Motility rate of Bull-C spermatozoa treated with or without DMSO during *in vitro* fertilization

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	24±5.3	23± 7.8[b]	26±12.1[b]	20± 8.8	11±5.5[b]	6±2.2[a]
5	23±8.5	25± 5.5[b]	20± 6.6[b]	19±10.8	12±5.8[b]	11±0.8[b]
50	21±5.8	20±12.9[b]	25±13.3[b]	17± 8.5	9±0.9[b]	7±3.0[a]
100	19±8.2	28± 3.1[b]	20± 8.0[b]	18±11.1	8±4.7[b]	8±2.0[b]
500	24±7.6	35± 7.6[b]	19± 5.0[b]	12± 8.8	8±5.0[a]	3±1.1[a]
CH	19±2.3	2± 0.9[a]	2± 0.4[a]	3± 0.8	1±0.5[a]	2±0.2[a]

[a,b] Within a column, values with different superscripts are significantly different ($p<0.05$).

Each figure represents mean±SE motility of frozen semen in 3 trials.

CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

Table 7. Percentage of live acrosome reacted spermatozoa Bull-A in the medium supplemented with DMSO or caffeine and heparin

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	3±1.6	7±2.2	14±3.0	6±1.0	5±2.1	5±1.8
5	7±0.1	15±4.7	16±3.7	8±2.5	7±1.3	5±0.8
50	8±3.0	12±3.8	12±2.5	8±1.5	6±1.8	3±1.2
100	5±1.7	13±4.3	11±4.1	7±1.9	5±1.5	5±0.9
500	7±0.7	13±5.4	13±1.3	7±1.0	6±1.4	5±1.0
CH	7±1.0	12±1.1	10±1.7	4±1.4	1±1.3	4±0.5

Each figure represents mean±SE motility of frozen semen in 3 trials.

CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

live spermatozoa for 3 bulls are shown in tables 7 to 9. At zero time, the numbers of acrosome reacted live spermatozoa for all bulls were almost the same, and no significant difference was found among treatment groups. From 0.5 to 2 h incubation of semen from all bulls, the percentage of reacted live spermatozoa was sightly increased in all groups except for CH group in Bull-B, and 0, 50 μM DMSO group and CH group in Bull-C. After 2 h incubation, the numbers of acrosome unreacted spermatozoa were decreased in time- dependent manner for all bulls. For Bull B groups treated with 5, 100 and 500 μM DMSO for 0.5 h incubation, the number of acrosome unreacted spermatozoa was significantly higher ($p<0.05$) compared to the 0 μM DMSO group (table 8).

DISCUSSION

In the present study, we failed to obtain the clear stimulus effects on motility and acrosome reaction tests for all bulls. For humans, DMSO may have a positive effect to stimulate the immotile spermatozoa treated by reactive oxygen species (de-Lamirande and Gagnon, 1992). They indicated that relieving the detrimental effect(s) of reactive oxygen species, especially hydroxyl radicals, from spermatozoa by DMSO resulted in recovery of motility of immotil spermatozoa. DMSO is a scavenger for reactive oxygen species, especially for hydroxyl radicals (Yu and Quinn, 1994). Reactive oxygen species including hydorxyl raidicals, which can be produced by the normal metabolism of spermatozoa (Aitken and Clatkson, 1987), and could decrease the human sperm motility (Chen et al., 1997). An excessive reactive oxygen species can inhibit the acrosome reaction in humans (Ichikawa et al., 1999). Furthermore, addition of the reactive oxygen species during *in vitro* fertilization inhibits embryonic development in bovine (Blondin et al., 1997). With these reports, it is postulated that the role of reactive oxygen species, especially hydroxyl radicals might be different for each species, and resulted in no effect in the motility and acrosome reaction of spermatozoa for all bulls in the present study.

In the present study, the sperm motility and the percentage of acrosome reacted spermatozoa were varied among 3 bulls, even with the same incubation period. It is well known that individual variation in sperm motility after ejaculation or freezing and thawing is present in mammals (Baker et al., 1993;

Table 8. Percentage of live acrosome reacted spermatozoa Bull-B in the medium supplemented with DMSO or caffeine and heparin

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	10±6.2	7±1.8^{a}	7±2.5	15±8.6^{ab}	8±1.7	2±0.6
5	15±6.9	17±2.8^{b}	19±6.7	12±4.0^{ab}	4±1.5	7±2.4
50	11±0.9	11±3.7^{ab}	17±5.7	7±3.5^{ab}	6±2.1	5±2.6
100	11±3.1	18±1.8^{b}	13±1.2	14±1.4^{a}	8±3.7	3±0.6
500	15±2.2	24±4.0^{b}	16±3.2	7±3.2^{ab}	6±2.6	4±0.8
CH	15±3.4	10±3.4^{ab}	9±3.9	7±1.1^{b}	7±1.9	4±1.7

^{a,b} Within a column, values with different superscripts are significantly different ($p<0.05$).
Each figure represents mean±SE motility of frozen semen in 3 trials.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

Table 9. Percentage of live acrosome reacted spermatozoa Bull-C in the medium supplemented with DMSO or caffeine and heparin

Treatments	Incubation period (h)					
	0	0.5	1	2	4	6
Conc. of DMSO (μM)						
0	6±1.1	5±1.3	6±2.2	7±3.5	6±2.0^{ab}	4±0.8
5	5±2.7	8±2.0	6±2.4	5±1.8	5±0.9^{a}	5±1.7
50	8±3.5	6±2.2	6±1.1	6±2.0	6±1.3^{ab}	5±2.0
100	3±1.3	10±4.6	6±1.2	9±4.9	5±0.8^{a}	5±2.1
500	8±5.8	15±6.7	6±0.9	5±1.4	5±1.8^{ab}	5±0.5
CH	9±6.6	7±1.5	4±1.3	6±0.9	2±0.4^{b}	5±1.6

^{a,b} Within a column, values with different superscripts are significantly different ($p<0.05$).
Each figure represents mean±SE motility of frozen semen in 3 trials.
CH=Represents the medium with 5 mM caffeine and 10 μg/ml heparin in fertilization medium.

Tsuzuki and Fujihara, 1998). A different frequency of the acrosome reaction was also present among bulls (Miller and Hunter, 1987). Our results were consistent with these reports.

Furthermore, embryonic development, cleavage rate and the percentage of development up to the blastocyst stage, also varied between bulls given the same treatment. It is well known that substantial variation in the development of embryos produced by *in vitro* fertilization with semen from different bulls (Iritani et al., 1986; Leibfried-Rutledge et al., 1989; Tsuzuki et al., 1991; Parrish et al., 1992; Larocca et al., 1997; Tsuzuki and Fujihara, 1998). It is postulated that differences among bulls in ability to produce embryos capable of development could explain the sperm contribution to the regulation for the zygotic cell cycle (Parrish et al., 1992). This variability might be caused by the stage of season, age of animal, ejaculated sperm quality and other factors (Iritani et al., 1986). These factors may have led to the variation in embryonic development among bulls in the present study.

The sperm motility of CH groups in all bulls sharply decreased after 0.5 to 6 h incubation compared to the other groups. Adenylate cyclase is required to produce cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (Morton and Albagli, 1973), and positive correlatios were found among the cAMP contents, the adenylate cyclase activity and sperm motility in humans (Ishikawa et al., 1989). Caffeine can inhibit the adenylate cyclase activity of bovine spermatozoa (Brown and Casillas, 1984). Therefore it is suggested that addition of caffeine in CH groups for all bulls may inhibit the adenylate cyclase activity and resulted in decreased sperm motility by the inhibition for cAMP of spermatozoa.

We failed to observe the clear relationship among embryonic development, the sperm motility and the acrosome reaction for all bulls. These findings suggest that increase of the embryonic development in some DMSO treated groups would not be caused by the activation of sperm motility and the acrosome reaction. It is suggested that the analysis of sperm motility to fertilizing ability *in vitro* with a computer-aided system which can analyze the detailed pattern of motility, is

not a powerful predictor in rat (Moore and Akhondi, 1996). In the present study, we observed the sperm motility by light microscopy. Therefore it is suggested that further study will be needed. On the other hand, the assessment for acrosome reaction is one of the methods to measure the sperm capacitation (Gordon, 1994). Cormier et al. (1997) indicated that bovine spermatozoa would have their capacitation initiated by freezing and thawing. Therefore, since we used frozen-thawed semen from 3 bulls in this study, a large proportion of spermatozoa which we used was almost capacitated just after freezing and thawing, so that they did not increase their acrosome reaction substantially during incubation periods.

The cleavage rate of oocytes fertilized with the sperm treated with 50 to 500 μM DMSO in Bull-B, and the percentage of blastocyst derived from the 100 μM DMSO treated group in Bull-A were significantly higher ($p<0.05$) than those of 0 μM groups. In addition, the percentage of cleaved oocytes developed to the blastocyst stage, fertilized with all concentrations of DMSO in Bull-A and 100 μM DMSO in Bull-C were also significantly higher than those of 0 μM DMSO groups, respectively. These results suggest that DMSO have some positive effects on the sperm-oocyte coculture. Furthermore, in Bull-A, the percentage of blastocysts fertilized by the sperm treated with 100 μM DMSO was significantly higher ($p<0.05$) than that of the CH group. Niwa and Ohgoda (1988) reported that caffeine and heparin mixture was useful for sperm penetration to bovine oocytes *in vitro*. Therefore, DMSO might be a useful reagent to stimulate the bovine sperm penetration to the oocytes as well as heparin and caffeine mixture.

It is not clear why DMSO can stimulate the embryonic development for some bulls. Some possibilities are as follows. Shatten et al. (1989) suggested that microtubule assembly during fertilization might be required for the progression from meiosis to first interphase of mouse oocytes. Concerning this, DMSO might have a profound effect on the polymerization of microtubules in mouse oocytes (Johnson and Pickering, 1987). Different formation of the centrosome, derived from spermatozoa, could explain the differences in subsequent embryonic development among bulls (Navara et al., 1996). The centrosome is known as the cell's microtubule-organizing center, and paternally derived during fertilization in most mammals (Navara et al., 1996). The centrosome, which could be activated soon after fertilization, can organize the microtubules of the sperm aster in bovines (Long et al., 1993). The sperm aster splits to form two asters, which serve as the poles for the first miotic spindle (Horiuchi and Numabe, 1999). With this system, the paternal centrosome may participate in the subsequent development of embryos (Horiuchi and Numabe, 1999). In the present study, spermatozoa of all bulls penetrated to the oocytes within the 6 h incubation period; spermatozoa can enter the oocytes within 3 h from the onset of *in vitro* fertilization in bovines (Park et al., 1989). Therefore, it is postulated that DMSO might induce the sperm aster formation by stimulating the microtubule polymerization for centrosome within a 6 h incubation of sperm-oocyte coculture, and result in inducing the first cell cycle earlier. These changes may increase the subsequent embryonic development after *in vitro* fertilization.

On the other hand, it is widely accepted that the bovine embryo is blocked at the 8 to 16 cell stage *in vitro*, resulting in the lower development of embryos to the blastocyst stage (Plante et al., 1994). At these stages, zygotic genome activation also seems to occur in bovines (Camous et al., 1986). This is likely to be one of the first critical events in early development (Schultz, 1993). It is suggested that bulls may influence events as a result of the mechanism by which the sperm activates the oocyte and triggers synthesis of compounds needed for the cell cycle (Gordon, 1994). It is possible that some factors, which are essential for the regulation of gene activation, are synthesized following fertilization (Aoki, 1997). Yu and Quinn (1994) showed that DMSO may influence to the transcriptional level of leukemia cells culture. These reports lead us to suspect that during *in vitro* fertilization, some compound including protein(s), which be needed for zygotic gene expression, may be synthesized by stimulation of mRNA by addition of DMSO, and result in increased subsequent development, especially after the 8 to 16 cell stage up to the blastocyst stage.

CONCLUSIONS

The addition of DMSO to the *in vitro* fertilization medium can stimulate subsequent embryo development in cattle.

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