The Effects of Dimethyl-Sulfoxide on the *In vitro* Maturation and Fertilization of Bovine Oocytes and the Subsequent Development

Y. Tsuzuki[1], D. H. Duran, Y. Kuroki, F. Uehara, K. Ashizawa and N. Fujihara[2]
Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan

ABSTRACT : The present studies were undertaken to evaluate the effects of a low concentration of dimethyl-sulfoxide (DMSO) on *in vitro* maturation and development of bovine oocytes fertilized *in vitro*.

Significantly more oocytes reached the metaphase stage of the second meiotic division in TCM-199 supplemented with 50 μM DMSO than in the control medium ($p < 0.05$), and the highest rates of development up to the blastocyst stage were obtained when 50 μM DMSO was added to the maturation and culture media ($p < 0.05$).

The avarage of cell numbers of the blastocysts, expanded and hatched blastocysts cultured with 50 μM DMSO were 81.7, 125.7 and 129.9 cells, respectively. The proportion of blastocysts with normal chromosome numbers was 90.5%.

These results suggest that the addition of 50 μM DMSO is beneficial for the maturation of bovine oocytes and production of the blastocysts with high quality.
(Key Words: DMSO, IVM, IVC, Bovine Oocytes, Embryos)

INTRODUCTION

Bovine oocytes collected from the ovary can be cultured *in vitro* to the blastocyst stage (Goto et al., 1988; Eyestone and First, 1989; Del Campo et al., 1993; Reed et al., 1996). However, the developmental capacity is still lower than that of *in vivo* developed embryos. The ideal culture condition for oocytes and embryos has not been found (Jonson and Nasr-Esfahani, 1994) although some attempts have been made to apply chemicals for culture system to improve development of embryos.

DMSO is widely used for cryopreservation of mammalian eggs, but high concentrations of DMSO in culture media are detrimental to maturation of bovine oocytes and arrest them at the metaphase stage of the first meiotic division (Chian and Niwa, 1994). However, it has been reported that DMSO at lower concentrations may have some positive effects on the cell differentiation in culture condition of mouse epidermal cells (Millar et al., 1981) and rat hepatocytes (Kojima et al., 1995).

In the present studies, we examined the effects of a low concentration of DMSO for the maturation and development of bovine eggs.

[1] Address reprint requests to Y. Tsuzuki.
[2] Laboratory of Animal Reproduction, Faculty of Agriculture, Kyushu University 46-06, Fukuoka 812-8581, Japan
Received November 6, 1997; Accepted January 26, 1998

MATERIALS AND METHODS

Oocyte maturation

Bovine ovaries of Japanese Black and Holstein cattle were obtained from a local slaughterhouse and brought to the laboratory in saline (0.9%, NaCl) maintained at 20-33℃ within 1-7 hours. The oocytes were aspirated from superficial small follicles (2-5 mm in diameter) with a 20-G needle (Terumo, Tokyo, Japan). The oocytes with attached cumulus cells were transferred to the maturation medium consisting of 25 mM Hepes buffered TCM-199 with earle salt (Gibco BRL Products, Gaithersburg, MD., U. S. A.), 0.12 iu/ml follicle stimulating hormone from pig pituitary (Sigma, St Louis, Mo. U.S.A.), antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin, Katayama Chemical Co., Osaka, Japan and 100 μg/ml dibekacin sulfate, Meiji Seika Co., Tokyo, Japan) 5% (V/V) calf serum (CS, Gibco BRL Products) and various concentrations of DMSO (0, 5, 50 and 100 μM, Nacalai Tequs, Kyoto, Japan). The oocytes were incubated for 24-25 hr in the atomosphere of 5% CO_2 in air at 39℃ with 100% humidity. After culture, nuclear maturation was assessed in some oocytes. The oocytes were placed in 0.2% hyaluronidase (Sigma) in PBS and denuded of cumulus cells using a mouth operated glass pipette, and then fixed in methanolacetic acid (3 : 1) and stained with 1% (W/V) neutral acetic orcein (Merk, Darmstadt, Germany).

Semen preparation and *in vitro* fertilization

Frozen semen from a Japanese Black Bull was thawed in a water bath 37-39℃ for one min. The semen was diluted 2, 4, 8, 16 and 32 times with BO solution (Brackett and Oliphant, 1975) in step-wise manner with 2 min interval. Then, the semen was diluted with an equal volume of BO solution supplemented with 10 mM caffeine sodium benzoate (50% w/w, Sigma), 20 μg/ml heparin (Acros Organics, NJ, U.S.A) and 15 mg/ml bovine serum albumin (Fraction V, Katayama Chemical Co.). The matured oocytes were introduced into the 100 μl semen drops (20-40 oocytes per drop) and incubated for 6 hr under light paraffin oil (Nacalai Tequs Inc.). The motility of spermatozoa at the onset of incubation ranged from 35 to 40%.

Embryo culture

After *in vitro* fertilization, the embryos were transferred to TCM-199 supplemented with 1% CS, 5 mM sodium lactate, 0.4 mM sodium pyruvate and DMSO. The concentration of DMSO in the *in vitro* culture (IVC) medium after insemination was 0, 5, 50 or 100 μM. The combination of DMSO concentration in the maturation (IVM) and culture (IVC) media was follows: IVM vs IVC (μM) = 0:0, 5:5, 5:0, 50:50, 50:0, 100:100 and 100:0. On the 3rd day after insemination, the embryos were removed from the cumulus cell layer and incubated for a further 7 days with a fresh medium.

Analysis of cell and chromosome number in the blastocysts

For the analysis of cell numbers, blastocysts at 6 to 10 days after insemination were transferred to 0.9% sodium citrate solution for 60 min at room temperature and fixed with acetic-ethanol solution (1:3). After fixation, embryos were mounted on glass slides and stained with 4% Giemsa (Merk) for 2 min at room temperature and observed by microscope. For examination of chromosomes, the blastocysts were incubated in drops containing 0.08 μg/ml vinblastin (Sigma) and phodophylotoxin (Sigma) for 8-12 hr. After incubation, the blastocysts were transferred to 1% sodium citrate for 30 min at room temperature and fixed with methanol-acetic acid. Thereafter, the oocytes were mounted on the glass slide and stained with Giemsa as mentioned above.

Statistical analysis

The results were analyzed by chi-square test (Snede-cor and Cochran, 1980).

RESULTS

Oocyte maturation and development *in vitro*

The maturation rates up to metaphase of the second meiotic division in each group are shown in table 1. The maturation rate was higher in DMSO treated groups than in the untreated control group. The highest rate was obtained in 50 μM group. Abnormal chromosomes were significantly more frequent ($p < 0.05$) in the control group than in 50 μM DMSO group.

Table 1. Nuclear maturation of bovine oocytes incubated in the medium supplemented with various concentraion of DMSO

Concentration of DMSO (μM)	N	Percent of oocytes in metaphase of the second meiotic division %
0	101	67.3[a]
5	77	80.5[b]
50	99	84.8[b]
100	92	79.3[ab]

[a,b] Different superscripts denote significant difference ($p < 0.05$).

The rates of development in each treatment group are shown in tables 2. The cleavage rates up to the 2 to 16 cell stage by the 3rd day after insemination significantly higher ($p < 0.05$) in all DMSO treated groups than in the control group. The rates of development up to the blastocyst stage by the 10th day after insemination was significantly higher ($p < 0.05$) in 50 μM DMSO group than in the control.

Table 2. Cleavage and development rates of bovine eggs matured and cultured with various concentrations of DMSO

Conc. of DMSO (μM) in IVM:IVC	N	% of embryos developed to 2 to 16 cells on the 3rd day after insemination	% of embryos developed up to the blastocyst stage by the 10th day after insemination
0 : 0	147	42.2[a]	5.4[a]
5 : 5	140	72.9[c]	14.3[b]
5 : 0	120	65.8[bc]	13.3[b]
50 : 50	142	63.4[bc]	23.9[c]
50 : 0	135	63.0[bc]	10.4[ab]
100 : 100	108	58.3[b]	10.2[ab]
100 : 0	110	59.1[b]	10.0[ab]

IVM, *In vitro* maturation; IVC, *In vitro* culture after insemination.
[a,b,c]; Percentage with different superscripts are significantly ($p < 0.05$) different within each column.

Cell numbers

The cell numbers of blastocysts obtained in the 50 μM DMSO group are shown in table 3. The average cell numbers of the embryos were 81.7, 125.7 and 129.9 in the early blastocyst, expanded and hatched blastocysts, respectively.

Table 3. Number of blastomeres of bovine blastocyst developed in the medium supplemented with 50 μM DMSO

Cell stage	N	Average numbers of blastomeres
Unexpanded blastocyst	27	81.7
Expanded blastocyst	21	125.7
Hatched blastocyst	16	129.9

Chromosome numbers

Table 4 shows the results of chromosome analysis for the blastocysts developed in the IVM and IVC media containing 50 μM DMSO. Over ninety percent of embryos had normal chromosome numbers (60).

Table 4. Chromosome numbers of bovine blastocyst developed in the IVM and IVC media with 50 μM DMSO

Cell stage	N	Abnormal %		Normal %
		Hyperploidy	Hypoploidy	
Unexpanded blastocyst	11	0.0	0.0	100.0
Expanded blastocyst	11	9.1	0.0	90.9
Hatched blastocyst	20	10.0	5.0	85.0
Total	42	7.1	2.4	90.5

DISCUSSION

The maturation and development rate of bovine eggs matured and cultured in the media supplemented with 50 μM DMSO was significantly higher than that of the control eggs, suggesting that a low concentration of DMSO can accelerate the nuclear maturation and embryonic development of bovine eggs. Although its intracellular molecular mechanisms are unknown, several possibilities as described below are suggested.

In mouse oocytes, microtubules and microfilaments are required for the process of spindle formation (Maro, 1986) and pronuclear migration (Schatten et al., 1986). Furthermore, DMSO can cause the polymerization of microtubules (Johnson and Pickering, 1987) and microfilaments (Vincent et al., 1990). It is postulated that small amount of DMSO (μM level) might have stimulated the polymerization of the microfilaments and microtubules in the cultured bovine eggs to improve their development.

Free radicals including hydroxy radicals, arising through cell respiration inhibit the development of mouse oocytes (Johnson and Nasr-Esfahani, 1994). Hydroxy radicals are detrimental to DNA and cause cell death (Meneghini and Martins, 1993). DMSO is a scavenger of hydroxy radicals (Yu and Quinn, 1994) and may inhibit harmful effect of hydroxy radicals to accelerate maturation and development of mammalian eggs.

The findings in the present study are similar to those in previous reports in respect of the cell numbers and percentage of blastocyst with normal chromosomes (Iwasaki and Nakahara, 1990; Goto et al., 1992).

In conclusion, small amount of DMSO (μM level) can stimulate the maturation and development of bovine eggs *in vitro* produce the embryos with high quality.

ACKNOWLEDGEMENTS

We express grateful thanks to the Miyazaki Prefectural Meat Inspector Center of Miyakonojou-division and Miyazaki Livestock Breeding Center for providing us with the ovaries and frozen semen.

REFERENCES

Brackett, B. G. and O. Oliphant. 1975. Capacitation of rabbit spermatozoa. Biol. Reprod. 32:101-107.

Chian, R. C. and K. Niwa. 1994. Completion of first meiotic division by sperm penetration *in vitro* of bovine oocytes inhibited at metaphase-I with dimethylsulfoxide. Theriogenology 42:55-64.

Del Campo, M. R., M. X. Donoso, A. T. Palasz, A. Garcia and R. J. Mapletoft. 1993. The effect of days in co-culture on survival of deep frozen bovine IVF blastocyst. Theriogenology 39:208 (abstr).

Eyestone, W. H. and N. L. First. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil. 85:715-720.

Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa. 1988. Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in vitro* matured follicular oocytes. J. Reprod. Fertil. 83:753-758.

Goto, K., N. Iwai, K. Ichikawa, A. Ishihara, Y. Takuma, M.

Motoyoshi, M. Tokumaru and Y. Nakanishi. 1992. *In vitro* culture of early bovine embryos produced by *in vitro* technique. J. Reprod. Dev. 38:j165-j171 (in Japanese).

Iwasaki, S. and T. Nakahara. 1990. Cell number and incidence of chromosomal anomalies in bovine blastocysts fertilized *in vitro* followed by culture *in vitro* or *in vitro* in rabbit oviducts. Theriogenology 33:669-675.

Johnson, M. H. and M. H. Nasr-Esfahani. 1994. Radical solutions and cultural problems: Could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryo *in vitro*? Bio Essays 16:31-38.

Johnson, M. H. and S. J. Pickering. 1987. The effect of dimethylsulphoxide on the microtubular system of the mouse oocyte. Development 100:313-324.

Kojima, T., T. Mitaka, D. L. Paul, M. Mori and Y. Mochizuki. 1995. Reappearance and long-term maintenance of connexin 32 in proliferated adult rat hepatocytes: use of serum-free L-15 medium supplemented with EGF and DMSO. J. Cell Sci. 108:1347-1357.

Maro, B., M. H. Johnson, M. Webb and G. Flach. 1986. Machanism of polar body function in the mouse oocytes: an interaction between the chromosomes, the cytoskeleton and the plasma membrane. J. Embryol. Morph. 92:11-32.

Meneghini, R. and E. L. Martins. 1993. Hydrogen peroxide and DNA damage. In: DNA and free radicals. (ed. Halliwell, B. and O. I. Aruoma), Ellis Horwood, Chichester, England pp. 83-93.

Miller, D. R., D. P. Allison, M. C. Rorvix and S. Slaga. 1991. Inhibited morphological terminal differentiation and enhanced proliferation of cultured mouse epidermal cells at different concentrations of dimethyl sulphoxide. Cell Prolif. 24:191-201.

Reed, W. A., T. K. Shu, T. D. Buch and K. L. White. 1996. Culture of *in vitro* fertilized bovine embryos with bovine oviductal epithelial cells, buffalo rat liver (BRL) cells, or BRL-Cell conditioned medium. Theriogenology. 45:439-449.

Shatten, H., G. Shatten, D. Mazia, R. Balczon and C. Simery. 1986. Behavior of centrosome during fertilization and cell division in mouse oocytes and in sea urchin eggs. Proc. Natl. Acad. Sci. U. S. A. 83:105-109.

Snedecor, G. W. and W. G. Cochran. 1980. Statistical methods. In: Testes of hypotheses. (7th ed. Snedecor, G. W. and W. G. Cochran). The Iowa State University Press, Ames, Iowa, U. S. A. pp. 64-82.

Vincent, C., S. J. Pickering, M. H. Jonson and S. J. Quick. 1990. Dimethyl sulfoxide affects the organization of microfilaments in the mouse oocytes. Mol. Reprod. Dev. 26:227-235.

Yu, Z. and P. J. Quinn. 1994. Dimethyl sulfoxide: A review of its applications in cell biology. Bioscience Reports 14:259-281.