EFFECTS OF PREINCUBATION AND INSEMINATION TIMES OF SPERMATOZOA ON THE DEVELOPMENT OF BOVINE OOCYTES FERTILIZED IN VITRO

Y. Tsuzuki, K. Ino, S. Kimura, N. M. Tanaka, N. Fujihara[1] and O. Koga

Department of Animal Science, College of Agriculture, University of Kyushu, Hakozaki, Fukuoka 812, Japan

Summary

Bovine in vitro fertilization experiment was carried out using ovary-derived follicular oocytes and frozen thawed spermatozoa to determine the optimal preincubation time of spermatozoa and the insemination time for successful in vitro fertilization rate. The possibility of parthenogenetic cell division of unfertilized oocytes during culture without spermatozoa was also examined. There was no significant ($p > 0.05$) difference in percent ratio of embryos developed to blastocyst stage between 0 and 3 h preincubation times of spermatozoa, showing a tendency to have higher percentage for 0 h of preincubation time. The 6 h insemination time seemed to be better for producing higher percentage of ova cleavage compared with those of 1 and 3 h treatments. Approximately 10% of unfertilized oocytes divided into 2 to 4-cell stages, and some of them cleaved to 5 up to 8-cells. The results obtained from this study suggested that 0 h of sperm preincubation time and 6 h of insemination time would be suitable for producing better in vitro fertilization rate of bovine oocytes. It is also likely that unfertilized bovine oocytes probably cleave to some cell stages with irregular divisions of the cells. On the one hand, considerable variation was also found in spermatozoal function among individual bulls.

(**Key Words**: Bovine, Oocytes, Spermatozoa, In vitro Fertilization)

Introduction

It is well accepted that mammalian spermatozoa require capacitational changes which are prerequisite for penetration of spermatozoa into the vitellus of ovum (Austin, 1951; Chang, 1951). Capacitation of spermatozoa is also needed for in vitro fertilization of domestic mammals.

In the case of in vitro fertilization of bovine ova, capacitation has generally been said to be induced by preincubation of spermatozoa in the media containing caffeine, heparin and some other chemicals. Preincubation time of bovine spermatozoa prior to in vitro insemination for inducing capacitation varied dependent upon individual bulls (Brackett et al., 1982; Iritani, 1985; Hanada, 1985; Iritani et al., 1986; Goto et al., 1989), suggesting that diversities of spermatozoa from individual males may affect capacitational changes of spermatozoa.

On the other hand, the time required to fertilize bovine ova in vitro, i. e. insemination time, was also variable, depending upon researchers. Large varieties of the time needed to fully fertilize ova were reported by lots of investigators, ranging from 5 (Saeki et al., 1990) to 22 h (Eystone et al., 1989).

Cell cleavage of unfertilized ova has also been shown in mammals (Kaufman, 1983). Parthenogenetic activation, fragmented or divided cells of unfertilized bovine oocytes has been reported (Hanada, 1985; Xu et al., 1986; Nagai, 1987; King et al., 1988; Fukui et al., 1989). It seems, however, very difficult to distinguish parthenogenetic or fragmented cells from normal cleavage of fertilized oocytes through light microscope. It may, thus, be necessary to examine the possibility of parthenogenetic development of unfertilized bovine oocytes in in vitro fertilization experiment.

In the present study, we have examined the optimal preincubation time of spermatozoa and the insemination time required to obtain better

[1]Address reprint requests to Dr. N. Fujihara, Department of Animal Science, College of Agriculture, University of Kyushu, Hakozaki, Fukuoka 812, Japan.

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in vitro fertilization rate of bovine ova. The cell cleavage of bovine oocytes cultured in the media free from spermatozoa was also studied.

Materials and Methods

We used follicular oocytes obtained from the ovaries which were collected from a local slaughterhouse. The oocytes were aspirated from the ovaries with follicles, 1 to 7 mm in diameter, using a syringe attached with a 20 G needle, which contained a small amount of TCM-199 supplemented with 2% inactivated calf serum (CS, Gibco), 10,000 iu/ml heparin (Katayama Chemical Co., Japan). After collection, the oocytes that were surrounded with cumulus cells were selected and centrifuged at 120 × g for 2 min at room temperature (24 ~ 27℃), and washed twice with TCM-199 added with 2% CS, without heparin. Oocyte maturation culture was carried out in a plastic culture dish (Coster, 35 m/m) containing 2.5 ml of TCM-199 added with 5% CS and antibiotics (penicillin 100 iu/ml and streptomycin 100 μg/ml, Meiji Seika Co., Japan), covered with paraffin oil, and incubated for 24 h at 39℃ under 5% CO_2 in air. After 24 h ova culture, in vitro fertilization technique was applied as follows: in this experiment, frozen semen from three bulls (P-216, PB-125 and PB-115) were obtained from Fukuoka Dairy Co-operative Association. These semen were thawed in a water bath at 37℃ dipping for 1 min and subsequently diluted 2, 4, 8, 16 times with BO solution (Brackett and Oliphant, 1975) stepwise per 2 min to remove glycerol. After the final dilution, semen were centrifuged at 600 × g for 8 min at room temperature (24 ~ 27℃), following by the adjustment of sperm concentration to be 1.8 ~ 1.9 × 10^7 cells/ml with the same medium as mentioned above, which was supplemented with 20μg/ml heparin, 5 mM caffeine (Katayama Chemical Co., Japan) and 15 mg/ml BSA (Katayama Chemical Co., Japan).

To determine the time required to induce sperm capacitation, spermatozoa were incubated for 0 and 3 h before insemination. A drop of 100 μl of sperm suspension was placed in a plastic culture dish. According to the scheduled time, several oocytes (20 to 30/drop) were introduced into the semen drops and cultured for 6 h at 39℃ in a CO_2-incubator. After 6 h of insemination time, the oocytes were transferred to 2.5 ml of TCM-199 containing 1% CS, 25 mM Hepes, and cultured further for embryo development. On the 3 day of culture, the oocytes were gently isolated from the cumulus cells that clung to the surface of the dishes, and media were replaced with fresh TCM-199 supplemented with 15% CS, and cultured for additional 7 days to observe the development of oocytes, exchanging media on the 3 and 7 days of culture. Developmental stages of oocytes were determined under everted microscope on a daily basis.

In order to examine the time of insemination, oocytes were incubated with spemratozoa for 1, 3 and 6 h. Experimetal procedures for this trial were carried out according to the same method as mentioned above, and the final developmental stages of embryos were scrutinized.

In this experiment, cell division of unfertilized oocytes was also studied. Following ovum maturation culture, the oocytes were further incubated for 6 h without spermatozoa in BO solution added with 10 μg/ml heparin, 2.5 mM caffeine and 7.5 mg/ml BSA. After 6 h incubation, the oocytes were moved to freshly prepared TCM-199 with 1% CS to continue further culture. On the 3 day of culture, media were exchanged with fresh TCM-199 added with 15% CS, and cultured for additional 7 days, exchanging media on the 3 and 7 days of culture. Developmental cell stages of oocytes were examined by the same procedures as described before.

All data were tested for significance using a chi square test (Snedecor and Cochran, 1980).

Results

Percentages of oocytes divided into the stages of 2 to 16 cells, which were determined on the 3 day after insemination, were higher for 0 h of preincubation time than that for 3 h of P-216 and PB-125, but PB-115 showed significantly ($p < 0.05$) higher percentage of embryonic development for 3 h preincubation than that for 0 h treatment, but significant ($p < 0.05$) difference was found in 0 h of treatment among individual bulls. As will be discussed in later section, some of the oocytes that divided into 2 to 16 cells might be from parthenogenetic development.

No significant ($p > 0.05$) difference was found in percent ratio of embryos developed to blas-

tocyst stage between 0 and 3 h of preincubation times in three semen samples (table 1).

As shown in table 2, significantly ($p < 0.05$) higher percentage of cleaved ova after 3 days of insemination was obtained in the groups of P-216 and PB-125 when the oocytes were incubated for 6 h in the presence of spermatozoa in comparison with those for 1 and 3 h cultures in the groups of P-216 and PB-125, but in the case of PB-115, 3 h of insemination brought about a successful result for early embryonic development. In clear contrast, percentages of embryos developed to the stage of blastocysts were noticeably ($p < 0.05$) higher for 6 h of insemination time in three semen groups than those for 1 or 3 h incubation (table 2). 1 h of insemination with spermatozoa from P-216 and PB-115 was not enough time to produce blastocysts (table 2). 6 h of insemination with PB-125 lead to an increased number of blastocysts, and even after 1 h of insemination, some of the ova developed to blastocyst stage (table 2).

Parthenogenetic cell division was also observed

TABLE 1. EFFECT OF SPERM PREINCUBATION TIME ON THE DEVELOPMENT OF BOVINE FOLLICULAR OOCYTES FERTILIZED IN VITRO

	% of oocytes developed to 2 to 16 cells*		% of oocytes developed to blastocysts**	
Semen No.	Preincubation time		Preincubation time	
	0 h	3 h	0 h	3 h
P - 216	59.4 ± 7.71^{ac}	49.8 ± 8.16^{ac}	13.7 ± 3.95^{ac}	14.0 ± 4.12^{ac}
PB - 115	36.3 ± 3.99^{bd}	55.1 ± 4.80^{ac}	13.1 ± 4.17^{ac}	9.2 ± 4.14^{ac}
PB - 125	72.2 ± 4.11^{ac}	54.2 ± 7.52^{ac}	22.4 ± 5.75^{ac}	$8.6 + 5.61^{ac}$

Values are means ± SE of five observations.

*,**: Determined on the 3 day (*) and within 10 days (**) after insemination.

[a,b]: Significantly different ($p < 0.05$) within a column.

[c,d]: Significantly different ($p < 0.05$) between 0 and 3 h.

TABLE 2. RELATIONSHIP BETWEEN INSEMINATION TIME AND EMBRYONIC DEVELOPMENT OF BOVINE FOLLICULAR OOCYTES FERTILIZED IN VITRO

	% of oocytes developed to 2 to 16 cells*			% of oocytes developed to blastocysts**		
Semen No.	Insemination time			Insemination time		
	1 h	3 h	6 h	1 h	3 h	6 h
P - 216	21.3 ± 10.55^{bc}	$52.3 + 6.50^{ad}$	59.4 ± 7.71^{ad}	0.0 ± 0.00	1.3 ± 1.34^{ac}	13.7 ± 3.95^{ad}
PB - 115	8.8 ± 7.05^{bc}	51.7 ± 11.56^{ad}	36.3 ± 3.99^{bd}	0.0 ± 0.00	$2.9 + 2.86^{ac}$	13.1 ± 4.17^{ad}
PB - 125	42.3 ± 4.59^{ac}	57.9 ± 9.84^{acd}	72.2 ± 4.11^{ad}	2.5 ± 2.50^{c}	7.8 ± 3.60^{ac}	22.4 ± 5.75^{ad}

Values are means ± SE of five observations.

*,**: Determined on the 3 day (*) and within 10 days (**) postinsemination.

[a,b]: Significantly different ($p < 0.05$) within a column.

[c,d]: Significantly different ($p < 0.05$) among insemination times.

in unfertilized bovine oocytes incubated without spermatozoa (table 3 and figure 1). Approximately 10% of the oocytes cleaved to 2- to 4-cell stages on the 4 day after incubation free from spermatozoa, and some of them developed to the stages of 5- to 8-cells (table 3), but the cell stages developed more than 9-cells were not observed in this experiment.

TABLE 3. PERCENT CLEAVAGE OF UNFERTILIZED BOVINE OOCYTES

Cell stages	Days after incubation			
	3 day	4 day	5 day	6 day
2 to 4 cells	6.3 ± 2.30*	10.3 ± 2.67	5.7 ± 1.62	3.5 ± 1.25
5 to 8 cells	0.4 ± 0.42	0.4 ± 0.42	0.0 ± 0.00	0.0 ± 0.00

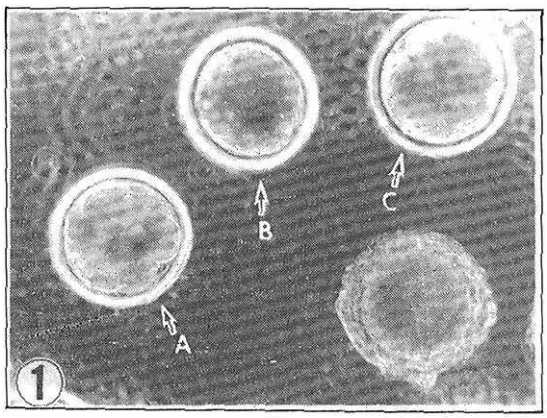

Figure 1. Unfertilized bovine oocytes on the 3 day after incubation without spermatozoa.
A: 8-cells oocyte, B: Undivided oocyte, C: 3-cells oocyte. × 200

Discussion

The results obtained from this study showed that 0 h of preincubation time of frozen-thawed bovine spermatozoa produced considerably higher developmental stages of oocytes than that of 3 h pretreatment, except for one of three semen samples (PB-115). This indicated that in the case of bovine in vitro fertilization, the incubation of frozen-thawed spermatozoa prior to insemination for inducing capacitation is not necessarily needed to fertilize bovine ova, though there may be some difference in spermatozoal function among individual bulls.

It has generally been accepted that even in vitro fertilization, the preincubation time of bovine spermatozoa to bring forth capacitation would be required to complete fertilization of ova, ranging from 1 min to 8 h dependent upon investigators. Iritani et al. (1984) obtained the highest fertility when bovine spermatozoa were incubated for 8 h prior to insemination in comparison with those of 4, 6 and 12 h of preincubation times. Koba et al. (1989) have also reported that 5 h preincubation time of bovine spermatozoa was successful for producing better fertility than 3 h treatment. On the one hand, Takagi et al. (1989) have successfully fertilized bovine ova by inseminating with spermatozoa which were treated with calcium ionophor for only one minute. Niwa et al. (1988) obtained considerably higher penetrability of bovine spermatozoa into oocytes employing spermatozoa which were not preincubated before insemination.

In our present work, the development of bovine oocytes to the stage of blastocysts was not influenced by the preincubation time of spermatozoa, suggesting that prolonged preincubation time of bovine spermatozoa before insemination is probably unnecessary for in vitro fertilization, depending upon individual bulls. This also meant that in this experiment, capacitational changes of frozen-thawed bovine spermatozoa may be brought about during 6 h incubation together with oocytes. The present results indicated that a considerable difference may be observed in fertilizing capacity of spermatozoa between individual males.

It has also been reported that fairly large variations were found in bovine in vitro fertility between the semen from individual bulls (Brackett et al., 1982; Hanada, 1985; Iritani et al., 1985, 1986, 1988; Leibfried-Rutledge et al., 1989). Parrish et al. (1986) has suggested some difference in cattle in vitro fertility between semen samples from the same males. On the other hand, Goto et al. (1989) reported no difference between the individuals of bulls with regard to fertilizing ability of spermatozoa and percent ratio of the development to blastocyst stages by use of frozen-thawed caudal epididymal spermatozoa. There is also suggestion that the oocytes from individual cows may have some different ability of development in vitro (Shioya et al., 1988; Goto et al., 1990), leading us to infer that further study will be needed to clarify these controversies.

In the present work, a higher percentage of embryonic development was shown when the oocytes were incubated with spermatozoa for 6 h, in comparison with those of 1 or 3 h treatments, suggesting the possibility that some capacitational changes of spermatozoa occurred during prolonged insemination times. Niwa et al. (1988) has reported that sperm penetrability into bovine oocytes increased as the insemination time was increased. Insemination times for bovine in vitro fertilization have been said to be around 3 to 6 h which are now widely accepted as a routine manner. In case of our present work, frozen-thawed bovine spermatozoa are considered to be capacitated during 6 h incubation with oocytes, because 1 or 3 h insemination times were not successful to obtain higher percentage of embryonic development. It has been said that no single explanation brings out clearly the various concepts involved in the complex phenomena of capacitation and/or acrosome reaction of mammalian spermatozoa (Sidhu and Guraya, 1989).

With regard to parthenogenetic cell division of unfertilized bovine oocytes, quite a number of cleaved cells was found in the present experiment, and developmental cell stages were 2- up to 8-cells. As already described in the section of the results, some of the oocytes developed to 2 to 16 cells in this study, arose probably from parthenogenetic cell division.

In the case of bovine ova, cytoplasmic fragments with 2-cells have been observed in in vitro culture, suggesting that 2 cell stages of embryonic development would not be better criteria for bovine in vitro fertility examination (Hanada, 1985). Fukui et al. (1989) have reported that about 3% of unfertilized bovine oocytes divided into 2 to 3-cell stages. On the one hand, electrical stimulus has also resulted in parthenogenetic activation of bovine oocytes matured in vitro (Kono et al., 1989). Parthenogenetic development of bovine oocytes observed in this study might be partly due to the culture of oocytes in the media containing caffeine and heparin which were used for inducing sperm capacitation. In particular, heparin has been reported to cause immediate acrosome reactions in vitro (Florman and First, 1988; Parrish et al., 1988), and to alter the nature of plasma membrane of bovine spermatozoa (Miller and Hunter, 1986; Miller and Ax, 1989).

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