Asian-Aust. J. Anim. Sci.
Vol. 22, No. 4 : 500 - 506
April 2009

www.ajas.info

Bovine Oocytes Can Be Penetrated in Modified Tris-buffered Medium

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ABSTRACT : A modified Tris-buffered medium (mTBM) has been widely used as an insemination medium for porcine *in vitro* fertilization (IVF). We examined whether mTBM could be used for bovine IVF. Bovine cumulus-oocyte complexes (COCs) were cultured in a serum-free medium containing 30 ng/ml EGF for 22 h. After culture, COCs were inseminated with spermatozoa for 12 h in mTBM containing 5 mM caffeine and 10 g/ml heparin. The penetration of oocytes increased significantly ($p<0.05$) as the sperm concentration increased from 0.1 (30%) to 1-10 (87-100%)$\times 10^6$ cells/ml. This was significantly different from values obtained at 1 (87%) and 10 (100%)$\times 10^6$ cells/ml. However, when COCs were inseminated with spermatozoa from different bulls, the proportions (62-100%) of oocytes penetrated varied according to the bull. The proportion (18%) of oocytes penetrated was significantly ($p<0.05$) lower in a fertilization medium without caffeine and heparin but increased with the addition of caffeine and/or heparin to the medium, and the proportion (93-96%) of oocytes penetrated increased significantly ($p<0.05$) when the medium was supplemented with heparin and caffeine. In this medium, sperm penetration was first observed at 3 h after insemination. Irrespective of the presence of glucose in the fertilization medium, the proportion (93-97%) of oocytes penetrated and the proportion (83-84%) of embryos at the ≥2-cell stage cultured in a chemically defined medium were not significantly different. However, the proportion of embryos developing to the blastocyst stage was significantly ($p<0.05$) higher in the presence (11%) of glucose in the fertilization medium than in its absence (2%). In conclusion, the present study demonstrated that bovine oocytes penetrated *in vitro* in mTBM can develop to the blastocyst stage and mTBM may be used for the *in vitro* production of bovine embryos. (**Key Words :** Bovine, *In vitro* Fertilization, Tris-buffer, Glucose, *In vitro* Culture)

INTRODUCTION

Since the first genuine success of the fertilization *in vitro* of bovine oocytes matured in culture was reported by Iritani and Niwa (1977), various techniques for producing bovine embryos *in vitro* have rapidly advanced (Tsuzuki et al., 2000; Hansen, 2006; Jang et al., 2008). Although the percentage of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) is high in cattle, the efficiency with which transferable embryos are produced *in vitro* is still low. Further improvements to the system are needed for embryo production *in vitro*.

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Received August 4, 2008; Accepted October 14, 2008

Most studies have used media buffered with bicarbonate (e.g. modified-Blackett and Oliphant medium and/or m-TALP) for IVF of bovine oocytes. Tajik et al. (1994) suggested the addition of bicarbonate to the IVF medium is a very important factor for the penetration of bovine oocytes matured *in vitro*. However, Clarke and Johnson (1987) and Berger and Horton (1988) have developed methods for the capacitation of fresh and frozen-thawed boar spermatozoa, respectively, in a Tris-buffered medium (TBM), without the addition of bicarbonate, which resulted in the successful penetration by boar spermatozoa of zona-free hamster oocytes. Recently, the successful *in vitro* penetration of pig oocytes with subsequent development to the blastocyst stage by using a modified-TBM (mTBM) has been reported (Abeydeera and Day, 1997ab, Abeydeera et al., 1998). However, little attention has been paid to the use of an mTBM as a bovine IVF medium.

In the present study, we i) examined the penetration of bovine oocytes in an mTBM and ii) determined the proper culture conditions for *in vitro* fertilization using an mTBM.

MATERIALS AND METHODS

Media

The medium used for the maturation of oocytes was a tissue culture medium (TCM) 199 (with Earle's salts buffered with 25 mM Hepes) supplemented with 0.1% (v:v) polyvinylalcohol, 30 ng/ml epidermal growth factor, 60 μg/ml sodium penicillin G and 100 μg/ml streptomycin sulfate.

The basic medium used for the treatment of spermatozoa and for the fertilization of oocytes was essentially the same as that used by Abeydeera and Day (1997) for the fertilization of porcine oocytes *in vitro* except for the addition of antibiotics. This medium, designated a modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 60 μg/ml sodium penicillin G and 100 μg/ml streptomycin sulfate.

The basic medium used for the culture of embryos was composed of 89.0 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 0.35 mM NaH_2PO_4, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 1% (v/v) of MEM non-essential amino acid solution (Gibco Laboratories, Grand Island, NY, USA), 2% (v/v) MEM amino acid solution (Gibco), 1 mM L-glutamine, and 1 mg/ml polyvinylalcohol (PVA). This medium, designated BECM-g, was essentially the same as that used by Park et al. (1997).

Maturation of oocytes

Ovaries collected from Japanese black or Holstein heifers or cows at a local abattoir were brought to the laboratory in 0.9% NaCl solution at 30 to 35°C within 2 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2 to 5 mm in diameter with an 18-gauge needle attached to a 10-ml disposable syringe. After washing 4 times with a maturation medium, 10 to 15 COCs were transferred into a 100-L drop of the same medium which had been previously covered with warm paraffin oil in 35×10 mm Falcon polystyrene culture dishes. After culture for 22 h at 39°C under 5% CO_2 in air, COCs were washed 4 times and placed into 50 μl mTBM supplemented with 20 mg/ml BSA (Cat. no. A-7030; Sigma Chemical Co., St. Louis, MO, USA) and 20 μg/ml porcine intestinal mucosal heparin (Sigma) under paraffin oil in culture dishes. The dishes were kept in a CO_2 incubator (5% CO2 in air at 39°C) for about 30 min until spermatozoa were added.

Sperm preparation for *in vitro* fertilization

One or 4 (Experiment 2) 0.5-ml straw(s) of frozen semen obtained from Japanese bulls was(were) thawed in water at 37°C. Spermatozoa were washed twice by centrifugation at 833×g for a period of 5 min each after dilution with an mTBM supplemented with 20 mM caffeine-sodium benzoate (Sigma; 10 mM caffeine) or an unsupplemented mTBM. The final sperm pellet was resuspended in the same medium as that used for washing to give a concentration of 2×10^6 or 0.2-20 (Experiment 1)$\times 10^6$ spermatozoa/ml. Then 50 μl of the sperm suspension was introduced into 50 μl of the medium that contained the COCs for insemination. The mixture was incubated at 39°C in air with 5% CO_2. Since the pH of the basic fertilization medium just after preparation was about 9.8-10.1, the final fertilization medium (an mTBM with caffeine, heparin and BSA) was preincubated for 16-18 h at 39°C in air with 5% CO_2 to stabilize the pH at 7.3-7.4 before use. At the end of the coculture of COCs and spermatozoa, the pH of the fertilization medium was 7.2-7.3. The method for *in vitro* fertilization was essentially the same as that used by Niwa and Ohgoda (1988) except for the replacement of the modified Blackett and Oliphant medium with an mTBM.

Culture of *in vitro* penetrated oocytes

At 12 h post insemination, oocytes were freed from the cumulus cells by vortexing for 2 min and washing 4 times with the culture medium. Ten to 15 denuded oocytes were then transferred to 100 μl of the same medium and the dishes were held in air with 5% CO_2 at 39°C. At 120 h post insemination, the embryos were transferred to a newly prepared medium supplemented with 2.78 mM glucose. Embryos were examined for the developmental stages at each intervals of 48-h post insemination under a dissecting microscope.

Experimental design

In Experiment 1, to examine if bovine COCs were penetrated by spermatozoa in a medium without the addition of a bicarbonate buffer and to determine the proper concentration of spermatozoa, oocytes were inseminated with a final concentration of 0.1 to 10×10^6 cells/ml for 12 h using spermatozoa from a bull (D64). After culture, the oocytes were freed from the cumulus cells by vortexing for 2 min, mounted, fixed in 25% (v:v) acetic alcohol for 48 to 72 h at room temperature, and stained with 1% (w:v) orcein in 45% (v:v) acetic acid. Sperm penetration into oocytes was examined under a phase-contrast microscope at a magnification of ×400. Oocytes were considered as penetrated when they had a decondensed sperm nucleus(ei) or pronucleus(ei) with the corresponding sperm tail(s) in the cytoplasm.

In Experiment 2, to compare the ability of spermatozoa from different bulls to penetrate, COCs were inseminated with spermatozoa from 4 different bulls for 12 h. From this

Table 1. Effect of sperm concentration on sperm penetration of bovine oocytes in modified tris-buffered medium (mTBM)

Sperm concentration ($\times 10^6$)	No. of oocytes inseminated	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total (%)	with enlarged sperm head (%)	with male and female pronuclei (%)	
0.1	52	29.9[a]	0[a]	100[a]	0[a]
1	46	86.9[b]	7.3[ab]	92.7[ab]	2.3[a]
5	54	92.3[bc]	0[a]	100[a]	39.9[b]
10	51	100[c]	5.6[b]	94.4[b]	46.5[b]

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

experiment, the concentration of spermatozoa was fixed at 1×10^6 spermatozoa/ml for all experiments.

In Experiment 3, to determine the proper concentration for caffeine and heparin, COCs were inseminated in media supplemented with 5 mM caffeine and/or 0 to 20 μg/ml heparin for 12 h under the same experimental conditions as in Experiment 1.

In Experiment 4, to estimate the time necessary for inducing sperm capacitation, COCs were inseminated under the same experimental conditions as in Experiment 1. Oocytes were examined 2 to 20 h post-insemination, as in Experiment 1, for evidence of sperm penetration and pronuclear formation. Experiments were repeated 2 times using spermatozoa obtained from D64.

In Experiment 5, to examine the effect of the presence of glucose in the fertilization medium on penetration, COCs were inseminated in the presence or absence of glucose for 12 h.

In Experiment 6, to examine the effect of the presence of glucose in the fertilization medium on the development of penetrated oocytes in a chemically-defined medium, oocytes inseminated for 12 h were cultured in BECM-g for 192 h.

Statistical analysis

Statistical analysis of the data obtained from four replicates, except for Experiment 4, was carried out using Analysis of Variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA) program. When ANOVA revealed a significant treatment effect, the treatments were compared with Fisher's protected least significant difference test. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The present study demonstrates, for the first time, that i) bovine oocytes are penetrated in a Tris-buffered medium without the addition of bicarbonate in an IVF medium, ii) oocytes penetrated in this medium are developed to the blastocyst stage and iii) a modified Tris-buffer medium can be used for the *in vitro* production of bovine embryos.

Experiment 1-sperm penetration in a medium without the addition of bicarbonate

COCs were inseminated in a Tris-buffered medium without a bicarbonate buffer (Table 1). The penetration of the oocytes increased significantly ($p<0.05$) as the sperm concentration increased from 0.1 (30%) to 1-10 (87-100%) $\times10^6$ cells/ml. This was significantly different from values obtained at 1 and 10×10^6 cells/ml. However, the proportion of oocytes penetrated and containing male and female pronuclei exceeded 93% in all treatments and the proportion of polyspermic oocytes was significantly higher as the sperm concentration increased from 0.1-1 to $5\text{-}10\times10^6$ cells/ml.

Bicarbonate is commonly used in most fertilization media. This is essential for *in vitro* fertilization (IVF) of mouse oocytes, and in its absence the acrosome reaction cannot occur (Lee and Storey, 1986). Since porcine sperm failed to penetrate in the presence of Hepes, bicarbonate is essential for the *in vitro* penetration of pig oocytes (Suzuki et al., 1994). At a constant pH, the concentration of bicarbonate may be critical for supporting the acrosome reaction in mice (Neill and Olds-Clarke, 1987). Recently, as determined by chlortetracycline analysis, varying concentrations of bicarbonate can significantly stimulate capacitation and/or the spontaneous acrosome reaction of boar spermatozoa (Abeydeera et al., 1997). Moreover, high penetration rates have been observed in a chemically defined protein-free medium by increasing the bicarbonate concentration with small change of pH in bovine (Tajik et al., 1994) and porcine (Wang et al., 1995) oocytes. Therefore it is postulated that bicarbonate has a function(s) in addition to being a buffering molecule (Bhattacharyya and Yanagimachi, 1988). However, in the case of guinea pigs, sperm penetration can occur in bicarbonate-free media supplemented with synthetic organic buffers such as Hepes, Tris or Mops but not as efficiently as in a bicarbonate buffered medium (Bhattacharyya and Yanagimachi, 1988). Clarke and Johnson (1987) and Berger and Horton (1988) have developed methods for capacitation of fresh and frozen-thawed boar spermatozoa, respectively, in a Tris-buffered medium (TBM), without the addition of bicarbonate, which resulted in the successful penetration by boar spermatozoa of zona-free hamster oocytes. Successful

Table 2. Penetration rate of spermatozoa obtained from different bulls in modified tris-buffered medium (mTBM)

No. of bulls	No. of oocytes inseminated	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total (%)	with enlarged sperm head (%)	with male and female pronuclei (%)	
D64	55	94.8[a]	1.8[a]	98.1[a]	11.7[a]
49-22F	53	100[b]	15.1[b]	87.4[b]	39.6[b]
51-15N	56	91.2[a]	9.6[b]	90.4[b]	10.0[a]
54-23F	50	61.9[c]	11.8[b]	87.0[b]	16.9[a]

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

in vitro penetration of pig oocytes with subsequent development to the blastocyst stage by using modified TBM (mTBM) has been reported (Abeydeera and Day, 1997ab; Abeydeera et al., 1998). Therefore the addition of bicarbonate to the IVF medium may not be essential for *in vitro* fertilization and it may be possible to replace it with Tris.

In the present study, the pH of the fertilization media ranged between 7.2 and 7.4 after coculture with COCs and spermatozoa and the proportion of oocytes penetrated was high. The pH of the modified-Blackett and Oliphant medium (mBO) is 7.7-7.9. This means that bovine spermatozoa tolerate a broad range of pH for the fertilization (7.2-7.9).

Experiment 2-penetration by spermatozoa from different bulls

The proportions (62-100%) of oocytes penetrated varied according to the bull (Table 2). Spermatozoa from 3 bulls (D64, 49-22F and 51-15N) had a significantly ($p<0.05$) higher ability to penetrate COCs than sperm from bull 54-23F. The proportions of oocytes penetrated and containing male and female pronuclei (87-98%) also varied according to the bull ($p<0.05$), but this variation was independent of the difference in penetration rate. The proportion of polyspermic oocytes (10-40%) also varied according to the bulls ($p<0.05$).

The proportions of sperm penetration from four different bulls was verified (Table 2). The varying ability of spermatozoa from different bulls to penetrate has already been reported (Tajik et al., 1993; Niwa and Ohgoda, 1988). The present results confirm this in a different medium without the addition of bicarbonate. Since the percentage of motile spermatozoa was not too different (30-50%; unpublished data), the differences did not arise from the sperm motility. They may be from the result of individual differences between the bulls.

Experiment 3-effect of caffeine and heparin

The proportions (18%) of oocytes penetrated were significantly ($p<0.05$) lower in fertilization media without caffeine and heparin (Table 3). However, this value increased with the addition of caffeine and/or heparin to the medium. The proportions (49-64%) of oocytes penetrated increased significantly ($p<0.05$) as heparin was added, but were not significantly different among concentrations of heparin without caffeine. The proportions (93-96%) of oocytes penetrated increased significantly ($p<0.05$) when the medium was supplemented with heparin and caffeine. However, the values were not significantly different among varying concentrations of heparin in the presence of caffeine. The proportions of oocytes penetrated and containing male and female pronuclei (54-98%) also varied among treatments ($p<0.05$), but this variation was independent of the differences in penetration rate. However, the proportions of polyspermic oocytes were not significantly different among the treatments.

The incubation of frozen-thawed spermatozoa in a medium with caffeine and heparin significantly increased the penetration rates of oocytes (Table 3). Caffeine may accelerate the rate of capacitation by precociously increasing the concentration of cyclic adenosine

Table 3. Effect of caffeine and heparin on sperm penetration of bovine oocytes in modified tris-buffered medium (mTBM)

CA con. (mM)	HP con. (μg/ml)	No. of oocytes inseminated	No. of oocytes penetrated			No. of polyspermic oocytes (%)
			Total (%)	with enlarged sperm head (%)	with male and female pronuclei (%)	
0	0	50	18.4[a]	45.8[a]	54.2[a]	8.3
	5	55	49.1[b]	15.9[ab]	84.1[ab]	2.8
	10	59	63.9[b]	16.1[ab]	83.9[ab]	3.1
	20	56	53.6[b]	8.1[a]	91.9[b]	4.6
5	0	51	64.5[b]	19.2[ab]	80.8[ab]	2.8
	5	54	92.5[c]	10.5[b]	89.6[b]	8.4
	10	53	92.7[c]	2.1[b]	97.9[b]	10.1
	20	55	96.3[c]	3.7[b]	96.3[b]	11.9

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

Table 4. The changes in sperm penetration of bovine oocytes through subsequential time in modified tris-buffered medium (mTBM)

Time of examination (hours after insemination)	No. of oocytes inseminated	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total (%)	with enlarged sperm head (%)	with male and female pronuclei (%)	
2	26	0	0	0	0
3	24	24	100	0	0
4	26	38.5	87.5	12.5	0
6	28	57.1	87.5	12.5	6.3
8	27	81.3	81.3	40.9	0
10	27	92.6	92.6	76	16.1
12	29	93.1	93.1	100	11.3
20	26	92.3	92.3	100	16.8

monophosphate in mice (Fraser, 1979). Caffeine is known to enhance and prolong the motility (Garbers et al., 1971; 1973), and to stimulate capacitation and penetration of bull (Niwa and Ohgoda, 1988) and boar spermatozoa (Wang et al., 1991). On the other hand, heparin can induce the acrosome reaction in bovine spermatozoa (Handrow et al., 1982; Parrish et al., 1985). Although the precise mechanism is unknown, heparin is considered to capacitate spermatozoa by binding to them and then causing an uptake of calcium into the spermatozoa (First and Parrish, 1988). Caffeine and heparin promote capacitation to about the same extent, but together, heparin and caffeine have an even more stimulatory effect (Fraser et al., 1985) and promote the penetration rate of bovine oocytes, suggesting a synergistic effect between the two chemicals in stimulating the IVF of bovine oocytes (Niwa and Ohgoda, 1988).

Experiment 4-time for sperm penetration

Sperm penetration was observed at 3 h post insemination (Table 4). The fist formation of male and female pronuclei was observed 4 h after insemination. Most of the oocytes (92-93%) were penetrated 10 h after insemination. The formation of male and female pronuclei was completed 12 h after insemination.

At 3 h post insemination, sperm penetration was first observed in a medium containing caffeine and heparin (Table 4). It took 6 h for bovine spermatozoa treated with heparin to start penetrating oocytes (Xu and Greve, 1988). However, in the presence of caffeine and heparin, the first penetration was observed at 3 h when spermatozoa were cocultured with COCs (Park et al., 1989). This result was similar to our report. It is concluded that capacitation and the acrosome reaction are induced within 3 h in mTBM with caffeine and heparin.

Experiment 5-effect of glucose on sperm penetration

Irrespective of the presence of glucose, the proportions (93-97%) of oocytes penetrated was high (Table 5). The formation of male and female pronuclei and polyspermic oocytes was not significantly different in the presence or absence of glucose either.

Irrespective of the presence of glucose, the proportion of penetration was high in a medium containing caffeine and heparin in the present study (Table 5). However, it has been reported that glucose is an essential component for inducing the acrosome reaction of spermatozoa and for supporting fertilization in mice (Fraser and Quinn, 1981). On the other hand, studies in the guinea pig (Hyne and Edwards, 1985) and goat (Kusunoki et al., 1989) suggested that glucose has an inhibitory effect on sperm capacitation and the fertilization of oocytes. In cattle, glucose may affect the fertilization of oocytes *in vitro* in different ways, depending on the media used. In media containing heparin, glucose inhibited or delayed the ability of heparin to induce sperm capacitation (Handrow et al., 1989; Parrish et al., 1989). Other reports (Niwa and Ohgoda, 1988; Shioya et al., 1988), showed that in a medium with caffeine or caffeine plus heparin, bovine spermatozoa can be capacitated, and penetrate in the presence of glucose. Finally, irrespective of the presence of glucose, a high penetration rate was obtained in a medium containing caffeine and heparin (Lim et al., 1993; Tajik et al., 1998). Our results confirm that the presence of glucose does not affect penetration by bovine spermatozoa in a medium containing caffeine and heparin or in a medium without bicarbonate.

Experiment 6-developmental capacity of penetrated oocytes

Blastocysts were obtained when oocytes penetrated *in vitro* were cultured in a chemically defined medium. The

Table 5. Effect of the presence or absence of glucose on sperm penetration of bovine oocytes in modified tris-buffered medium (mTBM)

Glucose (mM)	No. of oocytes inseminated	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total (%)	with enlarged sperm head (%)	with male and female pronuclei (%)	
10	54	96.7	0	100	9.8
0	58	93.1	2.1	97.9	14.7

Table 6. Effect of the presence or absence of glucose in modified tris-buffered medium (mTBM) on the development of bovine oocytes matured and fertilized *in vitro* in a chemically-defined medium

Glucose (mM)	No. of oocytes cultured	No. (%) of oocytes developed to				
		≥2-cell (48)	≥8-cell (96)	≥Morula (144)	Blastocyst (192)	
					Total	Hatching
10	54	83.7	44.3	40.5	11.2[a]	50.0
0	53	83.1	37.6	30.7	1.9[b]	0

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

proportions (83-84%) of oocytes that reached the 2-cell stage were not different in the presence or absence of glucose in the fertilization medium. The proportion (31-41%) of oocytes that reached the morula stage was not significantly different between two treatments either. However, the proportions of oocytes that reached the blastocyst stage ($p<0.05$) increased significantly in the presence of glucose. Half of the blastocysts obtained in the presence of glucose hatched (Table 6).

The presence of glucose during fertilization appeared not to affect the penetration of oocytes *in vitro* or early cleavage up to 144 h, but to promote further development to the blastocyst stages in the present study (Tables 5 and 6). Although glucose does not affect fertilization, the presence of glucose during fertilization may be needed for normal development to the blastocyst stage. However, it has been reported that the presence of glucose in the fertilization medium inhibited development to the morula and blastocyst stages (Lim et al., 1993). This discrepancy may arise from the different media for fertilization used in the two studies. Further experiments are required to clarify the role of glucose during fertilization for embryo development.

In conclusion, the present study demonstrated that bovine oocytes penetrated *in vitro* in a medium without the addition of bicarbonate can develop to the blastocyst stage. Although it was not investigated whether or not the blastocyst-stage embryos could develop into fetuses, mTBM may be used for the *in vitro* production of bovine embryos.

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