Effect of Tris, Sodium Bicarbonate and Caffeine in Fertilization Medium on *In Vitro* Fertilizability of Boar Spermatozoa Frozen in Straws

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ABSTRACT

The objective of this study was to examine the effect of caffeine and sodium bicarbonate in a fertilization medium on the fertilizability of boar spermatozoa that were frozen in straws. Boar spermatozoa were extended with Beltsville F5 extender and frozen in 0.25-ml straws. *In vitro* matured porcine oocytes were fertilized *in vitro* (IVF) with frozen-thawed boar spermatozoa for 6 h in a modified tris-buffered medium (mTBM) or in its modified medium by substituting the tris with 25 mM sodium bicarbonate (modified bicarbonate-buffered medium; mBBM). Some of inseminated oocytes were fixed and stained for examination of sperm penetration. IVF embryos were cultured in a North Carolina State University-23 medium for embryo development. The percentage of live sperm was 47±4% and morphological abnormality of acrosome was found in 14±3% of spermatozoa. Optimal sperm concentration for IVF was 0.75~1.0×10^6 sperms/ml when mTBM containing 5 mM caffeine was used as the fertilization medium. Sperm penetration was significantly (p<0.05) stimulated by increasing caffeine concentration in the IVF medium. In addition, mBBM significantly (p<0.05) increased sperm penetration (92%) compared to mTBM (65%). More (p<0.05) blastocysts (22% vs. 32%) developed from the oocytes that were fertilized in mBBM containing 1 mM caffeine than from those fertilized in mTBM with 5 mM caffeine. Our results indicate that boar spermatozoa can be frozen successfully in straws with holding their normal fertilizability and that caffeine and sodium bicarbonate stimulates sperm penetration *in vitro*.

(Key words : *In Vitro* fertilization, Frozen spermatozoa, Caffeine, Bicarbonate, Boar)

INTRODUCTION

The efficiency of *in vitro* production (IVP) of pig embryos has been greatly improved by the establishment of a successful *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) system. Currently, it is common to obtain 30~40% blastocysts from the culture of IVF pig embryos (Hao *et al.*, 2008; Kim *et al.*, 2008). Generally, the developmental capacity of pig embryos that were derived from IVM and IVF is lower than that of *in vivo*-derived embryos. There are many factors affecting the efficiency of pig IVP. Of those, polyspermic fertilization is the most common problem in the IVP of normal pig embryos (Yi *et al.*, 2007; Coy *et al.*, 2008). It has been known that pig oocytes are very weak in the physiological mechanism to block polyspermic fertilization compared to oocytes from other domestic species (Coy and Romar, 2002; Hao *et al.*, 2006). Furthermore, a higher rate of polyspermic fertilization is observed in *in vitro*-produced embryos than in embryos matured and fertilized *in vivo* (Hunter, 1991; Wang *et al.*, 1998). Many studies have attempted to overcome this problem by modifying IVM and/or IVF methods but the high frequency of polyspermic fertilization has been remained as a major obstacle in the pig IVP.

Sperm penetration and normal fertilization are influenced by various factors such as duration of sperm-oocyte coincubation, sperm concentration, or composition of IVF medium (Wang *et al.*, 1994; Hong *et al.*, 2004; Suzuki *et al.*, 2005; Wongsrikeao *et al.*, 2005). Various sources of spermatozoa are used for IVF in pigs but the optimal sperm concentration varies upon the batch of semen, the individual semen donor and the type of semen such as fresh or frozen semen (Koo *et al.*, 2005; Pelaez *et al.*, 2006; Coy *et al.*, 2008). Generally, unfrozen liquid semen shows higher rates of motile and live spermatozoa than frozen semen but the viability and fertilizability of spermatozoa are deteriorated as the storage period goes longer (Suzuki *et al.*, 2005). Changes in the sperm motility and viability due to the use of new batch of semen or liquid semen stored for an overly long period will cause inconsistent results in sperm penetration, normal fertilization, and subsequent embryo development. In contrast, although freezing and thawing process impairs acrosomal integrity and sperm viability, frozen spermatozoa can sustain their viability and fertilizability in a consistent le-

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vel even after a relatively long period of storage as long as the semen is stored under appropriate temperature. When different batch or type of semen is used for IVF, optimal sperm concentration to maximize sperm penetration but to minimize polyspermic fertilization should be re-determined before use. Therefore, use of frozen spermatozoa from a single individual would be an efficient way to minimize variations in IVF due to variations in sperm motility and viability. Boar spermatozoa can be frozen in the form of pellets or in straws. Frozen semen pellet is directly exposed to liquid nitrogen, which may act as a probable cause for bacterial contaminations in the IVF (Piasecka-Serafin, 1972). On the other hand, semen frozen in straws is not exposed directly to liquid nitrogen and therefore there are relatively few chances of contamination from the liquid nitrogen container. In addition, frozen semen straws are convenient to handle and therefore can be thawed easily by plunging them directly into warm water compared to frozen semen pellets. The objective of this study was to establish an efficient IVP system in pigs by optimizing the IVF method using boar spermatozoa that were frozen in straws. Boar spermatozoa frozen in straws were determined for the optimal sperm concentration for IVF by examining the effects of caffeine, tris and sodium bicarbonate in a fertilization medium on sperm penetration, pronuclear formation and subsequent embryo development *in vitro*.

MATERIALS AND METHODS

Semen Collection and Freezing

Sperm-rich fraction of ejaculated semen was collected by a gloved-hand method from an adult boar that was proven his fecundity. Semen was frozen by the method as previously described (Yoon *et al.*, 2000). Briefly, semen was cooled to 17℃ over 2 h and kept for 6 h at 17℃. Then, semen was diluted three times with Hülsenberger VIII diluent (Richter *et al.*, 1975) at 17℃ and centrifuged for 15 min at 300×g. The supernatant was discarded and the pellet of spermatozoa was resuspended in Beltsvill F5 (BF-5) extender (Pursel and Johnson, 1975) to give a concentration of 1×10^9 sperm/ml. After cooling to 5℃ over 1 h, sperm suspension was diluted with the same volume of BF-5 solution containing 2% (v:v) glycerol. The sperm suspension was aspirated into 0.25-ml plastic straws, exposed to liquid nitrogen vapor for 30 sec and stored in a liquid nitrogen container until use. Some semen straws were thawed and examined for viability and acrosomal integrity of frozen spermatozoa as previously described (Zou and Yang, 2000; Pelasz *et al.*, 2006). The rate of live spermatozoa was 47.0% and acrosomal deformity was found in 14% of frozen-thawed spermatozoa in this study.

Oocyte Collection and IVM

Ovaries were obtained from prepubertal gilts at a local slaughterhouse. Follicular contents were aspirated from follicles 3~8 mm in diameter, pooled into 15-ml conical tubes, and allowed to settle as sediment. The sediment was placed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA) (Bavister *et al.*, 1983). Only cumulus-oocyte complexes (COCs) with multi-layers of compact cumulus cells were selected. The base medium for IVM was tissue culture medium (TCM)-199 (Invitrogen, Grand Island, NY, USA), supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, and 10% (v/v) pig follicular fluid. After washing in IVM medium, a group of 50~80 COCs was placed into each well of a four-well multi-dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 10 IU/ ml eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/ml hCG (Intervet International BV). COCs were cultured at 39℃ in a humidified atmosphere of 5% CO_2. After 22 h in the maturation culture, the COCs were washed three times in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 22 h.

In Vitro Fertilization

Frozen-thawed boar semen was used for IVF in this study as previously described (Yoon *et al.*, 2000). The medium for sperm washing was calcium- and magnesium-free phosphate-buffered saline (PBS). Modified Tris-buffered medium (mTBM) or its modified medium by substituting the tris with 25 mM sodium bicarbonate (this medium was designated as modified bicarbonate-buffered medium; mBBM) was used as IVF medium (Table 1). A frozen semen straw was thawed at 39℃ for 1 min in a water bath and was then diluted in 10 ml of PBS. Sperm suspension was washed twice by centrifugation at 350×g for 3 min each, and the final sperm pellet was resuspended in mTBM or mBBM containing 1~5 mM caffeine to yield a concentration of 1.0×10^7 sperm/ml.

After IVM, expanded cumulus cells of COCs were removed by gentle repeated pipetting in a maturation medium containing 0.1% (w/v) hyaluronidase (Sigma-Aldrich). Denuded oocytes were washed three times in fertilization medium, and 20~25 oocytes in 5 μl of medium were introduced into a 40-μl drop of fertilization medium covered with warm mineral oil. Then, 5 μl of sperm suspension was added to each fertilization drop to give final sperm concentrations of 0.5×10^6, 0.75×10^6, 1.0×10^6 or 1.5×10^6 sperm/ml. Gametes were coincubated for 6 h in 5% CO_2 in a humidified

Table 1. Composition of modified tris-(mTBM) or bicarbonate-buffered (mBBM) medium used for *in vitro* fertilization

Components	mTBM (mM)	mBBM (mM)
NaCl	113.1	113.1
KCl	3.0	3.0
$CaCl_2$	7.5	7.5
Tris	20.0	-
$NaHCO_3$	-	25.0
Sodium pyruvate	5.0	5.0
Glucose	11.0	11.0
Caffeine	1~5	1~5
Bovine serum albumin[1]	0.8 % (w/v)	0.8 % (w/v)

[1] Cat. No.: A6003 (Sigma-Aldrich, St. Louis, MO, USA).

air atmosphere.

In Vitro Culture of Embryos

The medium for embryo development was North Carolina State University-23 (NCUS-23) medium (Petters and Wells, 1993), which was modified by replacing 5.5 mM glucose with 5.0 mM sodium lactate and 0.5 mM sodium pyruvate (Park *et al.*, 2005). After IVF, oocytes were washed three times in fertilization drops for removal of loosely attached spermatozoa and once in a culture medium containing 0.4% BSA (A-6003, fraction V; Sigma-Aldrich). Putative zygotes were transferred to a 30-μl droplet (10~15 zygotes/droplet) of culture medium covered with warm mineral oil and cultured for 150 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. After 48 and 156 h of culture, embryo cleavage and BL formation, respectively, were assessed under a stereomicroscope. Total blastocyst cell number was assessed using Hoechst 33342 staining under an epifluorescence microscope.

Examination of Sperm Penetration, Pronuclear Formation and Embryo Development

Sperm penetration and male pronuclear (MPN) formation of oocytes were examined by the standard protocol of our laboratory (Hong *et al.*, 2004). After 14~15 h of IVF, oocytes were mounted on a glass slide and fixed for 10 min in 25% (v/v) acetic acid in ethanol at 33℃ on a warm plate for the fast removal of lipids. They were then stained with 1% (w/v) orcein in 45% (v/v) acetic acid solution, and examined under a phase-contrast microscope (TS100; Nikon, Tokyo, Japan) at a 400× magnification. Oocytes were considered as penetrated when they had one or more swollen sperm heads or male pronuclei with their corresponding sperm tails. In this evaluation, oocytes that did not reach the metaphase-II stage were omitted from the analysis.

Experimental Design and Statistical Analysis

In Experiment 1, IVM oocytes were inseminated at concentrations of $0.5 \sim 1.5 \times 10^6$ spermatozoa/ml to determine the optimal concentration for IVF of the frozen spermatozoa used in this study. mTBM containing 5 mM caffeine was used as fertilization medium. In Experiment 2, effect of the addition of tris or sodium bicarbonate and caffeine (1 and 5 mM) to the fertilization medium was examined by a 2×2 factorial arrangement. Based on the results from Experiment 2, IVM oocytes were inseminated in mTBM or mBBM supplemented respectively with 5 mM and 1 mM caffeine in Experiment 3. Oocytes were inseminated at the concentration of 7.5×10^5 sperm/ml in Experiments 2 and 3.

All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data were analyzed using a general linear model, followed by the least significant difference mean separation procedure when treatments differed at $p<0.05$. Percentage data were arcsine transformed prior to analysis to maintain homogeneity of variances.

RESULTS

Experiment 1: Effect of Various Sperm Concentration on IVF

To determine the optimal sperm concentration for IVF, IVM oocytes were inseminated with frozen-thawed boar spermatozoa in mTBM containing 5 mM caffeine. Sperm penetration was significantly increased ($p<0.05$) by increasing the sperm concentration. Oocytes inseminated at 0.75×10^6 sperm/ml and 1.0×10^6 sperm/ml showed 52% and 69% of sperm penetration, respectively, while the rate of monospermic fertilization was 64% and 57%, respectively (Table 2). Based on the rates of sperm penetration and monospermic fertilization, $0.75 \sim 1.0 \times 10^6$ sperm/ml was found to be an optimal concentration for IVF in this study. The rates of MPN formation were not different among four sperm concentrations tested.

Experiment 2: Effect of Tris, Sodium Bicarbonate and Caffeine on IVF

Results on the sperm penetration and MPN formation of oocytes that were inseminated in mTBM and mBBM are shown in Table 3. When IVM oocytes were inseminated in mTBM, addition of 5 mM caffeine showed increased ($p<0.05$) sperm penetration (65%) than addition of 1 mM caffeine (28%). There was no diffe-

Table 2. Effect of sperm concentration on sperm penetration and male pronuclear formation of *in vitro* matured pig oocytes after *in vitro* fertilization

Sperm ($\times 10^6$/ml)	No. of oocytes examined[1]	No. (%) oocytes			Sperm /oocyte
		Penetrated	With monospermic fertilization[2]	MPN formed[2]	
0.5	88	16 (18)[a]	14 (88)[a]	14 (88)	1.14
0.75	91	47 (52)[b]	30 (64)[b]	40 (85)	1.55
1.0	87	60 (69)[c]	34 (57)[bc]	54 (90)	1.70
1.5	90	77 (86)[d]	17 (22)[c]	74 (96)	2.01

[1] Four replicates. Oocytes were inseminated at the sperm concentrations of $0.5 \sim 1.5 \times 10^6$ sperm/ml in a modified tris-buffered medium containing 5 mM caffeine.
[2] Percentage of the number of oocytes penetrated.
[a~d] Values with different superscripts within each column are significantly different ($p<0.05$).

Table 3. Effect of fertilization medium and caffeine concentration on sperm penetration and male pronuclear formation of pig oocytes after *in vitro* fertilization

IVF medium	Caffeine (mM)	No. of oocytes examined[1]	No. (%) oocytes			Sperm /oocyte
			Penetrated	With monospermic fertilization[2]	MPN formed[2]	
mTBM	1	86	24 (28)[a]	16 (67)[a]	21 (88)	1.38
	5	82	53 (65)[b]	29 (55)[ab]	44 (83)	1.58
mBBM	1	83	59 (71)[b]	19 (32)[bc]	52 (88)	1.93
	5	91	84 (92)[c]	14 (17)[c]	77 (92)	4.33

[1] Three replicates. Oocytes were inseminated at the sperm concentration of 7.5×10^5 sperm/ml.
[2] Percentage of the number of oocytes penetrated.
[a~c] Values with different superscripts within each column are significantly different ($p<0.05$).

Table 4. Sperm penetration and male pronuclear formation in pig oocytes fertilized *in vitro* with frozen-thawed boar spermatozoa in two fertilization media

IVF medium	Caffeine (mM)	No. of oocytes examined[1]	No. (%) oocytes			Sperm /oocyte
			Penetrated	With monospermic fertilization[2]	MPN formed[2]	
mTBM	5	93	60 (65)	36 (60)	45 (75)	1.73
mBBM	1	92	63 (68)	30 (48)	51 (81)	2.22

[1] Four replicates. Oocytes were inseminated at the concentration of 7.5×10^5 sperm/ml.
[2] Percentage of the number of oocytes penetrated.

rence in the rates of normal fertilization (55~67%) and MPN formation (83~88%). This stimulating effect of caffeine on sperm penetration was also shown when mBBM was used as a fertilization medium. Sperm penetration was higher ($p<0.05$) in oocytes that were inseminated in mBBM than in oocytes inseminated in mTBM.

Experiment 3: Effect of Tris and Sodium Bicarbonate in the IVF Medium on Sperm Penetration and Subsequent Embryo Development

When IVM oocytes were fertilized in mTBM and mBBM containing 5 mM and 1 mM caffeine, respectively, there were no differences in the rates of sperm

Table 5. *In vitro* development of pig embryos fertilized with frozen-thawed boar spermatozoa in two fertilization media

IVF medium	Caffeine (mM)	No. of embryos cultured[1]	No. (%) embryos developed to		Cell number /blastocyst
			≥ 2-cell	Blastocyst	
mTBM	5	120	64 (53)	26 (22)[a]	34
mBBM	1	120	68 (57)	38 (32)[b]	41

[1] Four replicates.
[ab] Values with different superscripts within each column are significantly different ($p<0.05$).

penetration, monospermic fertilization and MPN formation (Table 4). Embryo development to the two-cell stage was not altered by different fertilization medium. However, more blastocysts were developed from oocytes that were inseminated in mBBM containing 1 mM caffeine than from the oocytes inseminated in mTBM supplemented with 5 mM caffeine (22% vs. 32 %, $p<0.05$) (Table 5).

DISCUSSION

The results of this study clearly demonstrate that boar spermatozoa frozen in straws can be used successfully for the IVP of pig embryos after optimization of sperm concentration for IVF. In addition, it has been elucidated that sperm penetration is stimulated by the presence of sodium bicarbonate and caffeine in a fertilization medium, and mBBM can support *in vitro* development of IVF pig embryos to the blastocyst stage.

In this study, the optimal sperm concentration was determined as $0.75\sim1.0\times10^6$ sperm/ml when IVM pig oocytes were inseminated with frozen spermatozoa in straws. At these sperm concentrations, the rates of oocytes penetrated and with monospermic fertilization were 52~69% and 57~64%, respectively. Considering the proportions of live spermatozoa and those with morphological acrosomal deformity (47% and 14%, respectively) in the frozen-thawed semen used in this study, actual concentration of live spermatozoa with morphologically normal acrosome during IVF would be $3\sim4\times10^5$ sperm/ml. This sperm concentration was lower than the concentration of 2×10^6 /ml of frozen-thawed spermatozoa used for IVF in previous studies (Suzuki *et al.*, 2005; Coy *et al.*, 2008) but higher than 0.6×10^5 /ml of unfrozen spermatozoa in another study (Koo *et al.*, 2005) where sperm penetration and monospermic fertilization was 62% and 75%, respectively. It has been reported that sperm penetration and pronuclear formation varies depending on the IVF conditions such as type of semen, individual boar, and acrosomal integrity of spermatozoa (Suzuki *et al.*, 2005; Wongsrikeao *et al.*, 2005; Peláez *et al.*, 2006). From the results of the present and previous studies, it is suggested that optimal sperm concentration for IVF in pigs should be determined according to IVF conditions including individual batch of semen and fertilization media being employed.

For IVF in pigs, various media including TCM-199, mTBM or Tyrode's albumin lactate pyruvate (TALP) medium have been used as a fertilization medium (Abeydeera and Day, 1997; Coy *et al.*, 2008). It has been reported that several substances such as pH buffer, caffeine and heparin in a fertilization medium influences sperm penetration, incidence of polyspermy and subsequent embryo development (Bhattacharyya and Yanagimachi, 1988; Suzuki *et al.*, 1994; Abeydeera and Day, 1997). In this study, sperm penetration was stimulated by the supplementation of fertilization medium with sodium bicarbonate compared to tris supplementation. This result is similar with the previous finding (Suzuki *et al.*, 1994) that bicarbonate induces sperm capacitation and consequently increased sperm penetration in the pig IVF. In addition, caffeine stimulated sperm penetration during IVF. This stimulatory effect of caffeine was dose-dependent. Increased sperm penetration in the mBBM was further stimulated by increasing the caffeine concentration to 5 mM. It was not known in the present study whether the stimulatory effect of caffeine on capacitation and sperm penetration would be found in different batches of semen, because we used only one batch of semen. From the results, it is considered that spermatozoa with low fertilizability can be used properly for IVP of pig embryos by treating them with caffeine and sodium bicarbonate and then increasing the sperm penetrability.

Embryo development to the blastocyst stage was higher in oocytes that were fertilized in the mBBM than in oocytes fertilized in the mTBM although there were no differences in the sperm penetration, monospermic fertilization, and MPN formation. The only difference in the medium composition between two IVF media was the use of sodium bicarbonate and tris as a buffer in mBBM and in mTBM, respectively. Previously, it was reported that short-term (~20 min) exposure of two-cell hamster embryos to HEPES reduced embryo viability (Farrel and Bavister, 1984). Recently, Palasz *et al.* (2008) reported that different zwi-

tterionic buffers HEPES, TES and MOPS and PBS buffer affected *in vitro* development and mRNA expression of bovine embryos. Due to limited amount of information available concerning the effect of buffers on pig embryo development, we are not able to explain how the sodium bicarbonate in the fertilization medium stimulated blastocyst formation of IVF embryos in this study. The result of the increased blastocyst formation in oocytes that were fertilized in the presence of bicarbonate suggests that bicarbonate may have additional role(s) other than pH buffering in a fertilization process and influence subsequent embryo development.

In summary, results of our study indicate that boar spermatozoa can be frozen in straws with maintaining their fertilizability and that caffeine and sodium bicarbonate in the fertilization medium stimulates sperm penetration *in vitro*. From the results of this study, it is suggested that boar spermatozoa frozen in straws can be used efficiently for IVP of pig embryos after determination of the optimal sperm concentration for IVF of IVM pig oocytes.

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