FULL PAPER *Theriogenology*

Improved Monospermic Fertilization and Subsequent Blastocyst Formation of Bovine Oocytes Fertilized *In Vitro* in a Medium Containing NaCl of Decreased Concentration

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ABSTRACT. The objective of this study was to evaluate whether changes in NaCl concentration in a fertilization medium could improve normal fertilization and preimplantation development of bovine oocytes. *In vitro* matured bovine oocytes were inseminated with frozen-thawed semen for 18 hr in a Tyrode's medium with albumin, lactate and pyruvate (TALP), to which 114 (TALP-114), 96 (TALP-96) or 78 (TALP-78) mM NaCl was added. Presumptive zygotes were cultured for 192 hr in a modified TALP containing 90 mM NaCl, 1.5 mM glucose, 0.3% (w/v) BSA, minimal essential medium (MEM) essential and nonessential amino acids, and insulin-transferrin-selenium complex. Lower polyspermy rate was obtained by the insemination in TALP-96 (7.8 ± 2.3%) than by the insemination in TALP-114 (25.6 ± 1.4%), without decrease in male pronucleus (MPN) formation. Fertilization in TALP-78 also yielded decreased polyspermic fertilization (3.8 ± 1.5%), but significant decrease in MPN formation was found (63.1 ± 3.1%). In preimplantation development, more blastocysts developed from oocytes inseminated in TALP-96 (24.1 ± 1.7%) than from oocytes inseminated in TALP-114 (16.8 ± 1.4%). TALP-78, however, did not improve preimplantation development beyond the 8-cell stage compared with TALP-114. Mean cell number of blastocyst was higher when oocytes were fertilized in TALP-96 (137.0 ± 4.5) than in TALP-114 (123.1 ± 5.1) and in TALP-78 (102.3 ± 4.5). These results demonstrate that insemination of bovine oocytes in a TALP with decreased NaCl concentration (96 mM) improves blastocyst formation and embryo viability. Decrease in NaCl concentration below 96 mM, however, may delay or inhibit MPN formation, and inhibits subsequent development *in vitro*.

KEY WORDS: bovine, embryo development, *in vitro* fertilization, osmolarity, sodium chloride.

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Sodium chloride (NaCl) is one of the major components of culture media for oocyte maturation, sperm preparation and embryo development *in vitro*. Sodium and chloride ions derived from NaCl critically affect a variety of metabolism and high concentration of exogenous inorganic salts may raise the intracellular concentrations of these ions up to the level that causes conformational change of protein structure and the disruption of many cellular processes [19]. In embryo development, high NaCl concentration causes two-cell block, which occurs when outbred CF1 mouse embryos are cultured in medium M16 [18]. However, such developmental retardation disappears when NaCl concentration is reduced [8]. Another report suggests that the concentration of NaCl in media used to culture the pig embryos is too high to support embryo development [1]. Lim *et al.* [9] suggested that reduced concentration of NaCl to 89 mM in the modified Tyrode's medium is beneficial for *in vitro* development of bovine embryos. In the porcine, Funahashi *et al.* [6] found that *in vitro* maturation of immature oocytes in a low-salt medium elevated intracellular glutathione level and enhanced male pronuclear formation after sperm penetration.

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In contrast to the beneficial effect of reduced NaCl concentration in embryo culture media, it has been reported that a high concentration of NaCl in a fertilization medium is beneficial for the development of rat embryos [11]. In fact, the fertilizability of spermatozoa appears to be higher in hypertonic rather than in hypotonic solution in the rabbit [3], mouse, hamster [10] and man [13]. However, little is known regarding the effects of NaCl concentration in fertilization medium on bovine sperm penetration and subsequent *in vitro* development of bovine embryos. The objective of this study, therefore, was to evaluate the effects of NaCl concentration in IVF medium on sperm penetration and subsequent *in vitro* blastocyst formation of bovine oocytes matured and fertilized *in vitro*.

MATERIALS AND METHODS

Oocyte recovery and in vitro maturation (IVM): Ovaries were obtained from Holstein cows and heifers at a local abattoir and transported to the laboratory in physiological saline at 30–35°C. Cumulus-oocyte complexes (COCs) were aspirated with an 18-gauge needle attached to a 10-m*l* disposable syringe. Only COCs with unexpanded cumulus cells and evenly granulated cytoplasm were selected for IVM. Fifty to seventy COCs were cultured in a well of 4-well dishes (Nunclon, Roskilde, Denmark) containing 500

Table 1. IVF and IVC media used in this experiment

Components[a)]	Units	TALP-114 (for IVF)	TALP-96 (for IVF)	TALP-78 (for IVF)	mTALP (for IVC)
NaCl	mM	114.0	96.0	78.0	90.0
KCl	mM	3.2	3.2	3.2	3.2
$NaHCO_3$	mM	25.0	25.0	25.0	25.0
NaH_2PO_4	mM	0.4	0.4	0.4	0.4
Na-lactate	mM	10.0	10.0	10.0	10.0
Na-pyruvate	mM	0.5	0.5	0.5	0.5
$CaCl_2$	mM	2.0	2.0	2.0	2.0
$MgCl_2$	mM	0.5	0.5	0.5	0.5
Glucose	mM	–	–	–	1.5
ITS	%	–	–	–	1.0
BSA	mg/m*l*	6.0	6.0	6.0	3.0
EAA	%	–	–	–	2.0
NEAA	%	–	–	–	1.0
Osmolarity	mOsm	305 ± 3	270 ± 3	235 ± 3	260 ± 3

a) ITS: insulin (10 μg/m*l*), transferrin (5.5 μg/m*l*) and selenium (5 ng/m*l*) complex (100-strength); EAA: MEM essential amino acids (50-strength); NEAA: MEM non-essential amino acids (100-strength).

μl of IVM medium. The IVM medium comprised tissue culture medium 199 (TCM199; Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 0.02 AU/m*l* porcine FSH (Antrin®; Denka Chemical Co., Japan), and 1 μg/m*l* estradiol (Sigma, St. Louis, MO, U.S.A.). COCs were cultured for 24 hr at 39°C under a humidified atmosphere of 5% CO_2 in air.

Sperm preparation and in vitro fertilization (IVF): The medium used for oocyte washing, sperm preparation, and IVF was TALP containing 3 (for oocyte washing) or 6 mg/m*l* BSA, as described by Fukui [4]. To examine the effect of reduced NaCl concentration during fertilization process, the fertilization medium was modified by lowering NaCl concentration from 114 mM (TALP-114) to 96 (TALP-96) or 78 mM (TALP-78, Table 1). For IVF, frozen semen (0.5 m*l*) from a Holstein bull was thawed for 30 sec in 39°C water. An aliquot (about 0.2 m*l*) of thawed semen was carefully layered under 1 m*l* of modified calcium-free TALP (capacitation medium) in small round-bottom tubes (12 × 75 mm; Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) and held for 1 hr for swim-up procedure. Then, the top 0.8 m*l* from each tube was removed, pooled in a 15-m*l* conical tube, and centrifuged (500 × g, 10 min) twice. The final sperm pellet was resuspended in the capacitation medium to a concentration of 50×10^6 sperm/m*l*. An equal volume of 200 μg/m*l* heparin (Sigma) solution was added to the sperm suspension to yield spermatozoa and heparin concentrations of 25×10^6 sperm/m*l* and 100 μg/m*l*, respectively. Heparin-treated spermatozoa were incubated for 15 min at 39°C under a humidified atmosphere of 5% CO_2 in air. After IVM, oocytes with expanded cumulus cells were washed three times in a modified TALP (washing medium, pH 7.4) containing 2 mM $NaHCO_3$, 2 mM $CaCl_2$, and 10 mM Hepes. Five to six oocytes together with 3 μl of washing medium were introduced into a fertilization drop (43 μl) of each media (pH 7.8) under mineral oil (M-8410, Sigma). Then 4 μl of the heparin-treated sperm suspension was added to each fertilization drop to a final concentration of 2×10^6 sperm/m*l*. Gametes were incubated for 18 hr at 39°C under a humidified atmosphere of 5% CO_2 in air.

In vitro culture (IVC): Culture medium for embryo development was a modified TALP (mTALP) as described by Roh *et al.* [14] (Table 1). The basic culture medium was the TALP for fertilization as described by Fukui [4], but the concentrations glucose (1.5 mM) and BSA (3 mg/m*l*), and pH level (7.4) were modified from the original medium. The medium was supplemented with 2% (v/v) minimal essential medium (MEM) essential amino acids (EAA, 50-strength, Gibco), 1% (v/v) MEM nonessential amino acids (NEAA, 100-strength, Gibco) and 1% (v/v) insulin, transferrin and selenium mixture (100-strength, Sigma). Media were prepared with deionized high-purity water (Milli-Q; Milipore, Molsheim, France), sterilized by filtering through a 0.22-μm membrane filter (Gelman Sciences, Ann Arbor, MI, U.S.A.), and equilibrated for at least 2 hr at 39°C under a humidified atmosphere of 5% CO_2 in air until use. After IVF, loosely attached sperm and cumulus cells were removed by vortexing the oocytes for 5 min. Then, putative zygotes were washed three times in culture medium, placed in 30-μl microdrops (6–10 zygotes per microdrop) of culture medium under mineral oil and cultured for 174 hr in a humidified atmosphere of 5% CO_2 in air. At 48, 72, 144 and 192 hr after the start of insemination, embryo cleavage, development to the 8-cell, morula and blastocyst stage was evaluated, respectively.

Oocyte fixation and examination of sperm penetration: At 18 hr after insemination, oocytes were mounted on a glass slide, fixed for 72 hr in 25% (v/v) acetic acid in ethanol at 4°C, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined for evidence of sperm penetration as previously [12].

Examination of blastocyst cell number: At 192 hr after

Table 2. Effect of NaCl concentration in a fertilization medium on the sperm penetration and male pronuclear formation *in vitro*

NaCl in IVF medium (mM)	No. oocytes examined	% oocytes		
		Penetrated	Polyspermic[a)]	With male pronucleus[a)]
114	102	90.2 ± 1.2	25.6 ± 1.4[b)]	78.3 ± 1.5[b)]
96	126	92.7 ± 1.6	7.8 ± 2.3[c)]	80.9 ± 1.3[b)]
78	100	87.7 ± 2.2	3.8 ± 1.5[c)]	63.1 ± 3.1[c)]

a) Percentages were based on the number of oocytes penetrated by spermatozoa.
b)-c) Values (mean ± SEM) with different superscripts in the same column significantly differ (P<0.05).

Table 3. Effect of NaCl concentration in a fertilization medium on *in vitro* development of bovine embryos

NaCl in IVF medium (mM)	No. embryos cultured	% embryos				Cell number/ blastocyst
		Cleaved	8-cell	Morula	Blastocyst	
114	130	67.3 ± 3.4	36.6 ± 2.8[a)]	22.8 ± 2.3[a)]	16.8 ± 1.4[a)]	123.1 ± 5.1[a)]
96	120	67.6 ± 2.4	39.0 ± 2.9[a)]	26.6 ± 1.9[a)]	24.1 ± 1.7[b)]	137.0 ± 4.5[b)]
78	166	61.2 ± 5.1	12.6 ± 0.4[b)]	10.1 ± 0.6[b)]	7.1 ± 0.6[c)]	102.3 ± 4.5[c)]

a)-c) Values (mean ± SEM) with different superscripts in the same column significantly differ (P<0.05).

IVF, blastocysts were examined for cell number according to the procedure previously reported [5]. Briefly, a blastocyst was placed in 0.5% (w/v) sodium citrate solution for 15 min and fixed in a cold (4°C) fixative (methanol : acetic acid : water = 3:2:1) for 30–60 sec. The fixed blastocyst was mounted on a glass slide, dried, and then stained with 10% (v/v) Giemsa stain (Merck, Darmstadt, Germany) for 15 min. The cell number was determined by counting stained nuclei under a phase-contrast microscope.

Statistical analysis: Experiments were repeated four times and the model effect in each treatment was analyzed by a general linear model procedure. When a significant model effect was found, treatment effects in each parameter were compared by the least square method in the Statistical Analysis System (SAS, Cary NC, U.S.A.). All data on embryo development and mean cell number of blastocysts were expressed as mean ± SEM.

RESULTS

A significant ($p<0.05$) treatment effect was found in the rate of polyspermic fertilization and male pronucleus formation. Polyspermic fertilization was significantly lower ($p<0.05$) in TALP-96 (7.8 ± 2.3%, 10/118) and TALP-78 (3.8 ± 1.5%, 4/88) than in TALP-114 (25.6 ± 1.4%, 26/92), whereas male pronuclear formation was higher ($p<0.05$) in TALP-114 (78.3 ± 1.5%, 80/92) and TALP-96 (80.9 ± 1.3%, 102/118) than in TALP-78 (63.1 ± 3.1%, 62/88; Table 2). There were no significant differences in sperm penetration among treatment groups.

No significant difference in cleavage was found among groups after treatment. However, the developmental competence of oocytes fertilized in TALP-78 was significantly ($p<0.05$) decreased after 8-cell stage compared with that in other groups. Blastocyst formation was significantly higher ($p<0.05$) when oocytes were inseminated in TALP-96 (24.1 ± 1.7%, 29/120) than when in TALP-114 (16.8 ± 1.4%, 22/130) and TALP-78 (7.1 ± 0.6%, 12/166; Table 3). Mean cell number increased significantly ($p<0.05$) in the blastocyst developed from the oocytes inseminated in TALP-96 medium compared with other two media (Table 3).

DISCUSSION

The result of this study showed a fertilization medium containing 96 mM NaCl yielded better rates of monospermic fertilization and blastocyst formation than a fertilization medium containing 114 mM NaCl. Blastocysts developed from oocytes fertilized in 96 mM NaCl-containing medium had more blastomeres than blastocysts developed from oocytes fertilized in other fertilization media, which might show improved embryo viability. In a preliminary study, we used fertilization media containing NaCl of higher concentrations (132–150 mM) but showed decreased fertilization parameters and preimplantation development compared to using TALP-114 (data not shown). The results of our previous and current study are inconsistent with the results from the rabbit and rat, in which hypertonic solution (380 mOsm) was effective for capacitating rabbit spermatozoa [2], and sperm penetration was improved without increasing polyspermy when rat oocytes were fertilized in medium containing high NaCl concentrations (100–130 mM) [11]. Chang and Thorsteinsson [3] also reported that the fertilizability of spermatozoa appears to be higher in hypertonic rather than in hypotonic solution in the rabbit. Number of cellular mechanism occurred during fertilization could

affect our research outcome, although direct evaluation was not made in current study. Different response of fertilizing oocytes on extracellular osmosis among species might be one of major factors responsible for such inconsistency.

Some studies on bull-to-bull difference in sperm penetration and ability to support preimplantation development were reported previously [12,16]. Effect of heparin on sperm penetrability also differs in individual bull [15]. In this study, the same semen from a Holstein bull with proven fertility was used through the experiments, and therefore, it was not elucidated whether the IVF medium containing reduced NaCl (96 mM) will show the same effect as in this study even on different bull spermatozoa. Further research is needed to clarify the beneficial effect of reduced NaCl in fertilization medium on different bull spermatozoa.

In this study, further decrease in NaCl concentration from 96 mM to 78 mM did not improve embryo development into the blastocyst and decreased cell number of blastocysts. This result suggests that determination of an optimal NaCl concentration in fertilization medium might be considered to obtain increased monospermic fertilization without decreasing sperm penetration in bovine oocytes. Based on the previous result [3], improved developmental competence of oocytes fertilized in a medium with lowered NaCl concentration might reflect the fact that hypotonic solution reduces hyperactivity of sperm and thus contributes to inducing monospermic fertilization.

Monospermic embryos having diploid chromosome may have a better developmental competence than polyspermic ones having polyploid chromosome. Accordingly, our improved rate of blastocyst formation in a 96 mM NaCl group might be due to decreased rate of polyspermic fertilization. However, in pig IVF, Han *et al.* [7] showed that the development to the blastocyst stage was not different between polyploid or diploid embryos *in vivo* or *in vitro*. After karyotyping analysis of blastocysts was performed, we could not find any evidence on increased or decreased incidence of aneuploid blastocysts in the 96 mM group, compared with 114 mM group. On the other hand, epigenetic modification of embryos fertilized in the media with different salt concentration may affect the *in vitro* development of bovine IVF embryos.

Further decrease in NaCl concentration up to 78 mM did not further improve embryo development to the blastocyst stage. Such might result from decreased rate of pronuclear formation, since cleavage and development to the 4-cell stage were not different among three groups tested. However, it is possible that delayed formation of pronucleus is responsible for decreased competence of blastocyst formation.

In conclusion, insemination of bovine oocytes in a modified TALP medium with reduced NaCl concentration to 96 mM improves *in vitro* blastocyst formation and embryo viability, which might have resulted from increased monospermic fertilization. However, insemination in more hypotonic solution might delay male pronuclear formation, and inhibit development of bovine embryos *in vitro*.

REFERENCES

1. Beckmann, L.S. and Day, B.N. 1993. Effect of media NaCl concentration and osmolarity on culture of the early stage porcine embryo and viability of embryos cultured in a selected superior medium. *Theriogenology* **39**: 611–622.
2. Brackett, B.G. and Oliphant, G. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**: 260–274.
3. Chang, M.C. and Thorsteinsson, T. 1958. Effects of osmotic pressure and hydrogen-ion concentration on the motility and fertilizing capacity of rabbit spermatozoa. *Fertil. Steril.* **9**: 510–520.
4. Fukui, Y. 1990. Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine oocytes matured *in vitro*. *Mol. Reprod. Dev.* **26**: 40–46.
5. Fukui, Y. and Ono, H. 1989. Effects of sera, hormones, and granulose cells added to culture medium for *in-vitro* maturation, fertilization, cleavage and development of bovine oocytes. *J. Reprod. Fertil.* **86**: 501–506.
6. Funahashi, H., Cantley, T.C., Stumpf, T.T., Terlouw, S.L. and Day, B.N. 1994. Use of low-salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced pronuclear formation after *in vitro* fertilization. *Biol. Reprod.* **51**: 633–639.
7. Han, Y.M., Abeydeera, L.R., Kim, J.H., Moon, H.B., Cabot, R.A., Day, B.N. and Prather, R.S. 1999. Growth retardation of inner cell mass cells in polyspermic porcine embryos produced *in vitro*. *Biol. Reprod.* **60**: 1110–1113.
8. Lawitts, J.A. and Biggers, J.D. 1991. Overcoming the 2-cell block by modifying components in a mouse embryo culture medium. *Biol. Reprod.* **45**: 245–251.
9. Lim, J.M., Okitsu, O., Okuda, K. and Niwa, K. 1994. Effects of fetal calf serum in culture medium on development of bovine oocytes matured and fertilized *in vitro*. *Theriogenology* **41**: 1091–1098.
10. Miyamoto, H. and Chang, M.C. 1973. Effect of osmolarity on fertilization of mouse and golden hamster eggs *in vitro*. *J. Reprod. Fertil.* **33**: 481–487.
11. Oh, S.H., Miyoshi, K. and Funahashi, H. 1998. Rat oocytes fertilized in modified rat 1-cell embryo culture medium containing a high sodium chloride concentration and bovine serum albumin maintain developmental ability to the blastocyst stage. *Biol. Reprod.* **59**: 884–889.
12. Ohgoda, O., Niwa, K., Yuhara, M., Takahashi, S. and Kanoya, K. 1988. Variations in penetration rates *in vitro* of bovine follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. *Theriogenology* **29**: 1375–1381.
13. Pilikian, S., Moncharmont, P., Pinatel, M.C., Guerin, J.F., Mathieu, C. and Lornage, J. 1987. *In vitro* behavior of human nonfertile semen in isoosmotic and hyperosmotic media. *Arch. Androl.* **18**: 199–230.
14. Roh, S., Yoon, J.T., Han, K.Y., Lee, B.C. and Hwang, W.S. 1998. *In vitro* production of bovine embryos by modification of simple defined culture medium. *Korean J. Emb. Trans.* **13**: 235–243 (in Korean).
15. Saeki, K., Nagao, Y., Hoshi, M. and Nagai, M. 1995. Effects of heparin, sperm concentration and bull variation on *in vitro* fertilization of bovine oocytes in a protein-free medium. *Theriogenology* **43**: 751–759.
16. Sirard, M.A. and Lambert, R.D. 1985. *In vitro* fertilization of bovine follicular oocytes obtained by laparoscopy. *Biol. Reprod.* **33**: 487–494.

17. Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J.L., Callesen, H. and Thomsen, P.D. 1999. A high proportion of bovine blastocysts produced *in vitro* are mixoploid. *Biol. Reprod.* **60**: 1273–1278.
18. Whittingham, D.C. 1971. Culture of mouse ova. *J. Reprod. Fertil. Suppl.* **14**: 7–21.
19. Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. 1982. Living with water stress: evolution of osmolyte systems. *Science* **217**: 1214–1222.