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エピネフリンが受精に与える影響と
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Abbreviations

AI: artificial insemination

ALH: amplitude of lateral head

ANOVA: analysis of variance

ART: assisted reproductive technology

BCF: flagellar beat cross frequency

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CASA: computer assisted sperm analysis

COCs: cumulus-oocyte complexes

ET: embryo transfer

FCS: fetal calf serum

h: hour

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSD: honestly significant difference

IVC: *in vitro* culture

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

IVP: *in vitro* production

LIN: linearity

mBO: modified Brackett and Oliphant

min: minute

OPU: ovum pick-up

PHE: D-penicillamine, hypotaurine, and epinephrine

PN: pronucleus

SD: standard deviation

SEM: standard error of the mean

TCM 199: tissue culture medium 199

VAP: average path velocity

VCL: curvilinear velocity

VSL: straight line velocity

WOW: well of the well

Contents

Preface

Recently, assisted reproductive technologies (ART) such as artificial insemination (AI), embryo transfer (ET) and *in vitro* embryo production (IVP) including *in vitro* maturation, fertilization and culture (IVM, IVF and IVC) have been investigated to improve the efficacy of embryo production in cattle. In past two decades, the technology which produces sex-predetermined embryos by IVF of oocytes with X- or Y-chromosome bearing sperm (sex-sorted sperm) separated by flowcytometry has been developed. Embryos produced by using the sex-sorting technology were transferred to recipients and many offspring were obtained (Cran *et al.*, 1993; Cran *et al.*, 1995). It is thought that this technology is an effective method in production of sex-predetermined offspring (Seidel, 2007), and also meets demand in dairy and beef industries (Maxwell *et al.*, 2004). To produce sex-predetermined embryos derived from sex-sorted sperm by IVF, a kind of glycosaminoglycan, heparin has been usually used for sperm capacitation (Lu *et al.*, 1999; Lu & Seidel, 2004; Wilson *et al.*, 2006; Xu *et al.*, 2006; Xu *et al.*, 2009). However, IVF with heparin showed large variations of sperm penetration to oocytes (Niwa & Ohgoda, 1988) and embryo development (Lu & Seidel, 2004) between bulls. Therefore concentrations of heparin and sperm in IVF medium have to be optimized for individual bulls (Lu *et al.*, 1999) when practitioners use this IVF system. In addition, it was reported that the efficacy of embryo yields from IVF using sex-sorted sperm was lower than that using conventional non-sorted sperm; for example, unstable cleavage rate (19.6 to 69.1%) (Xu *et al.*, 2009), low cleavage rate (Zhang *et al.*, 2003), low blastocyst rate (Xu *et al.*, 2009) and big variations of blastocyst development (0.7 to 33.2%) (Xu *et al.*, 2006; Palma *et al.*, 2008). Therefore improvement of IVP efficiency using sex-sorted sperm is strongly required.

It was reported that the simultaneous addition of D-penicillamine, hypotaurine, and epinephrine (PHE) to the IVF medium improved sperm motility and parameters of sperm associated with bull fertility (Person *et al.*, 2007). Moreover, the addition of theophylline to IVF medium has been demonstrated as a stable and high fertilization rate (Takahashi & First, 1993) and blastocyst

developmental rates of more than 40% (Nagano *et al.*, 2013; Koyama *et al.*, 2014b). However, there is no report that has carried out IVF using sex-sorted sperm supplemented with PHE and theophylline.

Nowadays repeated oocyte collection by transvaginal ultrasound-guided follicular aspiration (Ovum pick-up: OPU) combined with IVP has become alternative and competitive to superovulation for embryo production in cattle. It can be alternative because it is possible to apply to animals successfully irrespective of the reproductive status of the donor, *i.e.* in pregnant and acyclic animals, in those having genital tract infections and in animals insensitive to superovulatory treatment (Boni, 2012). However, due to small numbers (about 5 to 6) of immature oocytes can be recovered by OPU (Merton *et al.*, 2009), numerous challenges of embryo culture systems for small numbers of embryos have been conducted (Krisher & Wheeler, 2010). Small numbers of embryos cultured in droplets showed low blastocyst development (Donnay *et al.*, 1997) compared to large number of embryos did. Thus, some novel embryo culture systems, handmade well of the well (WOW) system (Vajta *et al.*, 2000; Vajta *et al.*, 2008) and commercially available polystyrene-based WOW (Sugimura *et al.*, 2010) system had been developed for culturing individual or small group of embryos. The superiority of commercially available polystyrene-based WOW system (25 micro-wells) was proved (Sugimura *et al.*, 2013) when small numbers of embryos were cultured. However, the effect of adjacent embryos in a WOW system had not been examined, even though it was reported that adjacent number of embryos affect to embryo development (Gopichandran & Leese, 2006). Therefore, effect of adjacent embryos in a WOW system should be investigated for achieving stable embryo yields by culturing individual or small numbers of embryos.

To develop efficient IVP systems in cattle, the author conducted following studies. In chapter I, to establish IVF system that can produce stable and high blastocyst development derived from any bull, the effects of PHE and theophylline on fertilization and subsequent embryo development were examined. In addition, the effects of PHE and theophylline on sperm motility were also evaluated. In chapter II, to improve IVP outcome using sex-sorted sperm, the IVF system developed in chapter I was applied and the fertilization, embryo developmental kinetics and motility of sex-sorted sperm in

IVF medium supplemented with PHE mixture and theophylline were evaluated. In chapter III, to examine effect of number of adjacent embryos in a WOW system, small numbers of embryos were cultured and blastocyst development was examined.

Chapter I

Effects of simultaneous addition of D-penicillamine, hypotaurine, and epinephrine (PHE) to IVF medium including theophylline on motility and longevity of bovine sperm and fertilization

Introduction

For IVF of bovine oocytes, IVF medium supplemented with heparin was conventionally used (Parrish, 2014). Bovine sperm incubated with heparin *in vitro* can be induced to undergo the acrosome reaction and penetrate oocytes more effectively than sperm incubated without heparin (Parrish *et al.*, 1989). However, IVF with heparin was shown to require optimization of the sperm concentration for individual bulls (Parrish, 2014), and it exhibited large variations in penetration (Niwa & Ohgoda, 1988), cleavage and blastocyst rates in each bull (Lu & Seidel, 2004). For the practical use of a small number of valuable sperm such as sex-sorted sperm and cryopreserved sperm from dead bulls (Selokar *et al.*, 2014), it is difficult to optimize the sperm concentration in each bull. Therefore, I should establish an effective IVF system without the optimization of sperm concentration for individual bulls.

A mixture of PHE has often been added to IVF medium with heparin (Hasler & Stokes, 2013). This supplementation shortened the duration for oocyte penetration (Susko-Parrish *et al.*, 1990) and increased the cleavage rate compared with the results without PHE (Miller *et al.*, 1994). In addition, it was reported that the addition of PHE to the IVF medium improved motility and sperm parameters associated with bull fertility examined by computer-assisted sperm analysis (CASA) (Person *et al.*, 2007). Some reports have demonstrated that D-penicillamine in IVF medium extended the lifespan of bovine sperm (Pavlok, 2000), and that hypotaurine played a role as an inhibitor of reactive oxygen species and induced motile sperm reactivation in the golden hamster (Boatman *et al.*, 1990). Epinephrine, a kind of catecholamine, might stimulate soluble adenylyl cyclase and increase the

concentration of cAMP in the cytoplasm of sperm (Schuh *et al.*, 2007), and increased cAMP might accelerate the beating of flagella to promote motility in mice (Schuh *et al.*, 2006). However, the effects of a PHE mixture on sperm motility parameters and cAMP production in spermatozoa are not clear.

Theophylline also increases the concentration of cAMP in the cytoplasm of sperm by inhibiting phosphodiesterase (PDE) and stimulates sperm motility (Hoskins *et al.*, 1975). It has been demonstrated that a stable and high fertilization rate (Takahashi & First, 1993) and a blastocyst developmental rate of about 40% (Nagano *et al.*, 2013) were achieved by adding theophylline to bovine IVF medium at a fixed sperm concentration of 5×10^6 cells/ml recovered by a Percoll gradient (45 and 90%) procedure. However, in the case of IVF with a low dose of sperm, such as sex-sorted sperm (2.1×10^6 cells/straw) (Rath *et al.*, 2009), the number of motile sperm might be insufficient for an IVF protocol using medium supplemented only with theophylline. In a previous study, addition of a combination of PHE and theophylline to IVF medium resulted in high normal fertilization, cleavage and blastocyst rates (84.6, 81.1 and 51.6%, respectively) for bovine IVF using 2×10^6 cells/ml of sperm (Koyama *et al.*, 2014a). However, the previous report used the sperm derived from only one bull (Koyama *et al.*, 2014a). Also the effect of the combination of a PHE mixture and theophylline on fertilization was not examined in detail.

In chapter I, to establish an efficient system for bovine embryo production by IVF without optimization of the sperm concentration for sperm derived from any bulls, the author examined the effects of a PHE mixture, theophylline and sperm concentration (1, 2 or 5×10^6 cells/ml) on fertilization, cleavage and blastocyst development. In addition, I evaluated the effects of the PHE mixture and theophylline on sperm motility and the intracellular cAMP concentration of spermatozoa.

Materials and Methods

Chemicals

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation and fertilization

IVM of bovine oocytes was performed as described previously (Takahashi *et al.*, 1996). In brief, cumulus-oocyte complexes (COCs) aspirated from follicles (2 to 8 mm in diameter) of slaughterhouse-derived ovaries were cultured for 22 h in a droplet (about 10 COCs/50 μl) of maturation medium under a humidified atmosphere of 5% CO_2 in air at 39℃. The maturation medium consisted of HEPES-buffered TCM-199 (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/ml follicle-stimulating hormone (from porcine pituitary), 1 μg/ml estradiol-17β and 50 μg/ml gentamicin sulfate. IVF was conducted according to a procedure described previously (Takahashi & Kanagawa, 1998). Briefly, after the thawing of frozen semen from 9 bulls (A to I), motile sperm were separated using a Percoll (GE Healthcare, Buckinghamshire, UK) gradient (45 and 90%). Matured COCs were co-incubated with motile sperm in droplets (10-13 COCs/100 μl) of modified Brackett and Oliphant (mBO) isotonic medium (Takahashi & Kanagawa, 1998) containing 3 mg/ml fatty acid-free BSA and supplemented with 2.5 mM theophylline and/or PHE (20 μM D-penicillamine, 10 μM hypotaurine and 1 μM epinephrine) (Miller *et al.*, 1994) at 39℃ under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. All culture droplets were covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan). The final sperm concentration (1, 2 or 5 × 10^6 cells/ml), co-incubation time (12 or 18 h) and treatment with PHE and theophylline are described in the experimental design.

Examination of spermatozoon penetration and *in vitro* culture of presumptive zygotes after IVF

IVC of presumptive zygotes was performed using procedures that were basically the same as described previously (Takahashi & Kanagawa, 1998). After co-incubation with sperm, zygotes were freed from cumulus cells by vortexing. To evaluate sperm penetration, presumptive zygotes of each experiment group were fixed with ethanol:acetic acid at a ratio of 3:1 and stained with 1% aceto-orcein solution as described previously (Takahashi & First, 1992). Oocytes having an enlarged sperm head(s) or male pronucleus(ei) were defined as penetrated by sperm, and the following categories of oocytes penetrated by sperm were recorded: 1) oocytes with male and female pronuclei with a corresponding sperm tail (2PN), 2) oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy), and 3) oocytes other than 2PN and polyspermy, such as oocytes with an enlarged sperm head and anaphase II/telophase II chromosome or female pronucleus, or oocytes with a male pronucleus and telophase II chromosome (others). To evaluate the rates of development to blastocysts, cumulus-free presumptive zygotes were cultured for 150 h in droplets (25-30 presumptive zygotes/30 or 40 μl) using modified synthetic oviduct fluid, which contained 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium and 10 μg/ml insulin and was supplemented with 5 mM glycine, 5 mM taurine, and 1 mM glucose (Takahashi & Kanagawa, 1998), and 3 mg/ml fatty acid-free BSA instead of polyvinyl alcohol at 39℃ under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage and blastocyst rates were assessed after 48 and 168 h of IVF, respectively. All embryos that developed to blastocysts were subjected to counting of their cell numbers using an air-drying method (Takahashi & First, 1992).

Evaluation of sperm motility and sperm motility parameters by CASA

Motile sperm separated using a Percoll gradient (45 and 90%) and recovered motile sperm were incubated in 100-μl droplets of IVF medium (final concentration of 10×10^6 cells/ml in 100-μl droplets) at 39℃ in 5% CO_2, 5% O_2 and 90% N_2. After incubation, 3 μl of IVF medium from

droplets was placed onto 4-chamber slides with a depth 20 μm (Art. No. SC 20-01-04-B, Leja, Nieuw-Vennep, Netherlands) on a micro-warm plate (Kitazato Corporation, Shizuoka, Japan) at 37℃ for counting. Sperm in three fields (at least 100 spermatozoa) in a chamber were divided into motile and dead sperm, and the percentages of motile sperm and sperm motility parameters were evaluated using a CASA system (SMAS, DITECT Corporation, Tokyo, Japan). The evaluated sperm motility parameters were straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL × 100), flagellar beat cross frequency (BCF) and amplitude of lateral head (ALH).

Evaluation of intracellular cAMP concentration of spermatozoa

The intracellular cAMP concentration of sperm was measured using the cAMP Biotrak enzyme immunoassay system kit (RPN2251, Amersham, GE Healthcare, Life Sciences, UK) according to the protocol provided by the manufacturer. The microplate contained 12 × 8-well strips coated with donkey anti-rabbit IgG. In brief, motile sperm recovered using a Percoll gradient (45 and 90%) were incubated in 400 μl of IVF medium (final concentration of 10×10^6 cells/ml) in a 1.5-ml tube for 2 h at 39℃ under 5% CO_2, 5% O_2 and 90% N_2. After incubation, each aliquot was centrifuged at 500 × *g* for 5 min and the supernatant was removed. The sperm pellet was resuspended in 200 μl of lysis buffer and mixed on a microplate shaker for 10 min at room temperature. One hundred microliters of lysate and 100 μl of antiserum were added to the wells and incubated at 4℃ for 2 h. After incubation, 100 μl of cAMP-peroxidase conjugate was added to each well and incubated at 4℃ for 1 h. All supernatant was aspirated, and all wells washed four times with 400 μl of wash buffer using a microplate washer (Model 1575, Immunowash, Bio-Rad Laboratories, Tokyo, Japan). One hundred and fifty microliters of enzyme substrate was added to all wells and mixed on a microplate shaker at room temperature for 1 h. One hundred microliters of 1.0 M sulfuric acid was added to each well, the optical density was determined with in a plate reader (iMark, Bio-Rad Laboratories) at 450 nm, and cAMP concentrations in each well were evaluated.

Experimental design

In experiment 1-1, to examine the effect of the PHE mixture and different sperm concentrations on sperm penetration and blastocyst development rates in sperm derived from various bulls, IVF medium containing theophylline was used. Some of the matured COCs were co-incubated with sperm derived from bulls A, B and C at a concentration of 1, 2 or 5×10^6 cells/ml for 18 h, and fertilization status was examined. Other presumptive zygotes were cultured, and cleavage and blastocyst development were examined. As controls for sperm penetration and blastocyst development, oocytes incubated at a sperm concentration of 5×10^6 cells/ml supplemented only with theophylline were used. In addition, to confirm blastocyst development after IVF using a sperm concentration of 2×10^6 cells/ml in IVF medium containing a combination of theophylline and PHE, presumptive zygotes fertilized with sperm from six bulls (D to I) were cultured.

In experiment 1-2, to examine the effect of the PHE mixture and theophylline on sperm fertilizability in detail, matured COCs were co-incubated with sperm at a concentration of 1×10^6 cells/ml (bulls A and I) for 12 h in IVF medium with or without PHE and/or theophylline. Sperm of bull A showed similar 2PN rates among sperm concentrations of 1, 2 and 5×10^6 cells/ml for 18 h of IVF, irrespective of PHE and theophylline addition, in experiment 1-1. In addition, to confirm the effect of the PHE mixture and theophylline on sperm activity, after incubation (0, 2, 4, 6 and 8 h) of sperm of bull I, sperm motility was evaluated by CASA. For evaluation of sperm motility parameters, motile sperm with a VSL of ≥25 μm/sec (50.49 to 75.32%) were selected, as it has been recognized that motile sperm having a VSL of less than 25 μm/sec (25.51 to 40.51%) were probably not related to fertilization (Fig. 1-1).

To examine the effect of PHE and theophylline on the intracellular cAMP concentration of spermatozoa (bull I), the intracellular cAMP concentration of sperm was evaluated after 2 h of incubation in IVF medium with or without PHE and theophylline. As a control, sperm immediately after Percoll treatment (0 h) were used. All experiments in each group were carried with at least 3 replicates.

Statistical analysis

Sperm penetration rates (total penetration, 2PN and polyspermy rates) were compared among groups by Chi-square test. The percentages of cleavage and development to blastocysts, mean cell numbers in blastocysts, average sperm motility parameters and the concentration of intracellular cAMP after 2 h of incubation were compared by one-way ANOVA followed by Tukey-Kramer's HSD test as a *post hoc* test. Intracellular cAMP concentrations at 0 h and 2 h in each treatment were compared by Dunnett's test. All analyses were performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC, USA).

Results

Experiment 1-1

The effects of PHE and sperm concentrations on penetration are shown in Table 1-1. In bull A, the 2PN rates were similar among different sperm concentrations (1, 2 and 5 × 10^6 cells/ml), irrespective of PHE addition. In bulls B and C, at a sperm concentration of 1 × 10^6 cells/ml without PHE, the total penetration and 2PN rates were the lowest among all experimental groups. In bull C, at a sperm concentration of 1 × 10^6 cells/ml with the PHE mixture, the total penetration rate was significantly lower than that of the control (sperm concentration of 5 × 10^6 cells/ml without PHE; $P<0.05$). In all bulls, the highest polyspermy rates were observed when PHE was added to IVF medium including theophylline at a sperm concentration of 5 × 10^6 cells/ml.

The effects of PHE and different sperm concentration on embryonic development are shown in Table 1-2. At sperm concentrations of 1 and 2 × 10^6 cells/ml (bulls A, B and C), there were no significant differences in cleavage rate, blastocyst rate and mean cell number in blastocysts compared with those at 5 × 10^6 cells/ml without PHE (control). However, in bull C, the cleavage rate at a sperm concentrations of 1 x 10^6 cells/ml tended to be lower ($P=0.08$) than those at 2 × 10^6 cells/ml and in the control. In addition, the mean cell numbers of blastocysts at a sperm concentration of 1 × 10^6 cells/ml tended to be lower ($P=0.13$) than those at 2 × 10^6 cells/ml and in the control in bull C. As shown in Table 1-3, after fertilization with a sperm concentration of 2 × 10^6 cells/ml in the presence of PHE and theophylline, cleavage rates of 78.3 to 92.4% and blastocyst development rates of 31.9 to 62.0% were obtained from 6 bulls (D to I).

Experiment 1-2

In bulls A and I, after 12 h of co-incubation of COCs and sperm (1 × 10^6 cells/ml), the total penetration and 2PN rates in the presence of PHE and theophylline were higher than those in the other experimental groups (Table 1-4; $P<0.05$). The effects of PHE and theophylline on sperm motility

and sperm motility parameters are shown in Fig. 1-2. The total percentage of motile sperm decreased with increasing incubation period in all experimental groups; in particular, those at 6 and 8 h with only theophylline supplementation were significantly lower than those in the nontreatment group (Fig. 1-2 A; $P<0.05$). On the other hand, in terms of the percentages of sperm moving at more than 25 μm/sec in the VSL, only PHE addition showed stable motility (about 70%) during the experimental period, with low variation compared with the percentages in the nontreatment, theophylline addition, and combination of PHE and theophylline groups (Fig. 1-2 B). The average VAP levels of sperm incubated with theophylline regardless of PHE addition were significantly increased at 2 h and decreased at 6 h compared with those in the nontreatment group (Fig. 1-2 C; $P<0.05$). Regardless of PHE addition to IVF medium, the average LIN at 2 h with theophylline was significantly higher than those in the nontreatment group and the PHE addition only group (Fig. 1-2 D; $P<0.05$). The average BCF with PHE and theophylline at 6 h was higher than those in the nontreatment group and the PHE addition only group (Fig. 1-2 E; $P<0.05$). The average ALH levels at 6 and 8 h with PHE and theophylline were significantly lower than those in the nontreatment group and the PHE addition only group (Fig. 1-2 F; $P<0.05$).

As shown in Fig. 1-3, the mean intracellular cAMP concentration of sperm incubated with theophylline (167.5 ± 54.5 fmol/5 × 10^6 cells; mean ± SD) at 2 h was significantly higher than for those incubated with PHE (53.9 ± 54.7 fmol/5 × 10^6 cells) and in the nontreatment group (45.1 ± 47.9 fmol/5 × 10^6 cells; $P<0.05$). However, the cAMP concentration of sperm incubated with PHE and theophylline (117.3 ± 92.9 fmol/5 × 10^6 cells) showed an intermediate value between those of sperm incubated with theophylline or PHE. The mean intracellular cAMP concentration of sperm incubated with theophylline only showed a significantly higher value than that at 0 h (68.2 ± 19.9 fmol/5 × 10^6 cells; $P<0.05$).

Discussion

In bull A, addition of PHE to the IVF medium at different sperm concentrations (1, 2 and 5 × 10^6 cells/ml) did not affect 2PN rates at 18 h post insemination but did at 12 h post insemination. However, in bull B at 18 h post insemination, PHE addition was necessary to enhance the total penetration and normal fertilization at a sperm concentration of 1 × 10^6 cells/ml. Meanwhile, in bull C, the total penetration rate at a sperm concentration of 1 × 10^6 cells/ml was not enhanced compared with that at a sperm concentration of 2 × 10^6 cells/ml even though PHE was added to the IVF medium. In bulls A, B and C, addition of PHE to the IVF medium at a sperm concentration of 5 × 10^6 cells/ml showed the highest polyspermy rate. These results indicate that, when the combination of PHE and theophylline is added to IVF medium, the sperm concentration can be reduced from 5 × 10^6 cells/ml to 2 × 10^6 cells/ml, which can obtain a high and stable 2PN rate. In addition, after fertilization in the presence of PHE and theophylline with a sperm concentration of 2 × 10^6 cells/ml, I obtained a relatively high rate of normal fertilization (68.2 to 82.4%) from 3 bulls, and high cleavage (78.3 to 92.4%) and blastocyst rates (31.9 to 62.0%) from 9 bulls. From these results, a sperm concentration of 2 × 10^6 cells/ml is recommended for use in IVF in the presence of PHE and theophylline in IVF medium, which can stably provide high rates of normal fertilization and blastocyst development using sperm collected from any bull.

In the present study, the author found a synergistic effect of PHE and theophylline on the fertilizability of sperm; namely, PHE and theophylline enhanced the sperm penetration ability and shortened the time to penetration, especially at a low concentration of sperm. In agreement with the present results, it was reported that sperm penetration ability (Susko-Parrish, 1990) and cleavage rate (Miller *et al.*, 1994) were enhanced by the addition of PHE to IVF medium including heparin.

Analysis with the CASA system revealed that, in the presence of only theophylline, the percentage of total motile sperm became lower than that of the nontreatment group after 6 h of incubation; however, by the addition of PHE to the IVF medium with theophylline prevented the decrease in the

percentage of total motile sperm. Sperm incubated in IVF medium with only PHE maintained a stable percentage (about 70%) of motile sperm with a VSL of ≥25 μm/sec based on total sperm during the 8 h incubation period. I speculated that the addition of PHE to IVF medium maintains sperm motility. The averages of VAP and LIN at 2 h were increased in the presence of theophylline regardless of PHE addition compared with those in the nontreatment group. These results indicate that theophylline enhanced the progressive motility of sperm within a short period.

In the presence of PHE and theophylline at 6 h after incubation, average BCF was significantly higher compared with that in the nontreatment group. On the other hand, the average ALH of sperm incubated with the combination of PHE and theophylline at 6 and 8 h was significantly lower than that in the nontreatment group. This result is not consistent with a previous report describing that ejaculated sperm incubated in Tyrode's HEPES-buffered medium with heparin showed significantly high average ALH and BCF levels at 1 h to 4 h compared with those incubated in Tyrode's HEPES-buffered medium without heparin supplementation (Chamberland *et al.*, 2001). Chamberland *et al.* (2001) suggested that the increases in BCF and ALH of sperm incubated with heparin *in vitro* could be related to the hyperactivation of sperm. In addition, theophylline induced hyperactivation and the acrosome reaction of in sperm of dogs (Kawakami *et al.*, 1999) and enhanced the rate of sperm penetration into oocytes in humans (Loughlin & Agarwal, 1992). The increase in BCF and decrease in ALH observed in the present study probably indicate that sperm incubated with PHE and theophylline acquires a high level of activity without hyperactivation.

It was previously reported that adenosine and catecholamine agonists might increase cAMP in the cytoplasm of sperm and accelerate the beating of flagella related to BCF in mice (Schuh *et al.*, 2007). In the present study, theophylline, one of the PDE inhibitors, increased the intracellular cAMP. In humans, pentoxifylline, another PDE inhibitor, also increased intracellular cAMP content of sperm in a manner correlated to increases of BCF and ALH (Calogero *et al.*, 1998). However, in the present study, the ALH of sperm incubated with PHE and theophylline at 6 and 8 h was suppressed compared with that of sperm incubated with only theophylline. Because sperm incubated with PHE and

theophylline at 2 h showed a relatively low intracellular cAMP concentration compared with that of sperm incubated with only theophylline addition, I speculated that PHE activated an unknown signaling pathway to prevent the increase in intracellular cAMP and subsequently maintained sperm motility.

According to these hypotheses, sperm incubated with PHE and theophylline maintains high progressive motility. Therefore, these sperm have increased opportunities to encounter oocytes and subsequently penetrate them. Further study is needed to clarify the relationship between sperm motility parameters, intracellular cAMP content of sperm and not only IVF medium including theophylline and PHE mixture, but also D-penicillamine, hypotaurine and epinephrine separately.

In conclusion, the combination of a PHE mixture and theophylline synergistically accelerates sperm motility and sperm penetration of bovine oocytes. Theophylline activates sperm motility with increasing intracellular cAMP. However, PHE prevents an excessive increase in cAMP and maintains sperm motility without hyperactivation. When the combination of PHE and theophylline is added to IVF medium at a sperm concentration of 2×10^6 cells/ml, we can obtain stable normal fertilization and blastocyst development rates using sperm from any bull. Further study should be carried out that the present IVF system is effective for sperm penetration and development when sex-sorted sperm is used. Thus, in chapter II, the effect of PHE and theophylline addition to IVF medium at a sperm concentration of 2×10^6 cells/ml using sex-sorted sperm was investigated.

Table 1-1. The effects of the PHE mixture and sperm concentration on sperm penetration rate at 18 h after IVF using medium including theophylline

Bulls	Sperm concentration ($\times 10^6$ cells/ml)	PHE	No. of oocytes (replicates)	Percentages of			
				Total penetration	2PN	Poly	Others*
A	1	+	31 (3)	100.0^{a}	74.2	9.4^{ab}	16.4^{a}
		−	31 (3)	84.2^{b}	75.1	0^{b}	9.1^{ab}
	2	+	31 (3)	97.0^{ab}	68.2	13.0^{ab}	15.8^{a}
		−	31 (3)	96.7^{ab}	77.0	10.0^{ab}	9.7^{ab}
	5	+	42 (3)	97.8^{ab}	75.9	21.8^{a}	0^{b}
		−	37 (3)	100.0^{a}	87.0	13.0^{ab}	0^{b}
B	1	+	31 (3)	95.8^{a}	78.7^{a}	4.2^{a}	13.0^{a}
		−	32 (3)	59.4^{b}	50.0^{b}	0^{a}	9.4^{ab}
	2	+	34 (3)	93.9^{a}	82.5^{a}	11.5^{ab}	0^{b}
		−	33 (3)	97.0^{a}	84.8^{a}	6.4^{a}	5.8^{ab}
	5	+	31 (3)	100.0^{a}	73.6^{ab}	23.0^{b}	3.3^{ab}
		−	42 (4)	95.0^{a}	81.7^{a}	9.2^{ab}	4.2^{ab}
C	1	+	40 (3)	81.7^{ab}	73.3^{ab}	3.3^{a}	5.0^{ab}
		−	40 (3)	69.5^{a}	49.0^{b}	6.7^{a}	13.8^{a}
	2	+	51 (4)	97.9^{c}	82.4^{a}	15.7^{ab}	0^{b}
		−	48 (4)	89.2^{bc}	79.2^{ab}	3.9^{a}	9.4^{ab}
	5	+	35 (3)	97.0^{bc}	53.6^{b}	30.6^{b}	12.8^{a}
		−	32 (3)	100.0^{c}	80.8^{a}	15.9^{ab}	3.3^{ab}

$^{a, b, c}$ Values with different letters within each bull differ significantly ($P<0.05$).

*Others: an enlarged sperm head with anaphase II/telophase II chromosome or a male pronucleus was observed.

2PN: two pronuclei, Poly: penetrated with more than two sperm.

Table 1-2. The effects of PHE and sperm concentration on blastocyst developmental rate when using IVF medium including theophylline

Bulls	Sperm concentration ($\times 10^6$ cells/ml)	PHE	No. of oocytes (replicates)	% cleavage	% blastocysts / inseminated oocytes	Mean cell numbers in blastocysts (n)
A	1	+	72 (3)	81.9 ± 2.4	44.5 ± 8.7	144.9 ± 50.6 (33)
	2	+	71 (3)	86.2 ± 8.8	50.8 ± 11.3	148.9 ± 62.3 (36)
	5	−	70 (3)	87.5 ± 12.5	54.6 ± 11.5	165.9 ± 64.4 (38)
B	1	+	112 (4)	72.2 ± 6.3	36.6 ± 6.6	174.8 ± 60.3 (41)
	2	+	80 (3)	78.3 ± 4.4	50.0 ± 13.3	183.9 ± 63.8 (40)
	5	−	157 (6)	81.5 ± 9.7	39.2 ± 10.0	169.1 ± 69.6 (24)
C	1	+	65 (3)	68.0 ± 4.8	24.8 ± 15.2	148.3 ± 55.4 (16)
	2	+	91 (3)	86.8 ± 3.1	31.9 ± 5.0	191.2 ± 77.0 (29)
	5	−	189 (8)	80.5 ± 11.5	30.4 ± 8.0	188.1 ± 76.1 (58)

Values are means ± SD.

Embryos derived from bulls A to C were cultured in 40-μl droplets.

Table 1-3. Blastocyst development after IVF with a sperm concentration of 2×10^6 cells/ml in IVF medium containing a combination of theophylline and PHE

Bulls	No. of oocytes (replicates)	% cleavage	% blastocysts / inseminated oocytes	Mean cell numbers in blastocysts (n)
D	74 (3)	84.1 ± 4.4	50.6 ± 5.2	153.5 ± 62.6 (37)
E	80 (3)	92.4 ± 4.1	62.0 ± 12.2	143.1 ± 54.1 (50)
F	87 (3)	86.1 ± 3.9	39.2 ± 5.1	161.7 ± 59.4 (34)
G	200 (7)	80.8 ± 9.2	38.0 ± 10.9	170.3 ± 69.3 (76)
H	114 (4)	91.3 ± 10.3	37.9 ± 3.7	127.2 ± 68.9 (33)
I	125 (5)	87.9 ± 11.3	39.8 ± 7.7	153.0 ± 62.5 (49)

Values are means ± SD.

Embryos derived from bulls D to F and bulls G to I were cultured in 40-μl and 30-μl droplets, respectively.

Table 1-4. The effects of PHE and theophylline on sperm penetration rate of bulls A and I at 12 h after IVF with a sperm concentration of 1 × 10^6 cells/ml

Bulls	Treatment		No. of oocytes (replicates)	Percentages of			
	Theophylline	PHE		Total penetration	2PN	Poly	Others*
A	+	+	35 (3)	77.1[a]	42.9[a]	14.3	22.7
		−	33 (3)	45.5[bc]	12.1[b]	0	33.3
	−	+	33 (3)	54.5[b]	9.1[bc]	6.1	39.4
		−	32 (3)	28.1[c]	0[c]	0	27.9
I	+	+	55 (5)	60.3[a]	28.1[a]	0	32.2[a]
		−	57 (5)	39.0[b]	10.8[b]	0	28.6[a]
	−	+	56 (5)	8.8[c]	1.8[c]	0	6.7[b]
		−	57 (5)	1.7[c]	0[c]	0	1.7[b]

[a, b, c] Values with different letters within each bull differ significantly ($P<0.05$).

*Others: an enlarged sperm head with an anaphase II/telophase II chromosome or a male pronucleus was observed.

2PN: two pronuclei, Poly: penetrated with more than two sperm.

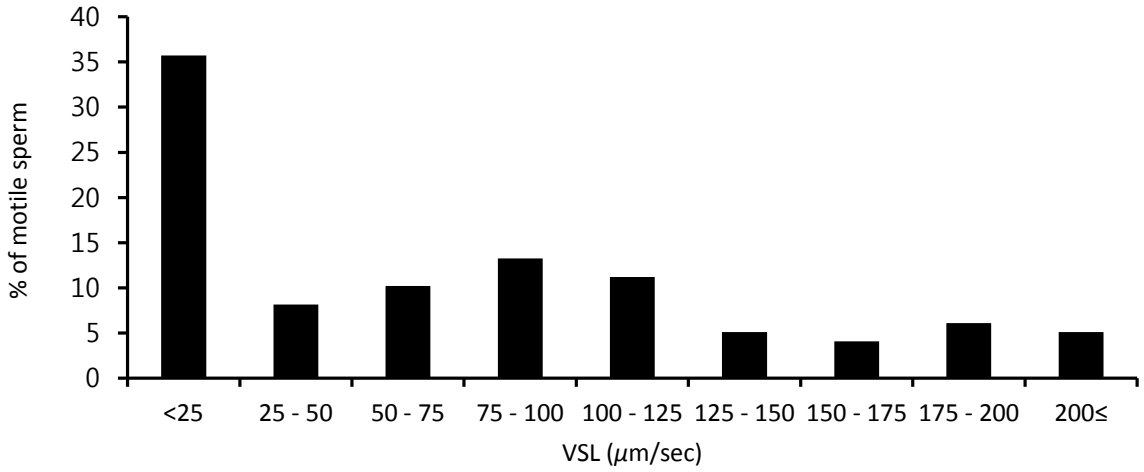

Fig. 1-1. Distribution of motile sperm based on straight line velocity (VSL) at 0 h of incubation. Sperm motility was analyzed immediately after treatment with a Percoll gradient (bull I).

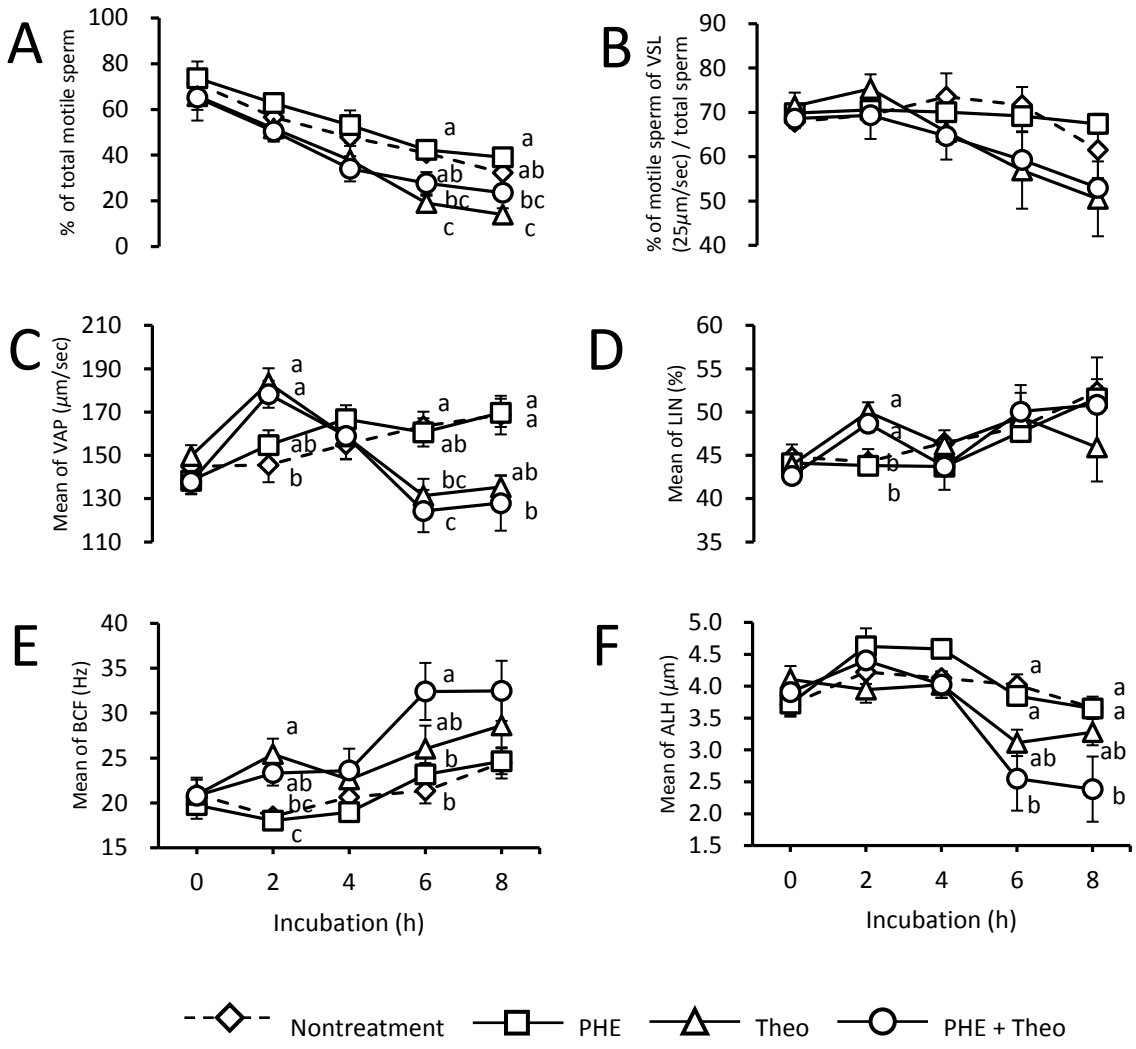

Fig. 1-2. The sperm motility and sperm motility parameters during incubation in IVF medium supplemented with or without PHE mixture and theophylline.

A, % of total motile sperm; B, % of motile sperm with a VSL of ≥25 μm/sec; C, mean VAP (μm/sec); D, mean LIN (%); E, mean BCF (Hz); F, mean ALH (μm).

Values in panels C to F were calculated by using sperm that showed a VSL of ≥25 μm/sec.

[a,b,c] Values with different letters differ significantly ($P<0.05$).

The error bar indicates the SEM. At least 4 replicates per group were performed.

Nontreatment, mBO medium only; PHE, mixture of D-penicillamine, hypotaurine, and epinephrine; Theo, theophylline.

VSL, straight line velocity; VAP, average path velocity; LIN, linearity (VSL/VCL [curvilinear velocity] × 100); BCF, flagellar beat cross frequency; ALH, amplitude of lateral head.

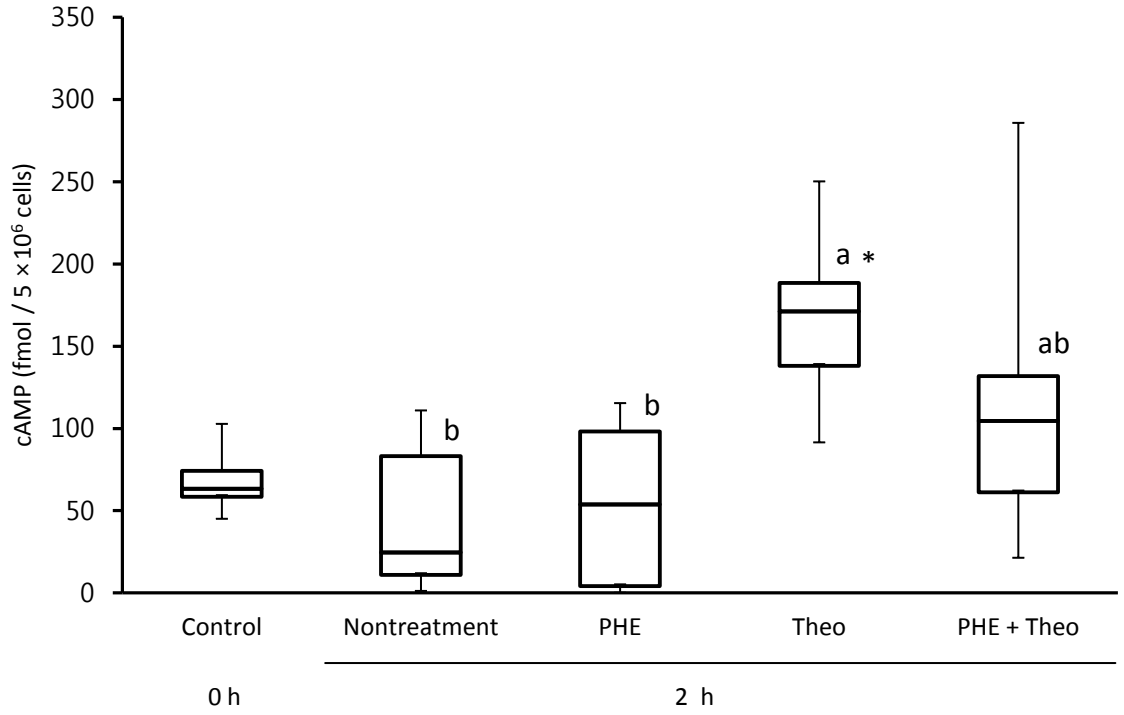

Fig. 1-3. Mean intracellular cAMP concentration in sperm at 2 h after incubation.

Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population.

[a, b] Different letters indicate a significant difference among groups ($P<0.05$).

* The asterisk indicates a significant difference from the control.

Six replicates per group were performed.

Control and nontreatment, mBO medium only; PHE, mixture of D-penicillamine, hypotaurine, and epinephrine; Theo, theophylline.

Summary

The present study aimed to establish an efficient system for bovine embryo production by *in vitro* fertilization (IVF) that can achieve stable normal fertilization and blastocyst developmental rates in any bull without optimization of the sperm concentration in IVF medium. I examined the effects of a PHE mixture (20 μM D-penicillamine, 10 μM hypotaurine and 1 μM epinephrine), theophylline (2.5 mM) and sperm concentration (1, 2 or 5 × 10^6 cells/ml) on fertilization and blastocyst developmental rates. High cleavage rates (78.3 to 92.4%) and blastocyst developmental rates (31.9 to 62.0%) at day 7 were obtained in the presence of PHE and theophylline in IVF medium with a sperm concentration of 2 × 10^6 cells/ml using sperm from 9 bulls. In addition, the synergistic effect of PHE and theophylline on normal fertilization (2 pronuclei) was clarified at 12 h after IVF with a sperm concentration of 1 × 10^6 cells/ml. Moreover, high linearity, high flagellar beat cross frequency and low amplitude of lateral head of motile sperm were found by computer-assisted sperm analysis. In conclusion, the combination of the PHE mixture and theophylline synergistically accelerates sperm motility and sperm penetration of bovine oocytes. Theophylline activates sperm motility with increasing intracellular cAMP. However, PHE prevents an excessive increase of cAMP and maintains sperm motility without hyperactivation. When the combination of PHE and theophylline is added to IVF medium at a sperm concentration of 2 × 10^6 cells/ml, we can achieve stable normal fertilization and blastocyst development in any bull.

Chapter II

Sperm penetration and developmental kinetics of embryos fertilized with sex-sorted sperm in IVF medium supplemented with PHE and theophylline

Introduction

Usage of sex-sorted sperm is a feasible way to produce offspring of a predetermined sex in the beef and dairy industries. Numerous studies using sex-sorted sperm have been reported for AI (Seidel *et al.*, 1999; Tubman *et al.*, 2004; Bodmer *et al.*, 2005; Andersson *et al.*, 2006; Cerchiaro *et al.*, 2007; DeJarnette *et al.*, 2011), embryo collection from superovulated cattle (Hayakawa *et al.*, 2009; Peippo *et al.*, 2009) and IVF (Cran *et al.*, 1995; Puglisi *et al.*, 2006; Wilson *et al.*, 2006; Xu *et al.*, 2006; Xu *et al.*, 2009). However, bovine oocytes fertilized with sex-sorted sperm capacitated by heparin showed unstable cleavage rate (19.6 to 69.1%) (Zhang *et al.*, 2003; Xu *et al.*, 2009) and low blastocyst rate (Xu *et al.*, 2009). In addition, there were big variations in blastocyst development (0.7 to 33.2%) among sex-sorted sperms derived from different bulls (Xu *et al.*, 2006; Palma *et al.*, 2008). Therefore, IVF system which can perform stable fertilization and blastocyst development using sex-sorted sperm derived from any bull, should be developed.

Recently, it was reported that IVF using sex-sorted sperm showed lower normal fertilization, cleavage, and blastocyst development than IVF using non-sorted sperm; however, intra-cytoplasmic sperm injection (ICSI) of sex-sorted sperm into oocytes showed similar results with IVF using non-sorted sperm (Jo *et al.*, 2014). These results indicate that if oocytes are normally fertilized and formed two pronuclei successfully by IVF using sex-sorted sperm, higher blastocyst development similar to IVF using non-sorted sperm will be achieved.

It was also reported that the first cleavage timing of zygotes fertilized with sex-sorted sperm was later, and the zygotes showed lower cleavage and blastocyst rates than those fertilized with non-sorted

sperm (Bermejo-Alvarez *et al.*, 2010). However, the reason of late first cleavage timing was not elucidated clearly. The author suspects that the penetration of sex-sorted sperm into oocytes occurs later than that of non-sorted sperm. When a PHE mixture and theophylline were added to IVF medium in chapter I, penetration of oocytes by non-sorted sperm was accelerated, and highly stable normal pronuclear formation and blastocyst development rates were achieved. Therefore, the author examined the timing of sperm penetration by sex-sorted sperm using IVF system developed in chapter I. In addition, developmental kinetics of embryos fertilized with sex-sorted sperm was examined. Moreover, to evaluate the characteristics of sex-sorted sperm in IVF medium with a PHE mixture and theophylline, sperm motility was also investigated.

Materials and Methods

Chemicals

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation and fertilization

IVM of bovine oocytes was performed as described in chapter I. In brief, COCs aspirated from follicles (2 to 8 mm in diameter) of slaughterhouse-derived ovaries were cultured for 22 h in a droplet (about 10 COCs/50 μl) of maturation medium under a humidified atmosphere of 5% CO_2 in air at 39℃. The maturation medium consisted of HEPES-buffered TCM-199 (Invitrogen) supplemented with 10% FCS (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/ml follicle-stimulating hormone (from porcine pituitary), 1 μg/ml estradiol-17β and 50 μg/ml gentamicin sulfate.

IVF with non-sorted sperm was conducted according to a procedure described in chapter I. In brief, after thawing, non-sorted frozen semen from 3 bulls (G, H and J) was layered on a Percoll (GE Healthcare, Buckinghamshire, UK) gradient (45 and 90%) and motile sperm was recovered. IVF with sex-sorted sperm (X-chromosome bearing sperm) was conducted according to a slightly modified procedure described in chapter I. Briefly, sex-sorted frozen semen (2 straws) from 3 bulls (G, H and J) was thawed and layered on a Percoll gradient (45 and 90%) containing 3 mg/ml fatty acid-free BSA in separable fine neck tube (Nipro Corporation, Osaka, Japan) and motile sperm was recovered. Matured COCs were co-incubated with motile sperm in droplets (10-13 COCs/100 μl) of mBO isotonic medium (Takahashi & Kanagawa, 1998) containing 3 mg/ml fatty acid-free BSA supplemented with 2.5 mM theophylline and/or PHE (Miller *et al.*, 1994) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39℃. All culture droplets were covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan). The final sperm concentration was adjusted to 2×10^6 cells/ml and co-incubated for 12 or 18 h as described in experimental design.

Examination of spermatozoon penetration after IVF

Examination of spermatozoon penetration after IVF was performed as described in chapter I. After co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing. Presumptive zygotes of each experiment group were fixed with ethanol:acetic acid at a ratio of 3:1 and stained with 1% aceto-orcein solution as described previously (Takahashi & First, 1992). Oocytes having an enlarged sperm head(s) or male pronucleus(ei) were defined as penetrated by sperm, and the following categories of oocytes penetrated by sperm were recorded: 1) oocytes with male and female pronuclei with a corresponding sperm tail (2PN), 2) oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy), and 3) oocytes other than 2PN and polyspermy, such as oocytes with an enlarged sperm head and anaphase II/telophase II chromosome or female pronucleus, or oocytes with a male pronucleus and telophase II chromosome (others).

***In vitro* culture of presumptive zygotes and evaluation of embryo developmental kinetics by time-lapse embryo observation system**

IVC of presumptive zygotes was performed using procedures that were basically the same as described previously (Takahashi & Kanagawa, 1998). Cumulus-free presumptive zygotes were cultured for 150 h in droplets (20-30 presumptive zygotes/30 μl) using a modified synthetic oviduct fluid, which contained 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium and 10 μg/ml insulin and was supplemented with 5 mM glycine, 5 mM taurine, and 1 mM glucose (Takahashi & Kanagawa, 1998), and 3 mg/ml fatty acid-free BSA instead of polyvinyl alcohol at 39℃ under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Development of presumptive zygotes was monitored by a time-lapse embryo observation system (CCM-IVF; Astec, Fukuoka, Japan) and photographs of presumptive zygotes were taken with × 4 magnification in every 30 min from 18 to 48 h after IVF. The percentages of cumulative cleaved embryos were analyzed based on sum of 3-h interval from 18 to 48 h after IVF. Cleavage and blastocyst rates were assessed after 48 and 168 h of IVF, respectively. All embryos

that developed to blastocysts were subjected to counting of their cell numbers using an air-drying method (Takahashi & First, 1992).

Evaluation of sperm motility and sperm motility parameters by CASA

Evaluation of sperm motility was performed as described in chapter I with a slight modification. Briefly, motile sperm separated using a Percoll gradient (45 and 90%) and recovered motile sperm were incubated in 50-μl droplets of IVF medium supplemented with PHE and theophylline (final concentration of 10×10^6 cells/ml in 50-μl droplets) under 5% CO_2, 5% O_2 and 90% N_2 at 39℃. After incubation, 3 μl of sperm dilution from droplets was placed onto 4-chamber slides with a depth 20 μm (Art. No. SC 20-01-04-B, Leja, Nieuw-Vennep, Netherlands) on a micro-warm plate (Kitazato Corporation, Shizuoka, Japan) at 37℃ for counting. Sperm in 6 fields (at least 100 spermatozoa) in a chamber were divided into motile and dead sperm, and the percentages of motile sperm and sperm motility parameters were evaluated using a CASA system (SMAS, DITECT Corporation, Tokyo, Japan). The evaluated sperm motility parameters were straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL × 100), flagellar beat cross frequency (BCF) and amplitude of lateral head (ALH). For evaluation of sperm motility parameters, motile sperm with a VSL of ≥25 μm/sec were selected, as it has been recognized that motile sperm having a VSL of less than 25 μm/sec are probably not penetrate to oocytes (Aitken, 1985; Holt *et al.*, 1985).

Experimental design

In experiment 2-1, to examine the effect of PHE mixture and theophylline on penetration of sex-sorted sperm to oocytes, matured COCs were co-incubated with sperm at a concentration of 2×10^6 cells/ml (bull G) for 12 h in IVF medium with or without PHE and/or theophylline.

In experiment 2-2, to examine the timing of sperm penetration, matured COCs were co-incubated with sex-sorted and non-sorted sperm at a concentration of 2×10^6 cells/ml (bulls G, H and J) for 12 and 18 h in IVF medium supplemented with PHE and theophylline.

In experiment 2-3, to examine sperm motility and sperm motility parameters, sex-sorted and non-sorted sperm (bull J) was cultured for 0, 2 and 4 h in IVF medium supplemented with PHE and theophylline.

In experiment 2-4, to evaluate embryonic development and developmental kinetics of embryos fertilized with sex-sorted and non-sorted sperm (bulls G, H and J), matured COCs were co-incubated with sperm at a concentration of 2×10^6 cells/ml for 18 h in IVF medium supplemented with PHE and theophylline. After IVF, presumptive zygotes were observed every 30 min until 48 h after IVF. Cell numbers in blastocysts were also examined.

Statistical analysis

Sperm penetration rates (total penetration, 2PN and polyspermy rates) were compared among groups by Chi-square test. The percentages of cleaved oocytes, development to blastocysts based on cleaved oocytes, mean cell numbers in blastocysts and average sperm motility parameters were compared by one-way ANOVA followed by Tukey-Kramer's HSD test as a *post hoc* test. All analyses were performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC).

Results

Experiment 2-1

Total penetration and 2PN rates in the presence of both theophylline and PHE in IVF medium were highest in all experimental groups (Table 2-1; $P<0.001$). When theophylline was added to IVF medium, total penetration rate was higher than those in the absence of theophylline ($P<0.001$).

Experiment 2-2

In bull G, total penetration rates in sex-sorted sperm at 12 and 18 h were lower than those in non-sorted sperm (Table 2-2; $P<0.05$). Percentage of 2PN in non-sorted sperm at 18 h was higher than that in sex-sorted sperm at 18 h ($P<0.05$). In bull H, total penetration and 2PN rates at 12 and 18 h in sex-sorted sperm were similar to those in non-sorted sperm. Polyspermy rate in sex-sorted sperm at 12 h was higher than that in non-sorted sperm ($P<0.05$); though there was no difference in the rate between sorted and non-sorted sperm after 18 h of incubation.

In bull J, total penetration and 2PN rates at 18 h in non-sorted sperm were tended to be higher than those in sex-sorted sperm ($P=0.09$ and 0.08, respectively). However, there were no differences in total penetration and 2PN rates at 12 h between sex-sorted and non-sorted sperm. Percentages of 2PN in sex-sorted and non-sorted sperm at 18 h were higher than those at 12 h ($P<0.05$). Total penetration rate in non-sorted sperm at 18 h was higher than that at 12 h ($P<0.05$).

Experiment 2-3

Sperm motility and sperm motility parameters of sex-sorted sperm (bull J) were shown in Fig. 2-1. Percentages of total motile sperm in sex-sorted and non-sorted sperm were similar in all incubation periods (Fig. 2-1 A). Percentage of motile sperm with a VSL of $\geq$25 μm/sec in sex-sorted sperm was similar to that in non-sorted sperm at 0 h (Fig. 2-1 B), but decreased after 2 h of incubation compared with non-sorted sperm ($P<0.05$). Means of VSL and LIN in sex-sorted sperm were lower than those

in non-sorted sperm at all incubation periods (Fig. 2-1 C and E; $P<0.05$), and mean of VCL in sex-sorted sperm at 2 h of incubation was lower than that in non-sorted sperm (Fig. 2-1 D; $P<0.05$). Mean of VAP in sex-sorted at 2 h of incubation was lower than that in non-sorted sperm (Fig. 2-1 F; $P<0.05$), and those at 0 and 4 h of incubation tended to be low ($P= 0.06$ and 0.06, respectively). At 0 h, mean of ALH in sex-sorted sperm was higher than that in non-sorted sperm (Fig. 2-1 G). At 2 and 4 h of incubation, means of BCF in sex-sorted sperm were lower than those in non-sorted sperm (Fig. 2-1 H; $P<0.05$), and that at 0 h was tended to be low ($P=0.08$).

Experiment 2-4

As shown in Table 2-3, in bulls G and J, cleavage rates and percentages of blastocysts (based on the number of cleaved oocytes) in sex-sorted sperm were lower than those in non-sorted sperm ($P<0.05$). In bull H, percentage of blastocysts in sex-sorted sperm was lower than that in non-sorted sperm ($P<0.01$), although the cleavage rate of oocytes inseminated with sex-sorted sperm were similar to that of non-sorted sperm.

The percentages of cumulative cleaved oocytes after fertilization were shown Fig. 2-2. In bull G, percentages of cumulative cleaved oocytes in sex-sorted sperm were lower than those in non-sorted sperm after 27 and 39 h of incubation ($P<0.05$). In bull H, percentages of cumulative cleaved oocytes between sex-sorted and non-sorted sperm were similar at all incubation periods. In bull J, percentages of cumulative cleaved oocytes in sex-sorted sperm were lower than those in non-sorted sperm after 33 h ($P<0.05$), except 42 h.

Discussion

In the present study, the addition of both PHE and theophylline to the IVF medium enhanced the penetration to oocytes of sex-sorted sperm. This result is consistent with the findings observed in chapter I using non-sorted sperm. However, in bull G, the total penetration rates in sex-sorted sperm at 12 and 18 h were significantly lower than those in non-sorted sperm. This indicates that the accelerating effect of PHE and theophylline on penetration to oocytes by sex-sorted sperm is low compared with that by non-sorted sperm. Also, percentage of 2PN in sex-sorted sperm in bull G did not increase from 12 to 18 h (43.4 to 49.6%), but that in non-sorted sperm increased significantly (59.8 to 82.4%). Therefore, pronuclear formation ability in sex-sorted sperm assumed to be lower than that in non-sorted sperm as described in a previous study (Jo *et al.*, 2014). In bull J, total penetration and 2PN rates at 12 h in sex-sorted sperm were similar to those in non-sorted sperm; however, total penetration rate in sex-sorted sperm did not increase from 12 to 18 h after IVF although that in non-sorted sperm increased. It indicates that sperm penetration is similarly accelerated in both sex-sorted and non-sorted sperms before 12 h of incubation, but sperm penetration ability of sex-sorted sperm is impaired from 12 to 18 h. In bull H, there were no differences in total penetration and 2PN rates in both kinds of sperms, but polyspermy rate in sex-sorted sperm at 12 h was higher than that in non-sorted sperm. The results indicate that sex-sorted sperm in bull H more easily penetrates to oocytes compared with non-sorted sperm. The author suspects that sex-sorted sperm of bull H may be easily capacitated in the IVF medium used in the study, and readily penetrate to oocytes compared with non-sorted sperm. The patterns of sperm penetration in 3 bulls indicate that sex-sorted sperm of individual bulls have different penetration ability to oocytes and some of them have low pronuclear formation ability compared with non-sorted sperm.

The results of sperm motility analyses showed that the similar level of motile sperm was recovered immediately after thawing in both sex-sorted and non-sorted sperm (59.7 and 64.7%, respectively). However, percentage of motile sperm (VSL of $\geq$25 μm/sec) in sex-sorted sperm was decreased

according to the duration of incubation were prolonged compared to that in non-sorted sperm. Hollinshead *et al.* (2003) also demonstrated that sex-sorted sperm had a lower motility and shorter lifespan *in vitro* than non-sorted sperm in ram. In addition, timed AI (Sales *et al.*, 2011) indicates that sex-sorted sperm have short longevity in oviduct compared to non-sorted sperm. Low LIN at 4 h in sex-sorted sperm also indicates lower progressive motility than that in non-sorted sperm in the present study. In addition, sex-sorted sperm showed higher ALH at 0 h, it means that sex-sorted sperm showed hyper activation-like changes immediately after thawing. These results indicate shorter longevity of sex-sorted sperm compared with non-sorted sperm in IVF medium supplemented with PHE and theophylline. In the present study, motility of sex-sorted sperm only from one bull was investigated, although the results of sperm penetration suggest that sex-sorted sperm may have various characteristics depending on bull. Therefore, in further study, characteristics of sex-sorted sperm from each bull should be investigated more in detail.

Embryos derived from the present IVF system using sex-sorted sperm showed lower cleavage and blastocyst rates (bulls G and J) or similar cleavage but lower blastocyst rates compared with non-sorted sperm (bull H). These results are consistent with a previous report that embryos fertilized with sex-sorted sperm showed low cleavage and low blastocyst development compared with those of non-sorted sperm (Bermejo-Alvarez *et al.*, 2010). Nevertheless, contrary to a previous study (Bermejo-Alvarez *et al.*, 2010), the obvious delay in the timing of first cleavage of embryos derived from sex-sorted sperm did not observed. It indicates that IVF system in the present study enhances penetration to oocytes by sex-sorted sperm, although blastocyst development of embryos derived from sex-sorted sperm has not been improved. The reasons of lower blastocyst development in sex-sorted sperm were not clear; however, one of the possibilities is a delay in PN formation in sex-sorted sperm.

In bull H, cleavage rate of oocytes fertilized with sex-sorted sperm was similar to that with non-sorted sperm, but blastocyst development became lower than that of non-sorted sperm. Fertilization status after 12 h of IVF showed higher polyspermy rate in sex-sorted sperm. The author speculates that this abnormality may become one of the causes of low blastocyst development in embryos

derived from sex-sorted sperm. It was reported that embryos derived from sex-sorted sperm showed similar cleavage rate, but lower blastocyst development than that from non-sorted sperm (Morton *et al.*, 2007; Blondin *et al.*, 2009). This result is comparable to the present result in bull H, although they did not examine the fertilization (Morton *et al.*, 2007; Blondin *et al.*, 2009). Morton *et al.* (2007) also reported that blastocysts derived from sex-sorted and non-sorted sperm showed different patterns of mRNA expression of developmentally important genes. Meanwhile, Bermejo-Alvarez *et al.* (2010) reported that there was no difference in the mRNA abundance of the genes related to embryo development. Thus, further investigations of mRNA expression in embryos derived from sex-sorted sperm should be carried out to elucidate impaired developmental competence.

In conclusion, addition of the combination of PHE and theophylline to IVF medium facilitates the penetration to oocytes by sex-sorted sperm. However, developmental competence of embryos derived from sex-sorted sperm was inferior to that of non-sorted sperm. Possible reasons for low blastocyst development are lower penetration and/or polyspermy caused by excessively enhanced fertilizability of sex-sorted sperm due to difference in the response to PHE and theophylline among bulls. Another possible reason of low blastocyst development may be due to low competence of pronuclear formation of oocytes fertilized with sex-sorted sperm. Therefore, for improving the normal fertilization, more suitable IVF system for sex-sorted sperm should be investigated.

Table 2-1. The effect of theophylline and PHE on sperm penetration with sex-sorted sperm at 12 h after IVF in bull G

Treatment		No. of oocytes (replicates)	Percentages of			
Theophylline	PHE		Total penetration	2PN	Poly	Others*
+	+	30 (3)	86.7[a]	43.3[a]	3.3	40.0[a]
+	−	30 (3)	33.3[b]	3.3[b]	0	30.0[a]
−	+	31 (3)	0[c]	0[b]	0	0[b]
−	−	31 (3)	0[c]	0[b]	0	0[b]

[a, b, c] Values with different scripts differ significantly (P<0.001).

*Others: an enlarged sperm head with anaphase II/telophase II chromosome or a male pronucleus was observed.

2PN: two pronuclei, Poly: penetrated with more than two sperm.

Table 2-2. Sperm penetration with sex-sorted and non-sorted sperm at 12 and 18 h after IVF in 3 bulls

Bulls	Sperm	IVF periods (h)	No. of oocytes (replicates)	Percentages of			
				Total penetration	2PN	Poly	Others*
G	Sex-sorted	12	42 (4)	79.2[a]	43.4	0	35.7
		18	52 (5)	78.2[a]	49.6[a]	9.3[a]	19.2
	Non-sorted	12	43 (4)	95.0[b]	59.8[x]	7.5	25.6
		18	61 (6)	95.0[b]	82.4[by]	0[b]	11.5
H	Sex-sorted	12	27 (3)	100	55.2	31.4[a]	14.8
		18	50 (5)	98.2	60.4	33.9	4.0
	Non-sorted	12	30 (3)	96.7	66.7	3.3[b]	26.7[x]
		18	32 (3)	97.0	75.5	21.5	0[y]
J	Sex-sorted	12	53 (5)	62.9	27.0[x]	8.0	28.3[x]
		18	75 (7)	68.3	47.9[y]	6.8	12.0[y]
	Non-sorted	12	40 (4)	59.6[x]	29.7[x]	14.5	17.5
		18	62 (6)	80.2[y]	62.6[y]	12.6	6.5

[a, b] Values with different letters differ significantly between sex-sorted and non-sorted sperm within same bulls at same incubation periods ($P<0.05$).

[x, y] Values with different letters differ significantly between different incubation periods within same bulls ($P<0.05$).

*Others: an enlarged sperm head with anaphase II/telophase II chromosome or a male pronucleus was observed. 2PN: two pronuclei, Poly: penetrated with more than two sperm.

Table 2-3. Embryonic development of oocytes fertilized with sex-sorted and non-sorted sperm in 3 bulls

Bulls	Sperm	No. of oocytes (replicates)	% cleavage	% blastocysts / cleaved oocytes	Mean cell numbers in blastocysts (n)
G	Sex-sorted	89 (3)	75.3 ± 1.7[a]	10.5 ± 7.1[a]	110.0 ± 41.6 (7)
	Non-sorted	90 (3)	83.3 ± 3.3[b]	38.5 ± 3.1[b]	133.2 ± 54.9 (22)
H	Sex-sorted	66 (3)	81.1 ± 6.3	10.2 ± 8.5[c]	105.4 ± 46.3 (5)
	Non-sorted	75 (3)	85.3 ± 2.3	37.4 ± 3.8[d]	128.9 ± 52.5 (24)
J	Sex-sorted	66 (3)	60.4 ± 5.1[a]	9.4 ± 11.0[a]	172.0 ± 77.8 (4)
	Non-sorted	67 (3)	80.5 ± 6.8[b]	43.3 ± 9.8[b]	145.1 ± 54.9 (23)

[a, b] Values (means ± standard deviation (SD)) with different scripts differ significantly in sex-sorted and non-sorted sperm of each bull ($P<0.05$).

[c, d] Values (means ± SD) with different scripts differ significantly in sex-sorted and non-sorted sperm of each bull ($P<0.01$).

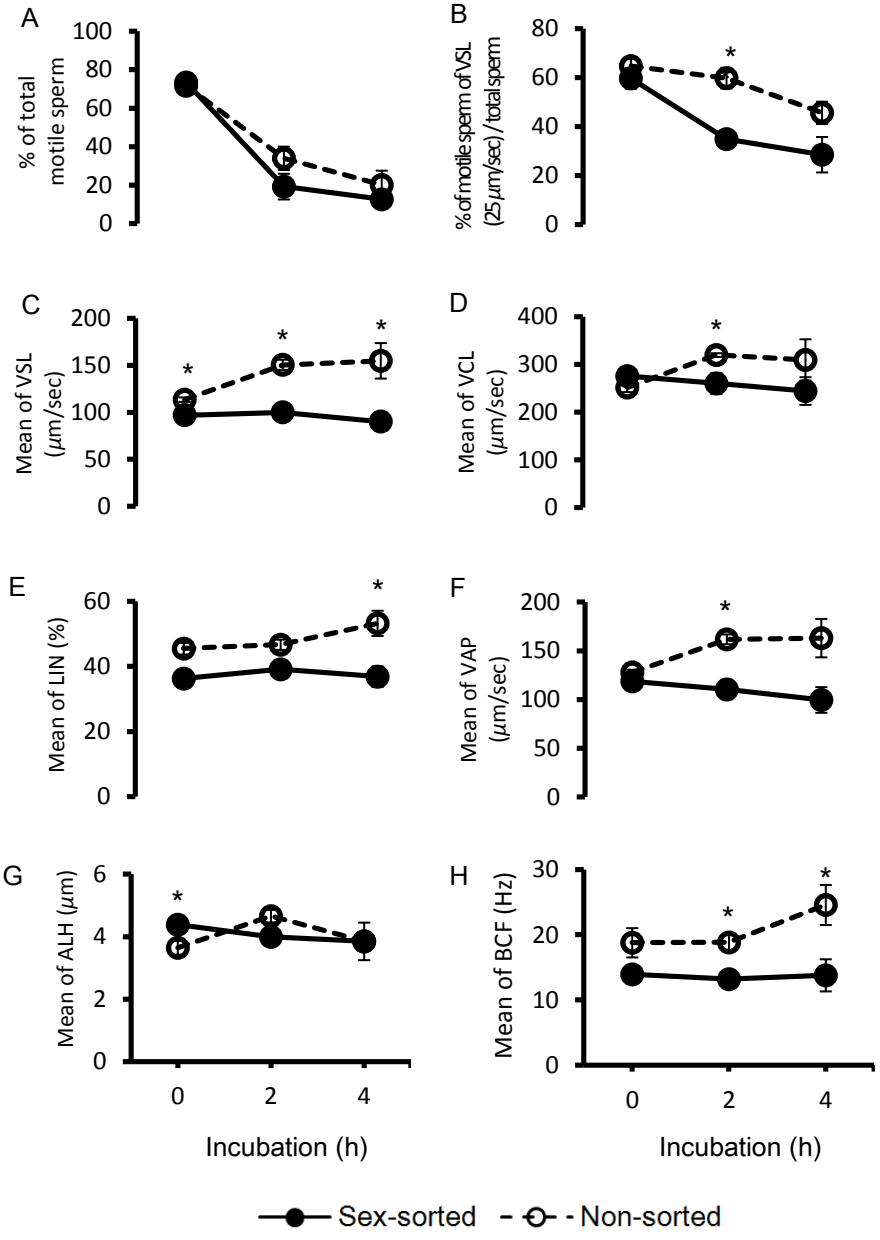

Fig. 2-1. The sperm motility and sperm motility parameters during incubation (bull J).

A, % of total motile sperm; B, % of motile sperm with a VSL of ≥25 μm/sec; C, mean VSL (μm/sec); D, mean VCL (μm/sec); E, mean LIN (%); F, mean VAP (μm/sec); G, mean ALH (μm); H, mean BCF (Hz).

Values in panels C to H were calculated by using sperm that showed a VSL of ≥25 μm/sec.

* Superscript means significant difference between sex-sorted and non-sorted sperm ($P<0.05$).

The error bar indicates the SEM. All data of experiments were conducted in 5 replicates.

VSL, straight line velocity; VCL, curvilinear velocity; VAP, average path velocity; LIN, linearity (VSL/VCL × 100); BCF, flagellar beat cross frequency; ALH, amplitude of lateral head.

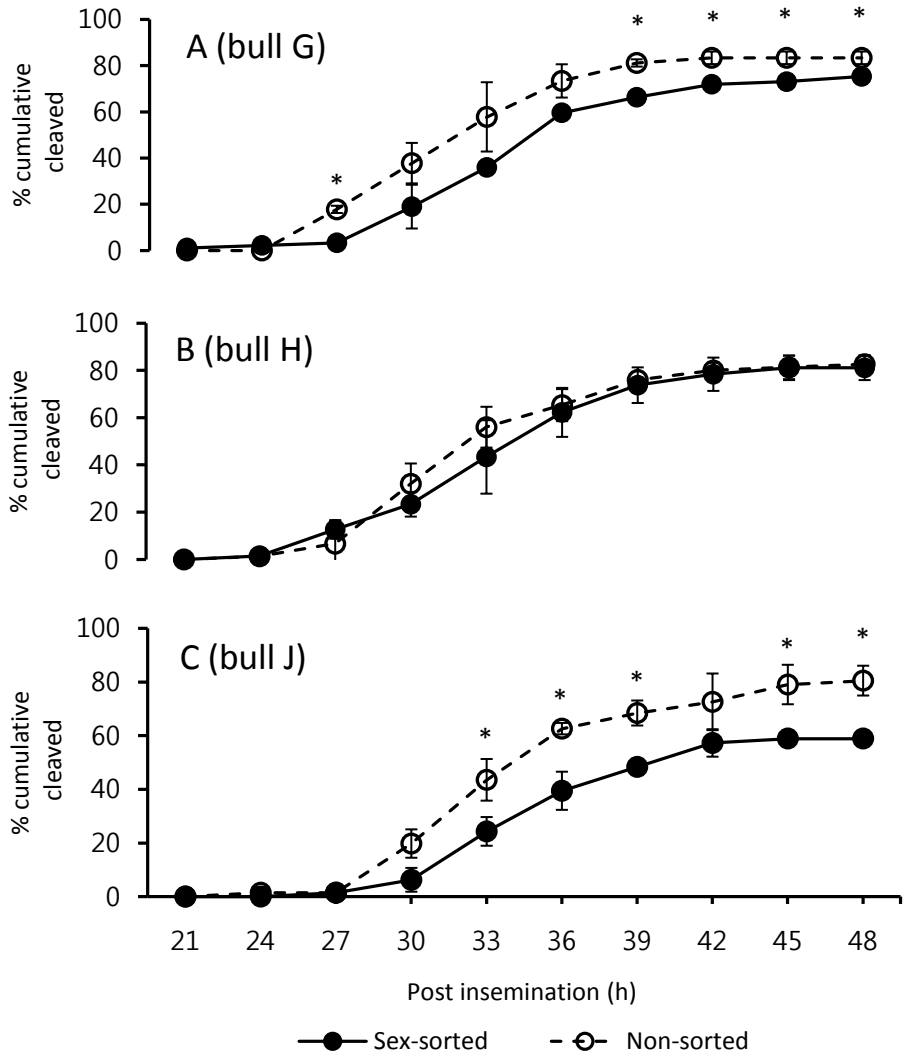

Fig. 2-2. The percentages of cumulative cleaved oocytes after fertilization with sex-sorted and non-sorted sperm from each of 3 bulls.

A, bull G (cleaved oocytes in sex-sorted *n*=67; non-sorted *n*=75); B, bull H (cleaved oocytes in sex-sorted *n*=53; non-sorted *n*=64); C, bull J (cleaved oocytes in sex-sorted *n*=40; non-sorted *n*=54). Percentage of cumulative cleaved oocytes at each incubation periods was calculated base on sum of 3 h interval. Percentage = (number of cleaved oocytes / number of fertilized oocytes) × 100.

* Superscript means significant difference between sex-sorted and non-sorted sperm ($P<0.05$).

All data of experiments were conducted in 3 replicates.

The error bar indicates the SEM.

Summary

The present study aimed to examine the sperm penetration of sex-sorted sperm using IVF system developed in chapter I and the developmental kinetics of embryos fertilized with sex-sorted sperm. In addition, to examine the characteristics of sex-sorted sperm in IVF medium with a PHE mixture and theophylline, motility of sex-sorted sperm was evaluated by computer assisted sperm analysis (CASA) system. IVF with a PHE mixture and theophylline synergistically enhanced total sperm penetration rate of sex-sorted sperm (86.7%) at 12 h compared to that with PHE or theophylline only ($P<0.001$; 0 or 33.3%, respectively). However, patterns of sperm penetration in sex-sorted sperm of 3 bulls indicate that individual bulls have different penetration ability and some of them have low pronuclear formation ability compared to non-sorted sperm. Evaluation of sperm quality by CASA revealed that sex-sorted sperm had short longevity compared to non-sorted sperm. The percentages of cleaved oocytes fertilized with sex-sorted sperm in 2 bulls were low compared those with non-sorted sperm, but that in one bull was similar. Blastocyst rates based on cleaved oocytes fertilized with sex-sorted sperm in 3 bulls were lower than those with non-sorted sperm ($P<0.05$). In conclusion, addition of both PHE and theophylline to IVF medium facilitates the penetration to oocytes by sex-sorted sperm. However, blastocyst development was not improved due to low fertilizability and/or polyspermy caused by excessively enhanced fertilizability of sex-sorted sperm, and may be due to low competence of pronuclear formation of oocytes fertilized with sex-sorted sperm.

Chapter III

The efficacy of well of the well (WOW) culture system on development of bovine embryos in a small group and the effect of number of adjacent embryos on their development

Introduction

During recent decades, ART in cattle have achieved considerable advances, such as a combination of IVP and OPU. An average of 4 to 6 available oocytes from a single cow is collected by OPU or a single slaughtered valuable oocyte donor cow (Merton *et al.*, 2009). However, most laboratories currently culture up to 20 embryos in a 20- to 50-μl droplet or up to 50 embryos in 400 to 500 μl in a well *in vitro* (Krisher & Wheeler, 2010; Vajta *et al.*, 2010) to obtain transferrable embryos, and small groups of 1 to 10 embryos in a droplet or a well have been shown to have low blastocyst rate and quality compared with large groups of 20 to 25 embryos (Donnay *et al.*, 1997; Ikeda *et al.*, 2000; Vajta *et al.*, 2000; Ward *et al.*, 2000; Nagao *et al.*, 2008; Senatore *et al.*, 2010). Therefore, a culture system for a small group of embryos is required to improve blastocyst yield. It was reported that, if embryos were cultured in a custom-made micro-well in a large well (WOW) (Vajta *et al.*, 2000; Matoba *et al.*, 2010) and in a commercially available polystyrene-based WOW (25 micro-wells) (Sugimura *et al.*, 2010), the blastocyst rate was significantly higher than that of droplet culture. In addition, Sugimura *et al.* (2013) demonstrated that the blastocyst rate in small group culture (5 embryos) in a WOW was higher than in a droplet.

Gopichandran & Leese (2006) reported that embryos surrounded by 3 or 8 adjacent embryos showed low blastocyst development compared with those surrounded by 5 adjacent embryos when bovine embryos were cultured in a group in a droplet. It was also reported that the number of adjacent embryos (3, 5 or 8) did not affect blastocyst development in a custom-made WOW dish (Matoba *et al.*, 2010). This discrepancy may have been caused by the large depth (500 μm) of

micro-wells of custom-made WOW dishes (Matoba *et al.*, 2010) because the overall diameter of embryos ranges from 150 to 190 μm (Linder & Wright, 1983). It is thought that usage of a commercially available WOW dish is preferable to achieve a high and stable blastocyst rate for culturing small numbers of embryos. However, an embryo in a micro-well (169 μm in depth) in the WOW dish developed by (Sugimura *et al.*, 2010) can be affected by factors outside of the micro-well. Thus, it should be examined the effect of the number of adjacent embryos on blastocyst production by a commercially available WOW dish.

In chapter III, the author examined the effects of the total number of embryos (5 vs. 25 embryos) and the number of adjacent embryos (0, 3, 5 and 8 embryos) in a WOW dish on blastocyst development.

Materials and Methods

Chemicals

All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation and fertilization

IVM of bovine oocytes was performed as described in previous chapters. In brief, COCs aspirated from follicles (2 to 8 mm in diameter) of slaughterhouse-derived ovaries were cultured for 22 h in a droplet (about 10 COCs/50 μl) of maturation medium under a humidified atmosphere of 5% CO_2 in air at 39˚C. Maturation medium consisted of HEPES-buffered TCM-199 (Invitrogen) supplemented with 10% FCS (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/ml follicle-stimulating hormone, 1 μg/ml estradiol-17β and 50 μg/ml gentamicin sulfate.

IVF was conducted according to a procedure described previously (Takahashi & Kanagawa, 1998). Briefly, after the thawing of frozen semen, motile sperm were separated using a Percoll gradient (45 and 90%). COCs were co-incubated with motile sperm (5×10^6 cells/ml) in droplets (10-13 COCs/100 μl) of mBO isotonic medium (Takahashi & First, 1992) containing 3 mg/ml fatty acid-free BSA and supplemented with 2.5 mM theophylline for 18 h at 39˚C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

In vitro culture of presumptive zygotes

IVC of presumptive zygotes was performed using the procedures that were basically the same as described previously (Takahashi & Kanagawa, 1998). After co-incubation with sperm, presumptive zygotes freed from cumulus cells by vortexing were washed 3 times and cultured for 150 h in droplets or a WOW dish using a modified synthetic oviduct fluid, which contained 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential

medium and 10 μg/ml insulin and was supplemented with 5 mM glycine, 5 mM taurine and 1 mM glucose (Takahashi & Kanagawa, 1998), and added 3 mg/ml fatty acid-free BSA instead of polyvinyl alcohol at 39˚C under 5% CO_2, 5% O_2 and 90% N_2. Polystyrene-based WOW dishes that have 25 micro-wells (5 × 5 micro-wells with 170-μm depth, 290-μm diameter and 400-μm distance between them; Dai Nippon Printing Co. Ltd., Tokyo, Japan) were prepared as described previously (Sugimura *et al.*, 2010). In brief, 125 μl of culture media were placed within the circular wall of WOW dish containing micro-wells and covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan). Twenty-five embryos were placed individually in each micro-well in a WOW dish. For the culturing of 5 embryos, 4 embryos were placed in the micro-well of 4 corners and the remaining one was put into the center micro-well in a WOW dish (no adjacent embryo).

To examine the effect of embryo number in a droplet and a WOW dish on the development to blastocysts, small or large numbers of embryos (5-6 or 25-26, respectively) inseminated by bull C sperm were cultured in a 40-μl droplet. Another large number of embryos inseminated by bull B sperm were cultured in a droplet (25-30 embryos/30 μl), and 5 or 25 embryos were cultured in a WOW. The effect of the number of adjacent embryos in a WOW was examined using data of 5- (0 adjacent embryos; Fig. 3 A) and 25-embryo culture with different numbers of adjacent embryos (3, 5 and 8 embryos; Fig. 3 B). Cleavage and blastocyst rates were assessed after 48 and 168 h of IVF, respectively. All embryos that developed to blastocysts were subjected to counting of their cell numbers using an air-drying method (Takahashi & First, 1992).

Statistical analysis

The frequencies of cleavage and development to blastocysts, and mean cell numbers in blastocysts were compared by one-way ANOVA followed by Tukey–Kramer's HSD as a *post hoc* test. Proportions of blastocysts among the embryo groups placed in different positions of a WOW dish (different numbers of adjacent embryos) were compared by Chi-square test. All analysis was performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC).

Results

As shown in Table 3-1, when embryos were cultured in a droplet, cleavage and blastocyst rates of the large-number group (25-26 embryos/droplet) tended to be higher than those of the small-number group (5-6 embryos/droplet) ($P=0.14$ and 0.07, respectively). In addition, the total cell number in blastocysts of the large-number group was significantly higher than that of the small-number group ($P<0.01$). When embryos were cultured in WOW, blastocyst rates were high regardless of embryo number in the WOW dish and similar to that of the large-number group in a droplet. Furthermore, the number of adjacent embryos in a WOW dish did not affect the development to blastocysts and total cell numbers in blastocysts, as shown in Table 3-2.

Discussion

Culturing small numbers of embryos in a droplet reduced the blastocyst rate and the quality of blastocysts (mean cell numbers in blastocysts) compared with those for embryos cultured with large numbers of embryos in a droplet (Nagao *et al.*, 2008; Senatore *et al.*, 2010). In the present study, there was no reduction in blastocyst rate and quality in a WOW dish. This result is in agreement with previous findings that the development of embryos to blastocysts was independent of the total number of embryos in a WOW dish (Sugimura *et al.*, 2013). The author speculated that diffusible factors like autocrine/paracrine growth factors released by embryos can be diffused in a droplet and influence the growth of their adjacent embryos (Stokes *et al.*, 2005). A small amount of autocrine/paracrine factors may be secreted by a small number of embryos, would be easily diluted in a droplet, and would show few effects on embryonic development. However, in this WOW dish, diffusible factors secreted by individual embryos probably accumulated in a micro-well, which may provide a suitable microenvironment for their development, as suggested in a previous study (Swain & Smith, 2011). Moreover, adjacent embryos in a WOW dish also did not affect blastocyst development and mean cell numbers in blastocysts in the present study, as previously described (Matoba *et al.*, 2010), even though the depth of the micro-well was different.

In conclusion, the polystyrene-based WOW dish used in this study is effective for individual embryo culture of small groups, and there is no reduction in embryo developments, regardless of the number of adjacent embryos (position of embryos) in micro-wells of the WOW.

Table 3-1. Effect of embryo number in a droplet and a WOW dish on embryonic development

Bulls	Culture system	No. of embryos cultured	No. of embryos (replicates)	% cleaved	% blastocysts / inseminated oocytes	Mean cell numbers in blastocysts (n)
C	droplet	25-26	102 (4)	85.4 ± 7.8	38.3 ± 1.7	186.1 ± 63.5[a] (39)
	droplet	5-6	97 (4)	71.0 ± 15.0	28.8 ± 8.5	142.1± 64.1[b] (28)
B	droplet	25-30	197 (7)	87.8 ± 4.4	41.2 ± 8.4	166.0 ± 59.9 (82)
	WOW	5	75 (3)	89.3 ± 10.1	41.3 ± 12.2	147.0 ± 56.7 (31)
	WOW	25	100 (4)	82.0 ± 5.2	45.0 ± 8.9	151.9 ± 45.7 (45)

[a,b] Values (means ± SD) with different superscripts differ significantly ($P<0.01$).

Table 3-2. Effect of number of adjacent embryos (position of embryos) in a WOW on the development of embryos to blastocysts and their cell numbers

No. of cultured embryos in a WOW	No. of adjacent embryos	No. of embryos	% blastocysts / inseminated oocytes (n)	Mean cell numbers in blastocysts (n)
25	8	36	41.7 (15)	151.3 ± 44.1 (15)
	5	48	47.9 (23)	150.9 ± 46.8 (23)
	3	16	43.8 (7)	156.6 ± 52.1 (7)
5	0	75	41.3 (31)	147.0 ± 56.7 (31)

Data were pooled from 4 replicates of culture of 25 embryos and 3 replicates of culture of 5 embryos with WOW (bull B) in Table 3-1.

Cell numbers in blastocysts are means ± SD.

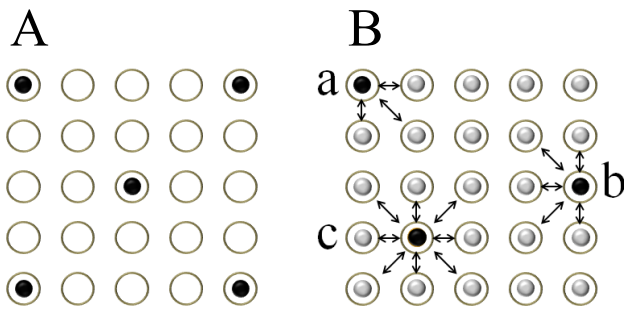

Fig. 3. Different numbers of adjacent embryos in 25 micro-wells in a WOW dish.

A: Embryo with no adjacent embryos.

B: a) Embryo with 3 adjacent embryos. b) Embryo with 5 adjacent embryos. c) Embryo with 8 adjacent embryos.

Summary

The aim of the present study was to clarify the efficacy of the WOW culture system for small numbers of embryos and the effect of number of adjacent embryos in a WOW dish on blastocyst development. In conventional droplet culture, embryos in the small-number group (5-6 embryos/droplet) showed low blastocyst development compared with a control group (25-26 embryos/droplet). However, small and large numbers of embryos (5-6 and 25 embryos, respectively) in a WOW dish showed no significant differences in cleavage, blastocyst rates, and mean cell number in blastocysts compared to the control group (25-30 embryos/droplet). In addition, the number of adjacent embryos in a WOW dish did not affect the development to blastocysts and cell number in blastocysts. In conclusion, a WOW dish can provide high and stable blastocyst development in small group culture wherever embryos are placed in micro-wells of the WOW dish.

Summary and Conclusions

Bovine embryo production by *in vitro* fertilization (IVF) with sex-sorted sperm is an effective method for production of sex-predetermined offspring and it meets demand of dairy and beef industries. In bovine IVF system, heparin has been widely used for capacitation of sperm. However, IVF with heparin shows big variations of sperm penetration to oocytes and embryo development between bulls, so the concentrations of sperm and heparin have to be optimized for individual bulls. If IVF system without optimization of concentration of sperm and heparin is developed, the efficiency of *in vitro* production (IVP) of embryo will be improved. In addition, repeated oocyte collection by transvaginal ultrasound-guided follicular aspiration (Ovum pick-up: OPU) combined with IVP has become alternative to superovulation for embryo production in cattle. However, due to small numbers (about 5 to 6) of immature oocytes can be recovered by OPU, an effective culture method for small numbers of embryos are required. In this thesis, the author conducted to establish new IVF system, which can obtain stable embryo development without optimization of sperm concentration in any bull. Also, influences of simultaneous addition of penicillamine, hypotaurine, and epinephrine (PHE) supplemented to IVF media including theophylline on fertilization and embryo development were investigated both for sex-sorted and non-sorted sperm. Moreover, the efficacy of well of the well (WOW) system was investigated for culturing of small numbers of embryos.

In chapter I, a PHE mixture was added to IVF media including theophylline and the effect on fertilization and embryo development were investigated using non-sorted sperm. Further, to estimate sperm quality in the IVF medium with PHE and theophylline, motility and sperm motility parameters were investigated by computer assisted sperm analysis (CASA) system. A combination of PHE and theophylline synergistically accelerates sperm motility and penetration ability. The synergistic effect of PHE and theophylline on normal fertilization was clarified after IVF with non-sorted sperm in concentration of 1×10^6 cells/ml at 12 h. Theophylline activates sperm motility with

increasing intracellular cAMP content in sperm and PHE prevents an excessive increase of intracellular cAMP contents in sperm. In addition, when a combination of PHE and theophylline is added to IVF medium at sperm concentration of 2×10^6 cells/ml, relatively high normal fertilization rate (68.2 to 82.4%) from 3 bulls at 18 h after IVF, and high cleavage rate (78.3 to 92.4%) and blastocyst developmental rate (31.9 to 62%) from 9 bulls were achieved. These results indicate that IVF medium with PHE and theophylline at sperm concentration of 2×10^6 cells/ml can produce stably high rate of normal fertilization and blastocyst development using sperm collected from any bull.

In chapter II, to confirm the efficacy of IVF system established in chapter I on IVF using sex-sorted sperm, sperm penetration rates and developmental kinetics of embryos fertilized with sex-sorted sperm in 3 bulls were investigated. Moreover, motility of sex-sorted sperm in supplemented IVF medium was evaluated by CASA system. IVF with PHE mixture and theophylline synergistically enhanced total sperm penetration rate of sex-sorted sperm compared to that with PHE or theophylline only. However, patterns of sperm penetration in sex-sorted sperm of 3 bulls indicate that individual bulls have different penetration abilities and some of them have low pronuclear formation ability compared to non-sorted sperm. The percentages of cleaved oocytes fertilized with sex-sorted sperm in 2 bulls were low compared with those in non-sorted sperm, but it was similar in one bull left. Blastocyst development based on the number of cleaved oocytes fertilized with sex-sorted sperm in 3 bulls was low compared to that with non-sorted sperm ($P<0.05$). Evaluation of sperm quality by CASA revealed that sex-sorted sperm have short longevity compared to non-sorted sperm. The results suggest that an addition of both PHE and theophylline to IVF medium facilitates the penetration to oocytes by sex-sorted sperm. However, blastocyst development for sorted sperm was inferior to that of non-sorted sperm due to low penetration and/or polyspermy caused by excessively enhanced fertilizability of sex-sorted sperm, and may be due to low competence of pronuclear formation of oocytes fertilized with sex-sorted sperm.

In chapter III, the efficacy of well of the well (WOW) system was investigated when small numbers of embryos were cultured in WOW. Culturing of small numbers of embryos in WOW showed high

and stable blastocyst development, and that embryos in WOW did not affected by number of adjacent embryos. Thus, a WOW system can provide high and stable blastocyst development in small group culture wherever embryos are placed in micro-wells of the WOW dish.

In the present study, the author has developed new IVF system which can achieve stable fertilization and blastocyst development using non-sorted sperm from any bull, and has clarified the efficiency of the WOW system to embryo development in individual and small number culture of bovine embryos. Practitioners can achieve high yields of bovine embryos by using the present IVF system and the WOW system without concerning the characteristics of the sperm they uses. However, when sex-sorted sperm was served to the present IVF system, the development to blastocysts was not enhanced although sperm penetration to oocytes was facilitated. It is considered to be necessary to improve the present IVF system for sex-sorted sperm.

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