CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD15289

Effect of spermatozoa motility hyperactivation factors and gamete coincubation duration on *in vitro* bovine embryo development using flow cytometrically sorted spermatozoa

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Abstract. The aim of the present study was to evaluate the effects of sperm motility enhancers and different IVF times on cleavage, polyspermy, blastocyst formation, embryo quality and hatching ability. In Experiment 1, sex-sorted X chromosome-bearing *Bos taurus* spermatozoa were incubated for 30 min before 18 h fertilisation with hyperactivating factors, namely 10 mM caffeine (CA), 5 mM theophylline (TH), 10 mM caffeine and 5 mM theophylline (CA + TH); and untreated spermatozoa (control). In Experiment 2, matured *B. taurus* oocytes were fertilised using a short (8 h) or standard (18 h) fertilisation length, comparing two different fertilisation media, namely synthetic oviducal fluid (SOF) fertilisation medium (SOF-FERT) and M199 fertilisation medium (M199-FERT). Cleavage and blastocyst formation rates were significantly higher in the CA + TH group (77% and 27%, respectively) compared with the control group (71% and 21%, respectively). Cleavage rates and blastocyst formation were significantly lower for the shortest fertilisation time (8 h) in M199-FERT medium (42% and 12%, respectively). The SOF-FERT medium with an 8 h fertilisation time resulted in the highest cleavage rates and blastocyst formation (74% and 29%, respectively). The SOF-FERT medium produced the highest embryo quality (50% Grade 1) and hatching rate (66%). Motility enhancers did not affect polyspermy rates, whereas polyspermy was affected when fertilisation length was extended from 8 h (3%) to 18 h (9%) and in M199-FERT (14%) compared with SOF-FERT (6%). We conclude that adding the motility enhancers CA and TH to sex sorted spermatozoa and Tyrode's albumin lactate pyruvate (TALP)-Sperm can improve cleavage and embryo development rates without increasing polyspermy. In addition, shortening the oocyte–sperm coincubation time (8 h) resulted in similar overall embryo performance rates compared with the prolonged (18 h) interval.

Additional keywords: IVF, sexed spermatozoa.

Received 17 July 2015, accepted 1 December 2015, published online 24 February 2016

Introduction

Unlike spermatozoa from other animal species, mammalian spermatozoa are unable to fertilise oocytes immediately after ejaculation. Spermatozoa must undergo a cascade of biochemical and physiological changes to acquire fertilising competence, called capacitation (Austin 1952; Bedford 1970). Capacitation gives the spermatozoa the ability to achieve hyperactive motility, interact with the zona pellucida (ZP), undergo the acrosome reaction and fuse with the oocyte's plasma membrane (Yanagimachi 1989, 1994*b*, 2011; Bailey 2010). Capacitation occurs in the female reproductive tract, but can also be realised *in vitro* (Coy *et al.* 2012; Parrish 2014).

Sperm capacitation is partially accomplished by removing decapacitating factors present in the seminal plasma. These factors modulate the fertilising ability of the spermatozoa by preventing the premature onset of capacitation (Bailey 2010;

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Fraser 2010). Furthermore, the removal of decapacitation factors adhering to the sperm plasma membrane causes biochemical and structural alterations leading to changes in the lipid composition of the sperm membrane and internal pH, increasing permeability to calcium and increased cellular metabolism and thus supporting the changing pattern of sperm motility and velocity (hyperactivation; Begley and Quinn 1982; Visconti *et al.* 1998; Suarez 2008).

Several biochemical candidates are recognised as capacitating factors. In Bovidae, the most commonly used *in vitro* capacitation factor is heparin (Parrish *et al.* 1988). Heparin stimulates the increase in intracellular calcium, pH and cAMP (Breininger *et al.* 2010). In addition to heparin, caffeine has been widely used as an *in vitro* fertilisation supplement to induce sperm capacitation and to increase sperm motility (Niwa and Ohgoda 1988; Park *et al.* 1989).

The enzyme phosphodiesterase (PDE) metabolises cAMP to 5′-AMP. PDE is present in mature spermatozoa (Visconti and Kopf 1998). Caffeine and theophylline are PDE inhibitors and their actions can lead to an increase in cAMP levels. Caffeine and heparin could act synergistically to induce sperm capacitation and, along with bovine serum albumin (BSA), promote the acrosome reaction and gamete fusion *in vitro* (Park *et al.* 1989; Parrish 2014). The use of xanthine derivatives, such as caffeine and theophylline, for pharmacological stimulation of sperm motility (hyperactivation) has had great impact on assisted reproduction techniques (ARTs) in humans when semen samples had slow sperm motility (Loughlin and Agarwal 1992; Lanzafame *et al.* 1994; Henkel and Schill 2003; Henkel 2012).

Two bovine IVF systems in particular are popular among research and commercial users: (1) a short sperm–oocyte coincubation time (≤8 h); and (2) a prolonged sperm–oocyte coincubation time (>16 h). Short exposure of oocytes to spermatozoa may improve IVF outcome by enhancing embryo quality (Berland *et al.* 2011), but can lead to lower cleavage and embryo development rates (Ward *et al.* 2002; Kochhar *et al.* 2003). Overnight sperm–oocyte coincubation is a routine practice in IVF laboratories. During this prolonged interaction (>16 h), oocytes and zygotes may be exposed to suboptimal culture conditions with increased reactive oxygen species (ROS) produced by dead spermatozoa (Baker and Aitken 2004; Dalvit *et al.* 2005; Tsunoda *et al.* 2014).

Currently, sex-sorted spermatozoa (SS) have been successfully used in most mammalian farm species (Johnson 1991; Hohenboken 1999; Lindsey *et al.* 2001; Evans *et al.* 2004), although the major positive economic impact has been noted in the dairy sector (Weigel 2004; De Vries *et al.* 2008). However, there are still some inefficiencies because of the sperm sex-sorting process (Seidel and Garner 2002; Rath *et al.* 2009; Gosalvez *et al.* 2011*a*, 2011*b*). Among them, after flow cytometric sorting of X and Y chromosome-bearing spermatozoa, the sorted sperm sample shows a marked reduction in post-thaw motility and average lifespan (Hollinshead *et al.* 2003; Suh and Schenk 2003; Suh *et al.* 2005). This could be a consequence of the DNA staining protocol, laser exposure, electrical charge disruption, flow cytometer pressure forces, a dilution effect, the freezing–thawing process and/or the accumulation of insults ('addition effect') that affect or compromise the essential sperm functions and fertilising capacity (Amann 1999; Schenk *et al.* 1999; Garner 2001, 2006, 2009; Suh *et al.* 2005; Mocé *et al.* 2006; Cran 2007; Schenk and Seidel 2007; Rath *et al.* 2009; Gosalvez *et al.* 2011*a*, 2011*b*; Garner *et al.* 2013). In addition, a high variation of sex-sorting efficiencies with regard to post sex-sorting sperm quality and embryo production among bulls has been reported (Zhang *et al.* 2003; Lu and Seidel 2004; Barceló-Fimbres *et al.* 2011). This variability may be reduced to acceptable levels using motility-stimulating factors and optimising sperm–oocyte incubation time. The main objective of the present study was to evaluate the effects of sperm motility enhancers and different exposure periods of bovine oocytes to sex-sorted spermatozoa in relation to cleavage, polyspermy, blastocyst formation, embryo quality and hatching ability.

Materials and methods

Unless otherwise stated, the chemicals used in the present study were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).

Oocyte recovery and IVM

Dairy cattle (*Bos taurus*) ovaries were collected from a slaughterhouse (Cargill, Fresno, CA, USA) and transported to the laboratory in an insulated container filled with prewarmed saline solution at approximately 32°C. The ovaries were washed several times and placed in a water bath at (37°C) in saline solution for oocyte aspiration. Oocytes were aspirated from 2–6-mm antral follicles using a 21-G butterfly needle connected to a vacuum pump. Cumulus–oocyte complexes (COCs) containing compact and complete cumulus cell layers were selected and matured in groups of 50 COCs in 400 μL M199 medium supplemented with alanyl-L-glutamine (ALA-glutamine) (0.1 mM), Na pyruvate (0.2 mM), gentamicin (5 μg mL^{-1}), epidermal growth factor (50 ng mL^{-1}), ovine (o) FSH (50 ng mL^{-1}), bovine (b) LH (3 μg mL^{-1}), cysteamine (0.1 mM) and 10% fetal bovine serum (FBS; Hyclone, GE Healthcare, South Logan, UT, USA). IVM was performed for 22–24 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

IVF

Fertilisation (Day 0) was performed using frozen–thawed X chromosome-bearing (hereafter referred to as 'female') sex-sorted Jersey (*B. taurus*) spermatozoa (Genex Cooperative, Shawano, WI, USA). Straws were thawed at 37°C for 45 s and then placed in an 80%–40% discontinuous density gradient (PureSperm; Spectrum Technologies, Healdsburg, CA, USA) for centrifugation (700*g*, 15 min) at room temperature. Next, the sperm sample was incubated for 30 min in Tyrode's albumin lactate pyruvate (TALP)-Sperm before fertilisation in one of the motility hyperactivation treated or untreated groups (a detailed description is provided below in the experimental design section). Then, a second centrifugation (300*g*, 5 min, 22–23°C) was performed after discarding the supernatant and resuspending the sperm pellet in TALP-Sperm (pH = 7.4, 295 mOsmol; Parrish *et al.* 1986, 1988). Matured groups of 15–20 COCs were washed twice with synthetic oviducal fluid (SOF)–HEPES and placed in

50 µL fertilisation medium. The final sperm concentration was adjusted to 1×10^6 spermatozoa mL^{-1} using a haemocytometer. The fertilisation medium was supplemented with BSA (essentially fatty acid free; 6 mg mL^{-1}), fructose (90 µg mL^{-1}), penicillamine (3 µg mL^{-1}), hypotaurine (11 µg mL^{-1}) and heparin (20 µg mL^{-1}; Ferré *et al.* 2015). Oocytes were coincubated with spermatozoa at 38.5°C in humidified atmosphere of 5% CO_2 in air.

Assessment of the nuclear status of presumptive zygotes

After IVF, presumptive zygotes were denuded mechanically by pipetting and washing three times in synthetic oviducal fluid (SOF)–HEPES. Thereafter, a minimum of 10 zygotes were collected from each treatment group and fixed for evaluation of polyspermy (Experiment 1, $n = 580$; Experiment 2, $n = 720$). Briefly, after washing three times in phosphate-buffered saline (PBS)–polyvinyl alcohol (PVA; 1 mg mL^{-1}), zygotes were fixed in 4% paraformaldehyde for 10 min and then washed in PBS-PVA five times. Nuclear staining was performed using 100 µg mL^{-1} Hoechst 33342 for 10 min, after which zygotes were washed three times with PBS-PVA and then mounted on glass slides. Slides were examined and zygotes were classified using an epifluorescent microscope at ×400 magnification. Embryos with two pronuclei (PN-2) were considered to have been fertilised normally, whereas those with three or more pronuclei (PN-3) were considered to be polyspermic.

*Embryo culture (*in vitro *culture)*

Presumptive zygotes were denuded mechanically by pipetting through a small-bore plastic pipette tip (Research Instruments, Cornwall, UK) and cultured in groups of 15–20 in 50-µL drops of potassium simplex optimised medium supplemented with amino acids (KSOMaa; pH = 7.4, 275 mOsmol; Evolve ZEBV-100; Zenith Biotech, Guilford, CT, USA) for 9 days (Biggers *et al.* 2000). On Day 3, 3% FBS was added to the culture medium containing the zygotes. The culture conditions were 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. On Days 7 and 9, blastocysts (BL) and hatched embryos, respectively, were evaluated morphologically according to International Embryo Transfer Society (Stringfellow *et al.* 2010) standards and recorded.

Experimental design

Experiment 1: effects of sperm motility hyperactivation factors on in vitro *embryo performance*

To examine the effect of different sperm motility hyperactivation factors on cleavage, embryo development, embryo quality and hatching ability, frozen–thawed sex-sorted spermatozoa that had been centrifuged and passed through a density gradient (as described above) were incubated for 30 min in TALP-Sperm before fertilisation in one of the following groups: (1) 10 mM caffeine (CA); (2) 5 mM theophylline (TH); (3) 10 mM caffeine and 5 mM theophylline (CA+TH); and (4) untreated (Control). After incubation, samples were centrifuged at 300*g* for 5 min at 22–23°C. The supernatant was then discarded and the sperm pellet was resuspended with the final fertilisation medium. All matured oocytes were fertilised using a modified SOF-FERT (Tervit *et al.* 1972) for 18 h. The final formulation of the SOF-FERT was as follows: 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM, $MgCl_2$, 1.17 mM $CaCl_2$, 5.3 mM sodium lactate, 25.07 mM $NaHCO_3$, 0.20 mM sodium pyruvate, 0.5 mM fructose, 5 µg mL^{-1} gentamicin, 20 µg mL^{-1} heparin and 6 mg mL^{-1} essentially fatty acid-free BSA. Sex-sorted X chromosome-bearing spermatozoa from three bulls were used to fertilise the COCs. Three replicates were performed for each motility hyperactivation factor and bull.

Experiment 2: in vitro *embryo production using short versus long gamete IVF duration co-incubation time and two different fertilisation media*

After maturation, oocytes were randomly divided into four groups according to fertilisation medium (M199-FERT and SOF-FERT) and oocyte–sperm coincubation time (8 and 18 h). The groups, M199:8 ($n = 1140$), M199:18 ($n = 1140$), SOF:8 ($n = 1190$) and SOF:18 ($n = 1280$), were supplemented with the sperm motility hyperactivation factor that produced the best results in the previous experiment. The main components of each fertilisation medium are given in Table 1. Sex-sorted X chromosome-bearing sperm samples from three bulls were used to fertilise the COCs. Four replicates were performed for all treatments and bulls.

Statistical analysis

Data were analysed using a generalised linear mixed-effects model with logit-link binomial distribution in order to consider the complete experimental design and technical constraints. InfoStat

Table 1. Components of the M199 and synthetic oviducal fluid (SOF) *in vitro* fertilisation media (FERT)

BSA-FAF, fatty acid-free bovine serum albumin

Component	M199-FERT (Gibco 11043–023)	SOF-FERT (Tervit *et al.* 1972)
NaCl (mM)	117.24	107.7
KCl (mM)	5.33	7.16
KH_2PO_4 (mM)	–	1.19
NaH_2PO_4 (mM)	1.01	–
$MgCl_26H_2O$ (mM)	–	0.49
$MgSO_47H_2O$ (mM)	0.814	–
$CaCl_22H_2O$ (mM)	1.8	1.17
$NaHCO_3$ (mM)	26.19	25.0
Glutathione (mM)	0.000163	–
Sodium lactate (mM)	–	5.3
L-Ascorbic acid (mM)	0.000284	–
L-Glutamine (mM)	0.685	–
Glucose (mM)	5.6	–
Amino acids (present)	Yes	No
Vitamin (present)	Yes	No
Gentamicin (µg mL^{-1})	5	5
Na-pyruvate (mM)	0.2	0.2
Fructose (mM)	0.5	0.5
Heparin (µg mL^{-1})	20	20
BSA-FAF (mg mL^{-1})	6	6
pH (adjusted)	7.4	7.4
Osmolality (mOsmol)	290–300	280–290

software version 2011 (Di Rienzo *et al.* 2011) and R (R Development Core Team 2014) packages were used for model estimation starting from the maximal linear predictor (η) for each output variable of interest (cleavage, blastocyst total and grade, hatching and polyspermy) according to the experimental setting:

$$\eta_{ijkl} = \mu + \tau_i + b_j + d_k \qquad \text{(Experiment 1)}$$

$$\eta_{mnkl} = \mu + \alpha_m + \beta_n + \alpha_m \times \beta_n + (1 + \alpha_m + \beta_n + \alpha_m \times \beta_n) \times d_k \qquad \text{(Experiment 2)}$$

where the linear predictor η_{ijkl} models an overall mean (μ) and the contribution of the *i*th motility enhancer treatment (τ_i) as fixed effects, whereas the random intercept effects consider the *j*th bull $b_j \sim N(0, \sigma_b^2)$ and day $d_k \sim N(0, \sigma_d^2)$ for the *l*th replicate for Experiment 1. In contrast, the linear predictor η_{mnkl} of Experiment 2 was modelled using as fixed effects an overall mean (μ), the *m*th fertilisation medium (α_m), the *n*th oocyte–sperm coincubation time (β_n) and the interaction term ($\alpha_m \times \beta_n$), whereas the random effects consider the *k*th day $d_k \sim N(0, \Sigma)$ jointly estimated for the intercept, double and triple interactions $(1 + \alpha_m + \beta_n + \alpha_m \times \beta_n) \times d_k$, respectively.

Fixed effects model assessment was undertaken using Type III analysis of variance (ANOVA) sum of squares using $P < 0.05$ and a likelihood ratio test approach for model selection, following a parsimony criterion (i.e. obtain the simplest model that copes with the data). Final model assumptions were validated using quantile–quantile and deviance versus predicted value plots.

Fixed effects model results for each output variable are reported as the mean proportion ± s.e.m. for sperm motility enhancers (Experiment 1), time, fertilisation medium and time × fertilisation medium interaction term (Experiment 2). Only significant terms were used to report the results (i.e. in some models the treatment results were similar). For example, if in Experiment 1 there was no contribution of motility enhancer treatment, only the overall mean would remain thus the proportion would be exactly the same no matter what enhancer was used. However, if significant terms were present, *a posteriori* proportion differences were determined using Fisher's least significant difference (l.s.d.) test with Bonferroni correction. Differences were considered significant at the 95% confidence level ($P < 0.05$) or a tendency at the 90% level ($P < 0.1$).

Results

In all, 3273 IVM bovine oocytes were fertilised with sex-sorted spermatozoa supplemented with different motility hyperactivation factors. The cleavage, blastocyst development, blastocyst quality and hatching rates of bovine oocytes coincubated for 16–18 h with sex-sorted spermatozoa are given in Table 2 (Experiment 1). The respective fixed-effects model results for sperm motility hyperactivation factors on oocyte cleavage, embryo development, embryo quality and hatching ability after oocyte fertilisation using sex-sorted spermatozoa are given in Table 3. There was a tendency for a difference in cleavage rates between the CA+TH and control groups ($P < 0.1$), whereas blastocyst formation was significantly higher in the CA+TH versus control group ($P < 0.05$). According to model results, blastocyst grades and embryo hatching rates (Table 3) did not differ significantly among different sperm motility hyperactivation factors.

Cleavage, blastocyst rates, blastocyst quality and hatching rates of bovine oocytes coincubated for different periods of time

Table 2. Effects of sperm motility hyperactivation factors on oocyte cleavage, embryo development, embryo quality and hatching ability after oocyte fertilisation using sex-sorted spermatozoa

Data are the mean ± s.d. CA, caffeine (10 mM); TH, theophylline (5 mM)

Motility enhancers	Oocytes (n)	Cleavage (%)	Blastocysts (%)				Hatching (%) (Day 9)
			Total (Day 7)	Grade I	Grade II	Grade III	
CA	842	55.36 ± 16.25	17.64 ± 6.05	9.55 ± 3.01	4.09 ± 1.51	4.00 ± 2.10	10.73 ± 3.13
TH	887	52.36 ± 16.66	15.91 ± 4.21	9.82 ± 2.68	3.45 ± 1.04	2.64 ± 1.12	10.91 ± 2.39
TH + CA	779	53.55 ± 17.37	18.36 ± 3.78	10.91 ± 2.34	4.36 ± 1.43	3.09 ± 1.14	12.45 ± 2.88
Control	765	57.36 ± 17.03	16.45 ± 4.30	8.73 ± 2.45	4.36 ± 1.63	3.36 ± 1.29	10.36 ± 3.23

Table 3. Fixed-effects model results (Experiment 1) for sperm motility hyperactivation factors on oocyte cleavage, embryo development, embryo quality and hatching ability after oocyte fertilisation using sex-sorted spermatozoa

Data adjusted by model are expressed as the mean proportion ± s.e.m. × 100. Within columns, values with different superscript lowercase letters differ significantly ($P < 0.05$); values with different uppercase letters exhibited a tendency to differ ($P < 0.1$). Statistical analyses were performed using Fisher's least significant difference tests with Bonferroni correction. CA, caffeine (10 mM); TH, theophylline (5 mM)

Motility enhancers	Oocytes (n)	Cleavage (%)	Blastocysts (%)				Hatching (%) (Day 9)
			Total (Day 7)	Grade I	Grade II	Grade III	
CA	842	72.36 ± 1.79^{AB}	23.94 ± 3.01^{ab}	57.06 ± 1.84	23.80 ± 1.55	19.06 ± 1.57	65.02 ± 1.73
TH	887	74.02 ± 1.80^{AB}	23.43 ± 3.01^{ab}				
TH + CA	779	77.13 ± 1.71^{A}	27.26 ± 3.28^{a}				
Control	765	71.12 ± 1.79^{B}	20.94 ± 2.75^{b}				

with sex-sorted spermatozoa (Experiment 2) are presented in Table 4. The respective fixed-effects model results for gamete coincubation time, medium and time × fertilisation medium interaction term (Experiment 2) on IVF performance and polyspermy after oocyte fertilisation with sex-sorted spermatozoa are given in Table 5. Based on model results, blastocyst grades and hatching rates were not significantly affected by sperm motility enhancers. Similar results were seen for gamete coincubation times (8 vs 18 h; Table 5), whereby total blastocyst, blastocysts Grades I and III and hatching rates were not significantly different between the two coincubation times. Furthermore, the blastocyst Grade II rate remained constant, showing no variation regardless of the coincubation time or fertilisation medium used. The cleavage rates in oocytes coincubated with sex-sorted spermatozoa were significantly lower ($P < 0.05$) with the shortest fertilisation time (8 h), M199-FERT and M199:8 h compared with the longer fertilisation time (18 h), SOF-FERT and the other coincubation groups, respectively. SOF:8 resulted in the highest cleavage rates and blastocyst formation. SOF-FERT produced the highest embryo quality and hatching rates compared with M199. Motility enhancers (Experiment 1; Table 6) did not affect polyspermy, whereas in Experiment 2 polyspermy rates were increased when fertilisation was extended to 18 h and in M199-FERT (Table 7). The lowest polyspermy rate was found in SOF-FERT with the shortest fertilisation time.

Discussion

The results of the present study demonstrate that the addition of TH and CA to sex-sorted spermatozoa before fertilisation can have a beneficial effect on embryo development and hatching ability. Sperm capacitation has been recognised as the necessary physiological changes that sperm must undergo to acquire fertilising capability (Parrish *et al.* 1988; Yanagimachi 1989; Yanagimachi 1994*b*; Yanagimachi 1994*a*; Bailey 2010; Aitken and Nixon 2013; Parrish 2014). Furthermore, the length of exposure of oocytes to spermatozoa and fertilisation medium also affect cleavage, embryo development, embryo quality and hatching ability.

The sperm capacitation mechanism is still poorly understood, but the process includes biochemical and ultrastructural changes that lead to the removal of adherents on the sperm membrane, changing the lipid composition of the sperm membrane, increased permeability to Ca^{2+}, changing internal pH and increasing cell permeability and metabolism (Fraser 1998; Visconti *et al.* 1998; Aitken and Nixon 2013). Motility plays a key role in the fertilisation process and is consequently considered an important functional parameter during pre- and post-freezing evaluation of spermatozoa. Motility is essential for successful cumulus cell and ZP penetration (Gordon 2003; Suarez and Ho 2003; Florman and Ducibella 2006), with the exception being ARTs like intracytoplasmic sperm injection. Increased sperm motility pattern and speed, called hypermotility, results as a consequence of the redistribution of membrane components during capacitation (Kay and Robertson 1998). Calcium and cAMP are the most important factors regulating hyperactivation of mammalian spermatozoa (Yanagimachi 1994*b*; Ho and Suarez 2001*b*). Ca^{2+} is a critical element in the signalling pathway to convert from the symmetrical to asymmetrical bending that is characteristic of hyperactivation (Suarez *et al.* 1991, 1993; Yanagimachi 1994*b*; Ho and Suarez 2001*b*; Marquez and Suarez 2007). TH and CA are methylxanthines that can cause intracellular accumulation of cAMP or activate Ca^{2+}-permeable cation channels in the sperm plasma membrane, and have been widely used in ART as motility stimulants (Niwa and Ohgoda 1988; Park *et al.* 1989; Loughlin and Agarwal 1992; Takahashi and First 1993; Ho and Suarez 2001*a*; Henkel and Schill 2003; Mortimer *et al.* 2013). Even though heparin alone can achieve similar cleavage and embryo development rates compared with previously published data using sex-sorted spermatozoa (Lu *et al.* 1999; Lu and Seidel 2004), the addition of CA and TH improved the results. The synergistic effects of xanthines have been reported in other studies (Niwa and Ohgoda 1988; Numabe *et al.* 2001; Kang *et al.* 2015). A higher concentration of CA (>2.5 mM) was shown to have adverse effects on embryo performance in Bovidae (Bird *et al.* 1989; Momozawa and Fukuda 2003). Therefore, in the present study we used a short incubation time (30 min) before fertilisation to avoid any potential toxic effect that xanthines can generate, especially after long fertilisation times (>16 h). In the present study, the

Table 4. Effects of gamete coincubation time and medium on IVF performance after oocyte fertilisation using sex-sorted spermatozoa

Data are the mean ± s.d. SOF, synthetic oviducal fluid

	Time (h)		Fertilisation medium		Fertilisation medium × time			
	8 (*n* = 2330)	18 (*n* = 2420)	SOF (*n* = 2470)	M199 (*n* = 2280)	SOF:8 (*n* = 1190)	SOF:18 (*n* = 1280)	M199:8 (*n* = 1140)	M199:18 (*n* = 1140)
Cleavage (%)	54.92 ± 24.22	65.50 ± 20.33	71.13 ± 18.48	49.29 ± 21.64	71.00 ± 16.18	71.25 ± 21.26	38.83 ± 20.01	59.75 ± 18.43
Blastocysts (%)								
Total (%) (Day 7)	21.13 ± 14.40	21.63 ± 11.07	27.21 ± 12.16	15.54 ± 10.54	29.67 ± 14.27	24.75 ± 9.61	12.58 ± 8.40	18.50 ± 11.94
Grade I	10.46 ± 9.85	10.04 ± 8.57	14.67 ± 9.21	5.83 ± 6.70	15.83 ± 10.46	13.50 ± 8.06	5.08 ± 5.50	6.58 ± 7.90
Grade II	5.92 ± 4.68	5.96 ± 2.63	7.67 ± 3.47	4.21 ± 3.24	8.67 ± 4.36	6.67 ± 2.02	3.17 ± 3.21	5.25 ± 3.05
Grade III	4.75 ± 2.29	5.63 ± 3.35	4.88 ± 2.31	5.50 ± 3.36	5.17 ± 2.92	4.58 ± 1.56	4.33 ± 1.44	6.67 ± 4.31
Hatching (%) (Day 9)	13.42 ± 11.96	12.50 ± 9.61	18.42 ± 10.83	7.50 ± 7.50	20.92 ± 12.24	15.92 ± 9.06	5.92 ± 5.16	9.08 ± 9.25

Table 5. Fixed-effects model results for gamete coincubation time, medium and time × fertilisation medium interaction term (Experiment 2) on IVF performance and polyspermy after oocyte fertilisation using sex-sorted spermatozoa

Data adjusted by model are expressed as the mean proportion ± s.e.m. × 100. Within rows, values with different superscript lowercase letters differ significantly ($P < 0.05$), as determined by Fisher's least significant difference test with Bonferroni correction. SOF, synthetic oviducal fluid

	Time (h)		Fertilisation medium		Fertilisation medium × time			
	8 ($n = 2330$)	18 ($n = 2420$)	SOF ($n = 2470$)	M199 ($n = 2280$)	SOF:8 ($n = 1190$)	SOF:18 ($n = 1280$)	M199:8 ($n = 1140$)	M199:18 ($n = 1140$)
Cleavage (%)	58.42 ± 3.09[b]	67.39 ± 3.73[a]	71.30 ± 3.29[a]	53.89 ± 3.72[b]	73.54 ± 2.96[a]	68.95 ± 4.20[a]	41.53 ± 5.43[b]	65.79 ± 3.88[a]
Blastocysts (%)								
Total (%) (Day 7)	18.21 ± 2.80		25.89 ± 3.64[a]	15.00 ± 2.13[b]	29.28 ± 4.82[a]	22.77 ± 2.90[ab]	12.27 ± 2.27[c]	18.21 ± 2.80[bc]
Grade I	35.04 ± 7.43		49.76 ± 5.33[a]	25.45 ± 8.60[b]	49.64 ± 5.45[a]	49.89 ± 6.36[a]	28.50 ± 10.01[ab]	22.62 ± 8.28[b]
Grade II	27.78 ± 1.40							
Grade III	28.41 ± 4.67		18.43 ± 2.80[b]	38.38 ± 5.98[a]	17.78 ± 3.23[b]	19.09 ± 3.34[b]	36.76 ± 5.68[a]	40.03 ± 7.73[a]
Hatching (%) (Day 9)	55.69 ± 4.34		65.56 ± 3.81[a]	45.34 ± 4.37[b]	65.56 ± 3.81[a]		45.34 ± 4.37[b]	

Table 6. Effect of motility enhancers on polyspermy rates after oocyte fertilisation using sex-sorted spermatozoa

Data adjusted by model are expressed as the mean proportion ± s.e.m. × 100. There were no significant differences among treatment groups in the rate of polyspermy, as determined by Fisher's least significant difference tests with Bonferroni correction. CA, caffeine (10 mM); TH, theophylline (5 mM)

Motility enhancers	Oocytes (n)	Polyspermy (%)
CA	145	4.83 ± 1.78[a]
TH	145	6.21 ± 2.00[a]
TH + CA	145	6.90 ± 2.10[a]
CONTROL	145	2.76 ± 1.36[a]

addition of CA and TH did not significantly increase the polyspermy rate (Table 6). In other studies, normal PN-2 rates were achieved using TH and a D-penicillamine, hypotaurine and adrenaline mixture, as well as increased cleavage rates and embryo development (Kang *et al.* 2015). In the present study, CA and TH may have enhanced sex-sorted spermatozoa penetration by shortening the fertilisation time through increased oocyte–sperm binding. The aforementioned effect may have more relevance in cases where low sperm concentration (e.g. sex-sorted spermatozoa vs conventional spermatozoa) and decreased motility are determinant factors during fertilisation.

Time of exposure during oocyte–sperm coincubation and the type of fertilisation medium used affect cleavage, polyspermy, embryo development, embryo quality and hatching rates. Usually, fertilising bovine COCs with conventional unsorted spermatozoa takes between 18 and 24 h at concentrations of 1–1.5 × 10^6 spermatozoa mL^{-1} (Rehman *et al.* 1994; Ward *et al.* 2002; Kochhar *et al.* 2003). This method has been widely used until now mainly because of the experimental practicality of leaving the oocyte–sperm coincubations overnight. However, shortening the sperm–oocyte coincubation to 9–10 h could also produce satisfactory results in terms of fertilisation rate and blastocyst development (Ward *et al.* 2002; Kochhar *et al.* 2003). Such flexibility with the duration of fertilisation could make embryo culture conditions more practical for commercial laboratory operations. Other fertilisation protocols reduced coincubation time even further (<6 h) and increased spermatozoa concentration up to 6 × 10^6 spermatozoa mL^{-1} (Brackett and Oliphant 1975; Nedambale *et al.* 2006). Both scenarios (i.e. high sperm concentration or extended coincubation time) may result in ROS release because of an increase in dead spermatozoa, which may induce ZP hardening and compromise the fertilisation rate and embryo developmental capacity (Guérin *et al.* 2001; Kattera and Chen 2003; Baker and Aitken 2004; Dalvit *et al.* 2005; Agarwal *et al.* 2006; Enkhmaa *et al.* 2009; Lopes *et al.* 2010; Tsunoda *et al.* 2014). The results of the present study revealed that prolonged coincubation time resulted in a significantly higher cleavage rate but did not significantly affect embryo development, blastocyst quality and hatching ability compared with the shorter fertilisation time (Table 5). This observation is in agreement with previous reports in which cleavage increased with oocyte-sperm exposure time and embryo development was not affected (Rehman *et al.* 1994; Ward *et al.* 2002; Barceló-Fimbres *et al.* 2011). However, other studies reported different findings than those of the present study. For example, shortening fertilisation time has been reported to increase both blastocyst rate (Kochhar *et al.* 2003; Nedambale *et al.* 2006) and quality, as measured by the ratio of total cell number : inner cell mass (Nedambale *et al.* 2006; Berland *et al.* 2011). This could be explained by the fact that we used a lower concentration (1 × 10^6 mL^{-1}) of sex-sorted spermatozoa instead of conventional semen, which may behave differently during fertilisation, oocyte interaction and subsequent fusion. Prolonged oocyte–sperm gamete coincubation (>18 h) has been reported to increase the rate of polyspermy (Long *et al.* 1994; Nedambale *et al.* 2006; Barceló-Fimbres *et al.* 2011), in agreement with the results of the present study (Table 7).

Koyama *et al.* (2014) demonstrated that bovine oocytes acquire their highest developmental competence at around 12 h after achieving nuclear maturation, or 30 h after the initiation of maturation. This finding would support the argument for coincubating oocytes for 8 h to achieve acceptable cleavage results. The results of the present study partially confirm this, but only cleavage differed significantly between the 8 h and 18 h fertilisation times, although a slight increase was noted between

Table 7. Effects of gamete coincubation duration and medium on polyspermy rates after oocyte fertilisation using sex-sorted spermatozoa
Data adjusted by model are expressed as the mean proportion ± s.e.m. × 100. Within rows, values with different superscript lowercase letters differ significantly ($P < 0.05$), as determined by Fisher's least significant difference test with Bonferroni correction. SOF, synthetic oviducal fluid

	Time (h)		Fertilisation medium		Fertilisation medium × time			
	8 ($n = 360$)	18 ($n = 360$)	SOF ($n = 360$)	M199 ($n = 360$)	SOF:8 ($n = 180$)	SOF:18 ($n = 180$)	M199:8 ($n = 180$)	M199:18 ($n = 180$)
Polyspermy	2.68 ± 0.91^{b}	8.87 ± 1.60^{a}	3.28 ± 1.09^{b}	7.32 ± 1.54^{a}	1.89 ± 1.04^{b}	5.64 ± 1.83^{ab}	3.79 ± 1.44^{b}	13.69 ± 2.67^{a}

cleavage and blastocyst formation with SOF:8 and SOF:18; whereas cleavage and embryo development decreased with M199:8 and M199:18 (Tables 4, 5).

In the case of regular human oocyte fertilisation, the current preference is for a shorter exposure time with spermatozoa in contrast with the traditional overnight protocol (Lin *et al.* 2000; Kattera and Chen 2003). However, it is common practice to leave a couple of layers of cumulus cells after a brief wash and before culture when short fertilisation protocols are used. This is primarily because mechanical removal of cumulus cells by repeated pipetting or vortexing is difficult when oocytes are at the early stages of fertilisation. Thus, it is probable that spermatozoa remain lodged or attached to the cumulus mass and penetrate the oocyte later. In the present study, the oocytes (and even the 8-h fertilised zygotes) were completely denuded by a specially designed small-bore plastic pipette tip with a 155-µm diameter, which ensured complete cumulus removal.

The fertilisation medium played a key role in overall cleavage, blastocyst development, embryo quality, hatching ability and polyspermy. In a previous study, when a short oocyte–sperm coincubation fertilisation medium, such as Bracket–Oliphant (BO), was extended from 6 to 18 h, blastocyst formation decreased markedly and polyspermy increased in a proportional manner (Nedambale *et al.* 2006). In addition, a different fertilisation medium (IVF-M199) showed no adverse effects on cleavage and embryo development rates after prolonging fertilisation coincubation to 18 h (Nedambale *et al.* 2006). Even though we reached the same conclusion regarding the effects of fertilisation medium, M199-FERT exhibited a poor performance in the case of all indicators assayed (cleavage, polyspermy, blastocyst, embryo quality and hatching rates) compared with SOF-FERT.

Nedambale *et al.* (2006) found that M199-FERT performed better than other traditional fertilisation media such as BO, KSOM-FERT or TALP, not only in cleavage rate (2-cell division), but also in advanced cell number stage at 48 h after fertilisation (8-cell stage), polyspermy, blastocyst formation, total cell number, hatching rate and post-thaw survival after vitrification. In a recent study using sex-sorted spermatozoa, SOF and TALP showed a significant improvement over M199 as fertilisation medium in terms of cleavage rate, blastocyst development, Grade 1 blastocysts, hatching rates and the number of inner cell mass and trophectoderm cells (Ferré *et al.* 2015).

It is interesting that M199-FERT did not perform as well as SOF-FERT in the present study, because M199 contains antioxidants such as glutathione and L-ascorbic acid, which are missing from the TALP and SOF media formulations (Nedambale *et al.* 2006). Antioxidants are important in mitigating the accumulation of ROS, which can generate oxidative stress and damage normal physiological sperm function (Bansal and Bilaspuri 2011; Aitken *et al.* 2012), impair sperm motility, affect membrane integrity and decrease oocyte penetration capacity (Chatterjee and Gagnon 2001; Aitken and Henkel 2011; Bansal and Bilaspuri 2011; Tsunoda *et al.* 2014). Antioxidants also contribute to sperm defence mechanisms, compromised by the freeze–thawing process, against hydrogen peroxides (Agarwal *et al.* 2006; Bansal and Bilaspuri 2011). Nevertheless, M199 also contains glucose and, as reported by Parrish *et al.* (1989), glucose may interfere with sperm capacitation, sperm–oocyte binding and ZP penetration, thus affecting early zygote division and subsequent embryo development. The assumption in the present study was that because M199 contains antioxidants, vitamins, amino acids and other cell-protective agents, the performance of sexed spermatozoa (having undergone a more rigorous treatment compared with conventional unsorted spermatozoa) may be improved to a point at which such benefits could outweigh any potentially negative effects from glucose; however, this was not the case.

We conclude that adding the sperm motility enhancers CA and TH can improve cleavage and embryo development rates without increasing polyspermy. In addition, shortening oocyte–sperm coincubation resulted in similar overall embryo performance rates compared with the prolonged (18 h) interval, facilitating the decision of choosing one particular protocol over another. Furthermore, the data from the present study suggest that SOF-FERT increases cleavage, blastocyst formation, embryo quality and hatching rates over M199-FERT.

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