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Comparison of different fertilisation media for an *in vitro* maturation–fertilisation–culture system using flow-cytometrically sorted X chromosome-bearing spermatozoa for bovine embryo production

Luis B. Ferré[A,F], *Yanina Bogliotti*[B], *James L. Chitwood*[B], *Cristóbal Fresno*[C], *Hugo H. Ortega*[D], *Michael E. Kjelland*[E] *and Pablo J. Ross*[B]

[A]Instituto Nacional de Tecnología Agropecuaria, Ruta Nacional 34, Km 227, Rafaela (2300), Santa Fe, Argentina.
[B]Department of Animal Science, One Shields Avenue, University of California, Davis, CA 95616, USA.
[C]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)/Facultad de Ingeniería, Universidad Católica de Córdoba, Av. Armada Argentina 3555, X5016DHK, Córdoba, Argentina.
[D]Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral/CONICET, R.P. Kreder 2805 (S3080HOF), Esperanza, Santa Fe, Argentina.
[E]Conservation, Genetics and Biotech, LLC, Vicksburg, MS 39182, USA.
[F]Corresponding author. Email: ferre.luis@inta.gob.ar

Abstract. High demand exists among commercial cattle producers for *in vitro*-derived bovine embryos fertilised with female sex-sorted spermatozoa from high-value breeding stock. The aim of this study was to evaluate three fertilisation media, namely M199, synthetic oviductal fluid (SOF) and Tyrode's albumin–lactate–pyruvate (TALP), on IVF performance using female sex-sorted spermatozoa. In all, 1143, 1220 and 1041 cumulus–oocyte complexes were fertilised in M199, SOF and TALP, respectively. There were significant differences among fertilisation media ($P < 0.05$) in cleavage rate (M199 = 57%, SOF = 71% and TALP = 72%), blastocyst formation (M199 = 9%, SOF = 20% and TALP = 19%), proportion of Grade 1 blastocysts (M199 = 15%, SOF = 52% and TALP = 51%), proportion of Grade 3 blastocysts (M199 = 58%, SOF = 21% and TALP = 20%) and hatching rates (M199 = 29%, SOF = 60% and TALP = 65%). The inner cell mass (ICM) and trophectoderm (TE) cells of Day 7 blastocysts were also affected by the fertilisation medium. Embryos derived from SOF and TALP fertilisation media had higher numbers of ICM, TE and total cells than those fertilised in M199. In conclusion, fertilisation media affected cleavage rate, as well as subsequent embryo development, quality and hatching ability. SOF and TALP fertilisation media produced significantly more embryos of higher quality than M199.

Additional keywords: *in vitro* produced, zona pellucida.

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Introduction

The demand for *in vitro*-derived bovine embryos (i.e. *in vitro* produced (IVP)) among commercial cattle producers has increased considerably in recent years, primarily because of the ability to combine IVP with X chromosome-bearing (hereafter referred to as "female") sex-sorted spermatozoa (SS) and high-speed genome-tested donor turnaround. Although sperm sex-sorting technology based on sperm DNA content is over a quarter of a century old (Johnson *et al.* 1989), the commercial impact of X and Y chromosome-bearing spermatozoa sorted by flow cytometry has only been realised in the past 10 years (Garner and Seidel 2008; Rath and Johnson 2008). The correct use of SS according to stud recommendations and high genomic total performance index (GTPI) sires allowed for its rapid expansion and market penetration (DeJarnette *et al.* 2009, 2011; Norman *et al.* 2010).

SS is currently used in AI (Crichton *et al.* 2006; DeJarnette *et al.* 2008, 2009, 2010), embryo transfer–multiple ovulation and embryo transfer (Sartori *et al.* 2004) and IVP (Lu *et al.* 1999; Zhang *et al.* 2003; Lu and Seidel 2004; Wilson *et al.* 2005, 2006;

Wheeler *et al.* 2006) programs, achieving a conception efficiency of 70%–90% compared with conventional semen (Seidel and Garner 2002; Garner and Seidel 2008). In addition to AI, IVP has grown remarkably due to ultrasound-guided transvaginal follicular aspiration for ovum pick-up (Pieterse *et al.* 1991; Galli *et al.* 2001; Chaubal *et al.* 2007), low-cost female embryos for herd expansion and improved pregnancy rates during summer or in repeat breeder cows (Ambrose *et al.* 1999; Rutledge 2001; Al-Katanani *et al.* 2002; Dochi *et al.* 2008; Stewart *et al.* 2011; Rasmussen *et al.* 2013).

The use of SS in IVP has shown mixed results (Lu *et al.* 1999; Zhang *et al.* 2003; Wheeler *et al.* 2006; Wilson *et al.* 2006; Blondin *et al.* 2009; Xu *et al.* 2009), likely due to laboratory-to-laboratory changes in production protocols, making it difficult to compare results across different studies. There has been little emphasis on defining proper (i.e. standardised) IVF conditions for SS.

Fertilisation is a multifactorial and complex process that includes sperm–ovum interaction, fusion and initiation of embryo development. Different fertilisation media have been used with SS with variable success (Xu *et al.* 2009). The most common fertilisation medium used with SS is Tyrode's albumin–lactate–pyruvate (TALP; Parrish *et al.* 1986). Popularity and acceptance of TALP medium among commercial IVP laboratories is based on its consistency and proven record, whereas synthetic oviductal fluid (SOF) has been used primarily for bovine *in vitro* culture (Tervit *et al.* 1972). In order to simplify the IVP process and to avoid transferring different components between fertilisation and culture media, SOF can be used as the fertilisation medium (Gandhi *et al.* 2000). A potential advantage of using the same medium base is that oocytes would be less exposed to osmotic change and environmental variation during the change from *in vitro* maturation (IVM) to IVF.

A commonly used medium for bovine oocyte maturation, and to a lesser extent for embryo culture, is M199 (Morgan *et al.* 1950). 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 reactive oxygen species (ROS) that can generate oxidative stress and damage normal physiological sperm function (Bansal and Bilaspuri 2010; Aitken *et al.* 2012), impair sperm motility, affect membrane integrity and decrease oocyte penetration capacity (Chatterjee and Gagnon 2001; Bansal and Bilaspuri 2010; Aitken and Henkel 2011; Tsunoda *et al.* 2013). Antioxidants also contribute to the sperm defence mechanism, compromised by the freeze–thawing process, against hydrogen peroxides (Agarwal *et al.* 2006; Bansal and Bilaspuri 2010). This situation may be further aggravated by the current commercial sperm sexing methodology (i.e. flow cytometry) that SS have undergone. The high speed during the sorting process could reduce sperm lifespan (Gosálvez *et al.* 2011) and increase ROS content during long fertilisation periods (Garner *et al.* 2013; Rath *et al.* 2013). A decrease in sperm chromatin stability could easily be associated with mechanical injury, enhanced via DNA exposure to Hoechst 33342 and/or laser illumination (Garner 2006).

Given the aforementioned considerations, and compared with other established bovine IVF media protocols (i.e. SOF and TALP), the use of M199 medium during fertilisation may also be beneficial to both the oocyte and SS. The primary aim of the present study was to compare the effects of different fertilisation media on fertilisation rates measured by cleavage rates and its subsequent implications on early embryonic development when using female sex-sorted semen.

Materials and methods

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

Oocyte recovery and IVM

Ovaries were collected during the spring and summer from an abattoir (Cargill, Fresno, CA, USA) and transported to the laboratory in an insulated container filled with prewarmed saline solution at approximately 32°C. Ovaries were washed several times and placed in a water bath (37°C) with saline for aspiration. Oocytes were aspirated from 2–6-mm antral follicles using a 21-gauge butterfly needle connected to the vacuum system at a flow rate of 15 mL water per minute. Cumulus–oocyte complexes (COCs) containing compact and complete cumulus cell layers were selected and matured in groups of 50 in 400 μL M199 medium (pH 7.35, 290 mOsmol) supplemented with ALA-glutamine (0.1 mM), Na pyruvate (0.2 mM), gentamicin (5 μg mL^{-1}), epidermal growth factor (50 ng mL^{-1}), ovine FSH (50 ng mL^{-1}), bovine 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.

In vitro *fertilisation*

Fertilisation (Day 0) was performed using frozen–thawed female sex-sorted semen (Genex Cooperative, Shawano, WI, USA). Semen straws were thawed at 37°C for 45 s. Straw contents were placed on an 80%/40% discontinuous density gradient (PureSperm; Spectrum Technologies, Healdsburg, CA, USA) for centrifugation (700*g*, 15 min) at room temperature. Next, a standard procedure for bovine sperm washing was performed using a second centrifugation (300*g*, 5 min, room temperature) after discarding the supernatant and resuspending the sperm pellet in TALP-Sperm (pH 7.4, 295 mOsmol). Groups of 15–20 matured COCs were washed twice the appropriated FERT media prior to being transferred to the final FERT media and placed in 50 μL fertilisation medium under mineral oil and spermatozoa were added to a final concentration of 1×10^6 spermatozoa mL^{-1}. All fertilisation media were 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}). Oocytes were coincubated with spermatozoa for 16–18 h at 38.5°C in humidified atmosphere of 5% CO_2 in air.

In vitro embryo culture

After 18 h, presumptive zygotes were mechanically denuded 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 optimized medium supplemented with amino acids (KSOMaa) (Evolve ZEBV-100 (Zenith Biotech, Guilford, CT, USA), pH 7.4, 275 mOsmol; Biggers *et al.* 2000), supplemented with 4 mg mL^{-1} BSA, under mineral oil for 9 days. On Day 3, 3% FBS was added. Culture conditions were 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Oxygen tension was reduced during *in vitro* culture (IVC) in order to increase the proportion of embryos developing to the blastocyst stage (Nakao and Nakatsuji 1990; Thompson *et al.* 1990; Voelkel and Hu 1992). Day 7 and Day 9 blastocysts and hatched embryos, respectively, were evaluated morphologically according to International Embryo Transfer Society (IETS) standards (Stringfellow *et al.* 2010).

Determination of inner cell mass and trophectoderm cell numbers in blastocysts

Day 7 blastocysts were subjected to immunofluorescent staining of inner cell mass (ICM) and trophectoderm (TE) cell markers (Goissis and Cibelli 2014). Briefly, after washing three times in phosphate-buffered saline (PBS)–polyvinyl alcohol (PVA; 1 mg mL^{-1}), blastocysts were fixed in 4% paraformaldehyde for 10 min, then washed in PBS-PVA five times and stored at 4°C in PBS-PVA until immunostaining (within 1 week). Blastocysts were permeabilised in Dulbecco's PBS (D-PBS) with 1% v/v Triton X-100 for 30 min at room temperature and then blocked in D-PBS with 0.1% Triton X-100, 1% BSA and 10% normal donkey serum for 2 h. Embryos were then incubated overnight with primary antibody at 4°C in D-PBS with 0.1% Triton X-100 and 1% BSA. The primary antibodies used were anti-caudal type homeobox 2 (CDX2) (AM392-5M; BioGenex, Fremont, CA, USA) and anti-sex determining region Y-box 2 (SOX2) (AN579-5M; BioGenex). After six washes with D-PBS containing 0.1% v/v Triton X-100, embryos were incubated with secondary antibodies (donkey anti-mouse Alexa Flour 568 (A10037) and donkey anti-rabbit Alexa Flour 488 (A21206); Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. DNA was stained with Hoechst 33342. After staining, the embryos were mounted in a drop of anti-fade solution (ProLong Gold; Invitrogen, Carlsbad, CA, USA) and covered with a coverslip. Images were taken using an Olympus FV1000 laser scanning confocal microscope (Olympus, Waltham, MA, USA) at 40× magnification with a step size of 1 μm. The numbers of TE and ICM nuclei were counted using National Institutes of Health (Bethesda, MD, USA; Schneider *et al.* 2012) ImageJ version 1.40.

Experimental design

To examine the effect of different fertilisation media on embryo development, embryo quality, hatching ability, total cell count and allocation, COCs were fertilised in M199-FERT (11043-023; Gibco, Carlsbad, CA, USA), SOF-FERT (Tervit *et al.* 1972) and TALP-FERT (Parrish *et al.* 1986). The main components of each of the fertilisation media are given in Table 1. Female SS from three different bulls were individually used to fertilise the COCs randomly assigned to all fertilisation media on the same day. For each oocyte batch, a single bull was used for IVF with all fertilisation media tested. Every combination of fertilisation media and SS from each bull was repeated three times. For each replicate, oocytes (Day 1), cleavage rate (Day 3), blastocyst and embryo grades (Day 7) and hatching rate (Day 9) were recorded. Independent samples were produced using the same protocol in order to obtain embryos for TE and ICM cell counts.

Table 1. Components of *in vitro* fertilisation (FERT) media
SOF, synthetic oviductal fluid; TALP, Tyrode's albumin–lactate–pyruvate; BSA-FAF, fatty acid-free bovine serum albumin

Component	M199-FERT	SOF-FERT	TALP-FERT
NaCl (mM)	117.24	107.7	114
KCl (mM)	5.33	7.16	3.2
KH_2PO_4 (mM)	–	1.19	–
NaH_2PO_4 (mM)	1.01	–	0.40
$MgCl_26H_2O$ (mM)	–	0.49	0.5
$MgSO_47H_2O$ (mM)	0.814	–	–
$CaCl_22H_2O$ (mM)	1.8	1.17	2.0
$NaHCO_3$ (mM)	26.19	25.0	25.0
Glutathione (mM)	0.000163	–	–
Sodium lactate (mM)	–	5.3	10
L-Ascorbic acid (mM)	0.000284	–	–
L-Glutamine (mM)	0.685	–	–
Glucose (mM)	5.6	–	–
Amino acids (present)	Yes	No	No
Vitamin (present)	Yes	No	No
Gentamicin (μg mL^{-1})	5	5	5
Na-Pyruvate (mM)	0.2	0.2	0.2
Fructose (mM)	0.5	0.5	0.5
Heparin (μg mL^{-1})	20	20	20
BSA-FAF (mg mL^{-1})	6	6	6
pH (adjusted)	7.4	7.4	7.4
Osmolarity (mOsmol)	292	289	298

Statistical analysis

Data were analysed using a generalised linear mixed effect model with logit-link binomial distribution or log-link Poisson distribution in order to consider the complete experimental design and technical constraints. InfoStat software version 2011 (Di Rienzo *et al.* 2011) and R packages (Development-Core-Team 2005) were used for model estimation starting from the maximal linear predictor (η) for each output variable of interest (cleavage, blastocyst, hatching, inner cell mass and trophectoderm cells):

$$\eta_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i \times \beta_j + b_k + b_k \times \alpha_i$$

where the linear predictor η_{ijk} models as fixed effects an overall mean (μ), the contribution of the *i*th fertilisation medium (α_i), the *j*th bull effect (β_j) and the interaction term ($\alpha_i \times \beta_j$), whereas the random effects consider the *k*th day $b_k \sim N(0, \sigma_d^2)$ intercept contribution and the interaction of the day with the fertilisation medium $b_k \times \alpha_i \sim N(0, \Sigma)$. A likelihood ratio test approach was used for model selection for the different recorded variables.

The significance of differences among medium treatment groups was determined using Fisher's least significant difference

(l.s.d.) test with the Bonferroni correction for every adjusted model. Significance in all tests was set at two-tailed $P < 0.05$ (i.e. the Type I error was set at $\alpha = 0.05$).

Results

Model effects for the different measured variables are given in Table 2. The results suggest that model selection is not a trivial matter; hence, the same model cannot be used across all the different stages. The blastocyst and hatching proportions include the medium as a fixed effect and day as a random intercept, whereas ICM and TE need to take into account bull and its interaction with medium as fixed effects in order to properly model the collected data.

Cleavage rate, blastocyst development quality and hatching proportions using the three different fertilisation media are given in Table 3. Interestingly, significant differences were found between M199 and SOF or TALP fertilisation media for cleavage rate, total blastocysts, Grades 1 and 3 blastocysts and hatching proportions. The proportion of Grade 2 blastocysts was the same ($P > 0.05$) regardless of the fertilisation medium used.

The ICM and TE of Day 7 blastocysts were affected by the fertilisation media (Table 4). Furthermore, ICM and TE proportions were significantly different between M199 and SOF or TALP fertilisation media. Embryos derived from SOF and TALP fertilisation media showed a lower ICM and higher TE adjusted model proportion compared with those derived from M199 fertilisation medium. *In vitro* parameters, such as cleavage, blastocyst formation, blastocyst Grade 1 and hatching rates after fertilisation with M199-FERT, SOF-FERT and TALP-FERT using SS are shown in Fig. 1.

Table 2. Likelihood ratio test *P*-values for selected linear predictor effects

a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$; d, $P < 0.1$; e, $P < 1$. Cleavage and total blastocyst proportions are considered with respect to oocyte counts, whereas hatching is in respect to total blastocysts

Distribution	Binomial								Poisson		
Model	Cleavage	Blastocyst				Hatching	ICM	TE	Counts		
		Total	Grade I	Grade II	Grade III				ICM	TE	Total
Medium (α)	a	a	a	e	a	a	a	a	a	a	a
Bull (β)	e		c		b		c	c	e	a	d
Media × Bull (α × β)	c						a	a	a		
Day (b)	a	a	a		a	a	a	a	a	a	a
Media × Day (α × b)		a								a	a

Table 3. Summary of *in vitro* production performance after oocyte fertilisation using sex-sorted spermatozoa

Data adjusted by model are expressed as the mean ± s.d. Results of Fisher's least significant difference tests are represented by superscript letters. Within columns, values with different superscript letters differ significantly (Bonferroni adjusted $P < 0.05$). SOF, synthetic oviductal fluid; TALP, Tyrode's albumin–lactate–pyruvate; FERT, fertilisation medium

Medium	*n*	Cleavage	Blastocyst				Hatching
			Total	Grade I	Grade II	Grade III	
M199-FERT	1143	0.57 ± 0.02^{a}	0.09 ± 0.02^{a}	0.15 ± 0.04^{a}	0.31 ± 0.04^{a}	0.58 ± 0.06^{a}	0.29 ± 0.07^{a}
SOF-FERT	1220	0.71 ± 0.01^{b}	0.20 ± 0.03^{b}	0.52 ± 0.05^{b}	0.25 ± 0.03^{a}	0.21 ± 0.04^{b}	0.60 ± 0.06^{b}
TALP-FERT	1041	0.72 ± 0.01^{b}	0.19 ± 0.02^{b}	0.51 ± 0.05^{b}	0.23 ± 0.03^{a}	0.20 ± 0.04^{b}	0.65 ± 0.06^{b}

Table 4. Summary of inner cell mass and trophectoderm cell counts in Day 7 blastocysts derived from different fertilisation media

Data are expressed as mean ± s.d. counts and proportions. Results of Fisher's least significant difference tests are represented by superscript letters. Within columns, values with different superscript letters differ significantly (Bonferroni adjusted $P < 0.05$). SOF, synthetic oviductal fluid; TALP, Tyrode's albumin–lactate–pyruvate; FERT, fertilisation medium; ICM, inner cell mass; TE, trophectoderm

Medium	*n*	Adjusted model counts			Adjusted model proportions	
		ICM	TE	Total	ICM/total	TE/total
M199-FERT	116	24.65 ± 0.95^{a}	58.73 ± 3.04^{a}	83.57 ± 4.12^{a}	0.295 ± 0.006^{a}	0.705 ± 0.006^{a}
SOF-FERT	105	25.94 ± 1.00^{a}	68.87 ± 1.32^{b}	95.07 ± 1.83^{b}	0.275 ± 0.005^{b}	0.725 ± 0.005^{b}
TALP-FERT	99	27.76 ± 1.07^{b}	71.81 ± 3.50^{b}	99.78 ± 4.25^{b}	0.278 ± 0.005^{b}	0.722 ± 0.005^{b}

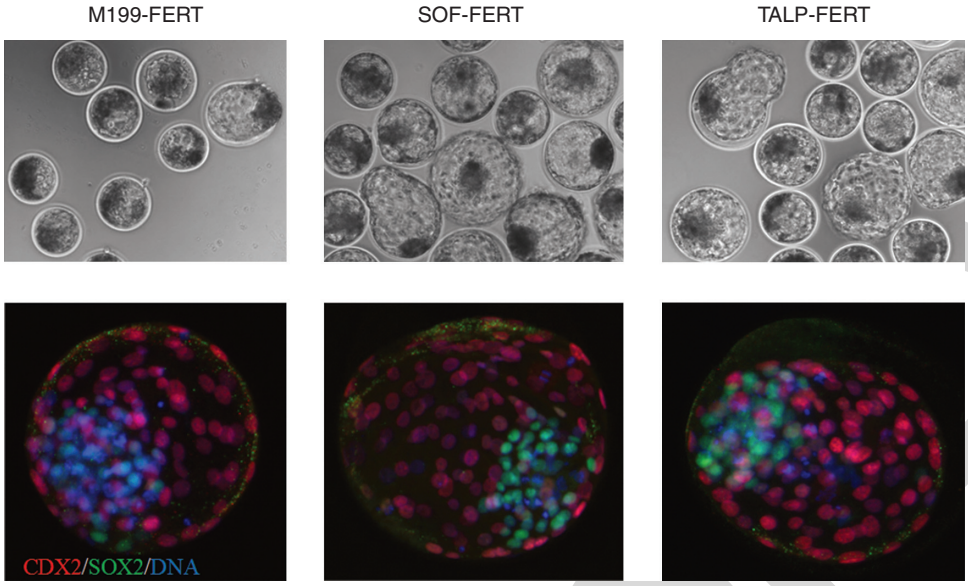

Fig. 1. Representative images of blastocysts produced using female sex-sorted spermatozoa under different fertilisation conditions. Top row: brightfield images of blastocysts (×20 objective). Bottom row, representative pictures of blastocysts stained for inner cell mass (ICM; anti-sex determining region Y-box 2 (SOX2)), trophectoderm (TE; anti-caudal type homeobox 2 (CDX2)) and DNA (Hoechst 33342 staining). These markers were used to determine the total cell number in blastocysts and the number of cells allocated to the TE and ICM (×40 objective).

Discussion

The production of high-quality blastocysts derived from an IVP system depends on the use of proper IVM, IVF and IVC systems that ensure full acquisition of developmental competence of the collected oocytes and contribute to producing viable embryos capable of full-term development after transfer to synchronised recipients. The fertilisation medium plays an important role in promoting favourable conditions for sperm capacitation, sperm–zona pellucida binding, acrosome reaction, penetration of the zona pellucida, sperm–oocyte binding and fusion, egg activation and the cortical reaction, zona reaction, pronuclei formation and initiation of cell division (Yanagimachi 2011; Parrish 2014). The medium formulation must also meet the metabolic requirements for both the spermatozoa and oocyte during the fertilisation incubation period.

The results of the present study demonstrate that bovine embryos derived from oocytes fertilised with female SS are affected by the fertilisation medium used in terms of developmental rates achieved, morphological quality and total cell number and ICM and TE allocation in blastocysts. Calculation of the developmental rate was based on the number of oocytes initially placed into IVM medium. M199 produced significantly less cleaved zygotes and embryos than SOF and TALP fertilisation media. Embryo quality, measured by IETS standards, was also lower in M199 compared with the other two medium formulations. The primary differences between M199 and SOF and TALP, used in the present study as fertilisation media, are antioxidants, carbohydrates and amino acids.

TALP is the most common IVF medium for bovine oocytes (Parrish *et al.* 1988); however, modifications to SOF (Choi *et al.* 1991; Gandhi *et al.* 2000) and Brackett and Oliphant (1975) media have also been successful for cattle IVF. Routinely used for bovine oocyte maturation, M199 has a more complex formulation than TALP and SOF. One component missing in the TALP and SOF formulations that is present in M199 is an antioxidant (Nedambale *et al.* 2006). Accumulation of ROS could generate oxidative stress and damage normal physiological sperm functions (Bansal and Bilaspuri 2010; Aitken *et al.* 2012). The high-speed flow cytometry sorting process could reduce sperm lifespan (e.g. in terms of DNA fragmentation rates, as observed by Gosálvez *et al.* 2011) and increase ROS content during long fertilisation times (Johnson *et al.* 1999; Garner 2006). The initial hypothesis was that M199 medium could have an advantage over the other two media in terms of minimising osmotic stress to MII oocytes as result of passage from IVM to IVF, protecting oocytes and sperm against oxidative stress through antioxidants and preventing oocyte aging. These potential advantages of M199 were not reflected in the developmental parameters measured.

M199 contains glucose. As reported by Parrish *et al.* (1989), glucose may interfere with sperm capacitation, sperm–oocyte binding and zona pellucida penetration, thus affecting early zygote division and subsequent embryo development. In the present study, SS were used to determine whether a similar effect would be produced and to what extent, compared with the results of non-sorted spermatozoa presented in Parrish *et al.* (1989). The idea was that because M199 contains antioxidants, vitamins, amino acids and other cell-protective agents, the performance of SS (having undergone a more rigorous treatment compared with conventional non-sorted spermatozoa) may be

improved to a point at which such benefits could outweigh any potentially negative effects from glucose. During the glycolysis of glucose substrate by bull spermatozoa, acidification of intracellular pH (pH_i) occurs due to the generation of H^+ (Parrish *et al.* 1989, 1994; Vredenburgh-Wilberg and Parrish 1995; Galantino-Homer *et al.* 2004). It was suggested that bull sperm capacitation is, in fact, associated with a slight alkalisation of pH_i and, consequently, glucose is typically not included in bovine fertilisation media.

Non-essential amino acids (NEAA) could have a positive effect during oocyte cleavage and the length of the first cell cycle (Rosenkrans and First 1994; Pinyopummintr and Bavister 1996*b*; Pinyopummintr and Bavister 1996*a*; Van Winkle 2001). Higher blastocyst rates can be achieved from zygotes that cleave earlier (24–30 h) than those cleaving later (>30–40 h; Dinnyés *et al.* 1999; Lechniak *et al.* 2008). The initial supplementation of NEAA could stimulate cleavage rate, blastocyst formation and hatching, whereas the addition of essential amino acids (EAA) would be necessary to stimulate cell division after the 8-cell stage (Partridge and Leese 1996; Lane and Gardner 1997*a*. 1997*b*; Lu *et al.* 1999; Steeves and Gardner 1999; Van Winkle 2001; Zhang *et al.* 2003). M199-FERT, which contains NEAA and EAA, resulted in reduced cleavage division, blastocyst formation, embryo grade and hatching ability compared with TALP and SOF (both amino acid free).

It has been reported that cleavage rates may be correlated with blastocyst performance (Holm *et al.* 1998; Lonergan *et al.* 1999; Ward *et al.* 2001; Alomar *et al.* 2006; Barceló-Fimbres *et al.* 2011). This suggests retardation of the first cell cycle could negatively impact passing the 8- to 16-cell stage (embryonic genome activation), and therefore result in a lower blastocyst formation rate. The success or failure to overcome this critical stage depends not only on the contribution of oocyte competence to blastocyst development, but also on sperm integrity, which has a direct effect on embryo formation and quality (Parinaud *et al.* 1993; Ward *et al.* 2001). Time-lapse monitoring confirmed that zygote cleavage rates were correlated with blastocyst yield (Arav *et al.* 2008; Pribenszky *et al.* 2010; Kirkegaard *et al.* 2012). These observations are in accordance with our findings, whereby the low cleavage in M199-FERT affected all subsequent stages, such as blastocyst, embryo quality and hatching ability.

The ICM and TE counts and TE cell proportion showed that M199 fertilised embryo quality is compromised compared with embryos derived from SOF and TALP fertilisation media. Reduced ICM and TE cell numbers and the inability of a blastocyst to hatch from the zona pellucida could be signs of developmental incompetence and negatively impact cryosurvival outcome, and both implantation and pregnancy maintenance rates. That developmental competence and viability of IVP bovine embryos is inferior to that of their *in vivo*-derived counterparts is well known, and is possibly due to reduced embryo quality resulting from suboptimal IVM, IVF and/or IVC conditions (Rizos *et al.* 2002). At the moment, ICM and TE cell numbers have been used as indicators of quality and viability for IVP preimplantation embryos, although stereoscopic morphological criteria alone could be insufficient for prediction of developmental competence (van Soom *et al.* 1997).

There is an optimal window for fertilisation after maturation, and if fertilisation does not occur within that time, unfertilised oocytes will undergo time-dependent deterioration in quality (oocyte aging; Ottolenghi *et al.* 2004; Miao *et al.* 2009; Koyama *et al.* 2014). The effect of oocyte quality on embryonic developmental potential after fertilisation is significant (Krisher 2004; Wang and Sun 2007). The M199 medium may be suitable for oocyte maturation, but perhaps it is delaying fertilisation by affecting sperm capacitation, sperm–oocyte binding and fusion or both. The final consequence could be an aged, unfertilised oocyte or compromised fertilised zygote from which blastocyst formation, quality and hatching rates could be substandard. Future research may include specifically checking for the kinetics of cleavage or embryo development using M199 medium compared with the other media. Such experiments performed under a time-lapse embryo monitoring video system would help elucidate whether the use of M199 is associated with delayed fertilisation.

The low cleavage rate obtained with M199 medium significantly reduced the number of embryos in each drop that continued development and could interact within the dish microenvironment. Developmental success of group-cultured embryos can be attributed to exposure to embryotrophic autocrine and paracrine factors (Paria and Dey 1990). Embryos produce and secrete various factors that could affect the rate of compaction, cavitation, zona hatching, ICM and TE cell number (Gopichandran and Leese 2006; Richter 2008; Hegde and Behr 2012). In the present study, embryos derived from SOF and TALP fertilisation media showed higher embryo development, Grade 1 blastocysts, hatching ability and cell allocation than embryos fertilised with M199, suggesting that the SOF and TALP fertilisation media are more capable of supporting the development of higher-quality embryos.

The main objective after using SS in IVP is to produce the highest percentage of Grade 1 blastocyst embryos on Day 7. To achieve the maximum percentage of high-quality blastocysts, SS must undergo capacitation in order to reach a fertilisation-competent state. Sperm capacitation changes involve reorganisation of membrane proteins and lipids, which affects plasma membrane fluidity, and changes in intracellular ion concentrations, metabolism and motility, among others. The capacitation process ends with the acrosome reaction and hyperactivation (Parrish *et al.* 1988; Visconti *et al.* 1998; Parrish 2014). During the flow cytometry sorting process, spermatozoa are subjected to different insults, such as DNA staining, dilution effect, centrifugation forces, high pressure, electrical charge, laser emissions and cryopreservation (Schenk *et al.* 1999; Suh *et al.* 2005; Schenk and Seidel 2007; Rath *et al.* 2009; Garner *et al.* 2013). Consequently, post-thaw survival, sperm integrity, acrosome status, membrane functionality, motility pattern and DNA quality, among others, could be seriously affected. In addition, the sperm-sorting process may induce partial capacitation (Lu and Seidel 2004). Therefore, current IVF protocols developed for unsorted semen (i.e. conventional semen) may not be ideally suited for SS.

In conclusion, the different fertilisation media used with SS affected cleavage rate and subsequent embryo development, quality and hatching ability. In the present study, SOF and TALP

fertilisation media produced significantly more and higher-quality embryos than M199.

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References

Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., and Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertil. Steril.* **86**, 503–512. doi:10.1016/J.FERTNSTERT.2006.02.088

Aitken, R. J., and Henkel, R. R. (2011). Sperm cell biology: current perspectives and future prospects. *Asian J. Androl.* **13**, 3–5. doi:10.1038/AJA.2010.155

Aitken, R. J., Jones, K. T., and Robertson, S. A. (2012). Reactive oxygen species and sperm function: in sickness and in health. *J. Androl.* **33**, 1096–1106. doi:10.2164/JANDROL.112.016535

Al-Katanani, Y. M., Drost, M., Monson, R. L., Rutledge, J. J., Krininger, C. E., 3rd, Block, J., Thatcher, W. W., and Hanse, P. J. (2002). Pregnancy rates following timed embryo transfer with fresh or vitrified *in vitro* produced embryos in lactating dairy cows under heat stress conditions. *Theriogenology* **58**, 171–182. doi:10.1016/S0093-691X(02)00916-0

Alomar, M., Mahieu, J., Verhaeghe, B., Defoin, L., and Donnay, I. (2006). Assessment of sperm quality parameters of six bulls showing different abilities to promote embryo development *in vitro*. *Reprod. Fertil. Dev.* **18**, 395–402. doi:10.1071/RD05132

Ambrose, J. D., Drost, M., Monson, R. L., Rutledge, J. J., Leibfried-Rutledge, M. L., Thatcher, M. J., Kassa, T., Binelli, M., Hansen, P. J., Chenoweth, P. J., and Thatcher, W. W. (1999). Efficacy of timed embryo transfer with fresh and frozen *in vitro* produced embryos to increase pregnancy rates in heat-stressed dairy cattle. *J. Dairy Sci.* **82**, 2369–2376. doi:10.3168/jds.S0022-0302(99)75487-1

Arav, A., Aroyo, A., Yavin, S., and Roth, Z. (2008). Prediction of embryonic developmental competence by time-lapse observation and 'shortest-half' analysis. *Reprod. Biomed. Online* **17**, 669–675. doi:10.1016/S1472-6483(10)60314-8

Bansal, A. K., and Bilaspuri, G. S. (2010). Impacts of oxidative stress and antioxidants on semen functions. *Vet. Med. Int.* **2010**, 686137. doi:10.4061/2011/686137

Barceló-Fimbres, M., Campos-Chillón, L. F., and Seidel, G. E., Jr (2011). *In vitro* fertilization using non-sexed and sexed bovine sperm: sperm concentration, sorter pressure, and bull effects. *Reprod. Domest. Anim.* **46**, 495–502. doi:10.1111/J.1439-0531.2010.01696.X

Biggers, J. D., McGinnis, L. K., and Raffin, M. (2000). Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biol. Reprod.* **63**, 281–293. doi:10.1095/BIOLREPROD63.1.281

Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P., and King, W. A. (2009). Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* **71**, 30–38. doi:10.1016/J.THERIOGENOLOGY.2008.09.017

Brackett, B. G., and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260–274. doi:10.1095/BIOLREPROD12.2.260

Chatterjee, S., and Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol. Reprod. Dev.* **59**, 451–458. doi:10.1002/MRD.1052

Chaubal, S. A., Ferre, L. B., Molina, J. A., Faber, D. C., Bols, P. E. J., Rezamand, P., Tian, X., and Yang, X. (2007). Hormonal treatments for increasing the oocyte and embryo production in an OPU–IVP system. *Theriogenology* **67**, 719–728. doi:10.1016/J.THERIOGENOLOGY.2006.07.022

Choi, Y. H., Fukui, Y., and Ono, H. (1991). Effects of media and the presence of bovine oviduct epithelial cells during *in vitro* fertilization on fertilizability and developmental capacity of bovine oocytes. *Theriogenology* **36**, 863–873. doi:10.1016/0093-691X(91)90352-E

Crichton, E. H. S., McSweeney, K., and Schenk, J. (2006). Artificial insemination of lactating Holstein cows with sexed sperm. *Reprod. Fertil. Dev.* **18**, 281. doi:10.1071/RDV18N2AB347

DeJarnette, J. M., Nebel, R. L., Marshall, C. E., Moreno, J. F., McCleary, C. R., and Lenz, R. W. (2008). Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows. *J. Dairy Sci.* **91**, 1778–1785. doi:10.3168/JDS.2007-0964

DeJarnette, J. M., Nebel, R. L., and Marshall, C. E. (2009). Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology* **71**, 49–58. doi:10.1016/J.THERIOGENOLOGY.2008.09.042

DeJarnette, J. M., McCleary, C. R., Leach, M. A., Moreno, J. F., Nebel, R. L., and Marshall, C. E. (2010). Effects of 2.1 and 3.5x10(6) sex-sorted sperm dosages on conception rates of Holstein cows and heifers. *J. Dairy Sci.* **93**, 4079–4085. doi:10.3168/JDS.2010-3181

DeJarnette, J. M., Leach, M. A., Nebel, R. L., Marshall, C. E., McCleary, C. R., and Moreno, J. F. (2011). Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? *J. Dairy Sci.* **94**, 3477–3483. doi:10.3168/JDS.2011-4214

Development-Core-Team (2005). 'R: A Language and Environment for Statistical Computing.' (R Foundation for Statistical Computing: Vienna.)

Di Rienzo, J., Casanoves, F., Balzarini, M., Gonzalez, L., Tablada, M., and Robledo, C. (2011). 'InfoStat v2011.' (InfoStat Group, College of Agricultural Sciences, National University of Córdoba: Argentina.)

Dinnyés, A., Lonergan, P., Fair, T., Boland, M. P., and Yang, X. (1999). Timing of the first cleavage post-insemination affects cryosurvival of *in vitro*-produced bovine blastocysts. *Mol. Reprod. Dev.* **53**, 318–324. doi:10.1002/(SICI)1098-2795(199907)53:3<318::AID-MRD7>3.0.CO;2-O

Dochi, O., Takahashi, K., Hirai, T., Hayakawa, H., Tanisawa, M., Yamamoto, Y., and Koyama, H. (2008). The use of embryo transfer to produce pregnancies in repeat-breeding dairy cattle. *Theriogenology* **69**, 124–128. doi:10.1016/J.THERIOGENOLOGY.2007.09.001

Donnay, I., Verhaeghe, B., and Neirinckx, G. (2004). Enriching a defined maturation medium improves subsequent embryonic development of bovine oocytes cultured in small and large groups. *Reprod. Fertil. Dev.* **16**, 274. doi:10.1071/RDV16N1AB310

Galantino-Homer, H. L., Florman, H. M., Storey, B. T., Dobrinski, I., and Kopf, G. S. (2004). Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated protein tyrosine phosphorylation. *Mol. Reprod. Dev.* **67**, 487–500. doi:10.1002/MRD.20034

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R., and Lazzari, G. (2001). Embryo production by ovum pick up from live donors. *Theriogenology* **55**, 1341–1357. doi:10.1016/S0093-691X(01)00486-1

Gandhi, A. P., Lane, M., Gardner, D. K., and Krisher, R. L. (2000). A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Hum. Reprod.* **15**, 395–401. doi:10.1093/HUMREP/15.2.395

Garner, D. L. (2006). Flow cytometric sexing of mammalian sperm. *Theriogenology* **65**, 943–957. doi:10.1016/J.THERIOGENOLOGY.2005.09.009

Garner, D. L., and Seidel, G. E., Jr (2008). History of commercializing sexed semen for cattle. *Theriogenology* **69**, 886–895. doi:10.1016/J.THERIOGENOLOGY.2008.01.006

Garner, D. L., Evans, K. M., and Seidel, G. E. (2013). Sex-sorting sperm using flow cytometry/cell sorting. *Methods Mol. Biol.* **927**, 279–295. doi:10.1007/978-1-62703-038-0_26

Goissis, M. D., and Cibelli, J. B. (2014). Functional characterization of SOX2 in bovine preimplantation embryos. *Biol. Reprod.* **90**, 30. doi:10.1095/BIOLREPROD.113.111526

Gopichandran, N., and Leese, H. J. (2006). The effect of paracrine/autocrine interactions on the *in vitro* culture of bovine preimplantation embryos. *Reproduction* **131**, 269–277. doi:10.1530/REP.1.00677

Gosálvez, J., Ramirez, M. A., López-Fernández, C., Crespo, F., Evans, K. M., Kjelland, M. E., and Moreno, J. F. (2011). Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. *Theriogenology* **75**, 206–211. doi:10.1016/J.THERIOGENOLOGY.2010.09.011

Hegde, A., and Behr, B. (2012). Media composition: growth factors. *Methods Mol. Biol.* **912**, 177–198.

Holm, P., Shukri, N. N., Vajta, G., Booth, P., Bendixen, C., and Callesen, H. (1998). Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their *in vitro* viability and sex. *Theriogenology* **50**, 1285–1299. doi:10.1016/S0093-691X(98)00227-1

Johnson, L. A., Flook, J. P., and Hawk, H. W. (1989). Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod.* **41**, 199–203. doi:10.1095/BIOLREPROD41.2.199

Johnson, L. A., Welch, G. R., and Rens, W. (1999). The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for *in vitro* fertilization and AI. *J. Anim. Sci.* **77**(Suppl. 2), 213–220.

Kirkegaard, K., Agerholm, I. E., and Ingerslev, H. J. (2012). Time-lapse monitoring as a tool for clinical embryo assessment. *Hum. Reprod.* **27**, 1277–1285. doi:10.1093/HUMREP/DES079

Koyama, K., Kang, S. S., Huang, W., Yanagawa, Y., Takahashi, Y., and Nagano, M. (2014). Aging-related changes in in vitro-matured bovine oocytes: oxidative stress, mitochondrial activity and ATP content after nuclear maturation. *J. Reprod. Dev.* **60**, 136–142. doi:10.1262/JRD.2013-115

Krisher, R. L. (2004). The effect of oocyte quality on development. *J. Anim. Sci.* **82**(E-Suppl.), E14–E23.

Lane, M., and Gardner, D. (1997*a*). Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes *in vitro*. *J. Assist. Reprod. Genet.* **14**, 398–403. doi:10.1007/BF02766148

Lane, M., and Gardner, D. K. (1997*b*). Differential regulation of mouse embryo development and viability by amino acids. *J. Reprod. Fertil.* **109**, 153–164. doi:10.1530/JRF.0.1090153

Lechniak, D., Pers-Kamczyc, E., and Pawlak, P. (2008). Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reprod. Biol.* **8**, 23–42. doi:10.1016/S1642-431X(12)60002-3

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., and Boland, M. P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* **117**, 159–167. doi:10.1530/JRF.0.1170159

Lu, K. H., and Seidel, G. E., Jr (2004). Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically-sorted sperm. *Theriogenology* **62**, 819–830. doi:10.1016/J.THERIOGENOLOGY.2003.12.029

Lu, K. H., Cran, D. G., and Seidel, G. E., Jr. (1999). *In vitro* fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology* **52**, 1393–1405. doi:10.1016/S0093-691X(99)00225-3

Miao, Y. L., Kikuchi, K., Sun, Q. Y., and Schatten, H. (2009). Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum. Reprod. Update* **15**, 573–585. doi:10.1093/HUMUPD/DMP014

Morgan, J. F., Morton, H. J., and Parker, R. C. (1950). Nutrition of animal cells in tissue culture; initial studies on a synthetic medium. *Proc. Soc. Exp. Biol. Med.* **73**, 1–8. doi:10.3181/00379727-73-17557

Nakao, H., and Nakatsuji, N. (1990). Effects of co-culture, medium components and gas phase on *in vitro* culture of *in vitro* matured and *in vitro* fertilized bovine embryos. *Theriogenology* **33**, 591–600. doi:10.1016/0093-691X(90)90536-3

Nedambale, T. L., Du, F., Xu, J., Chaubal, S. A., Dinnyes, A., Groen, W., Faber, D., Dobrinsky, J. R., Yang, X., and Tian, X. C. (2006). Prolonging bovine sperm–oocyte incubation in modified medium 199 improves embryo development rate and the viability of vitrified blastocysts. *Theriogenology* **66**, 1951–1960. doi:10.1016/J.THERIOGENOLOGY.2006.04.044

Norman, H. D., Hutchison, J. L., and Miller, R. H. (2010). Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. *J. Dairy Sci.* **93**, 3880–3890. doi:10.3168/JDS.2009-2781

Ottolenghi, C., Uda, M., Hamatani, T., Crisponi, L., Garcia, J.-E., Ko, M., Pilia, G., Sforza, C., Schlessinger, D., and Forabosco, A. (2004). Aging of oocyte, ovary, and human reproduction. *Ann. N. Y. Acad. Sci.* **1034**, 117–131. doi:10.1196/ANNALS.1335.015

Paria, B. C., and Dey, S. K. (1990). Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. *Proc. Natl Acad. Sci. USA* **87**, 4756–4760. doi:10.1073/PNAS.87.12.4756

Parinaud, J., Mieusset, R., Vieitez, G., Labal, B., and Richoilley, G. (1993). Influence of sperm parameters on embryo quality. *Fertil. Steril.* **60**, 888–892.

Parrish, J. J. (2014). Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. *Theriogenology* **81**, 67–73. doi:10.1016/J.THERIOGENOLOGY.2013.08.005

Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Critser, E. S., Eyestone, W. H., and First, N. L. (1986). Bovine *in vitro* fertilization with frozen–thawed semen. *Theriogenology* **25**, 591–600. doi:10.1016/0093-691X(86)90143-3

Parrish, J. J., Susko-Parrish, J., Winer, M. A., and First, N. L. (1988). Capacitation of bovine sperm by heparin. *Biol. Reprod.* **38**, 1171–1180. doi:10.1095/BIOLREPROD38.5.1171

Parrish, J. J., Susko-Parrish, J. L., and First, N. L. (1989). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. *Biol. Reprod.* **41**, 683–699. doi:10.1095/BIOLREPROD41.4.683

Parrish, J. J., Susko-Parrish, J. L., Uguz, C., and First, N. L. (1994). Differences in the role of cyclic adenosine 3′,5′-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. *Biol. Reprod.* **51**, 1099–1108. doi:10.1095/BIOLREPROD51.6.1099

Partridge, R. J., and Leese, H. J. (1996). Consumption of amino acids by bovine preimplantation embryos. *Reprod. Fertil. Dev.* **8**, 945–950. doi:10.1071/RD9960945

Pieterse, M. C., Vos, P. L. A. M., Kruip, T. A. M., Wurth, Y. A., van Beneden, T. H., Willemse, A. H., and Taverne, M. A. M. (1991). Transvaginal ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology* **35**, 857–862. doi:10.1016/0093-691X(91)90426-E

Pinyopummintr, T., and Bavister, B. D. (1996*a*). Effects of amino acids on development *in vitro* of cleavage-stage bovine embryos into blastocysts. *Reprod. Fertil. Dev.* **8**, 835–841. doi:10.1071/RD9960835

Pinyopummintr, T., and Bavister, B. D. (1996*b*). Energy substrate requirements for *in vitro* development of early cleavage-stage bovine embryos. *Mol. Reprod. Dev.* **44**, 193–199. doi:10.1002/(SICI)1098-2795(199606)44:2<193::AID-MRD8>3.0.CO;2-I

Pribenszky, C., Losonczi, E., Molnar, M., Lang, Z., Matyas, S., Rajczy, K., Molnar, K., Kovacs, P., Nagy, P., Conceicao, J., and Vajta, G. (2010). Prediction of *in-vitro* developmental competence of early cleavage-stage mouse embryos with compact time-lapse equipment. *Reprod. Biomed. Online* **20**, 371–379. doi:10.1016/J.RBMO.2009.12.007

Rasmussen, S., Block, J., Seidel, G. E., Jr, Brink, Z., McSweeney, K., Farin, P. W., Bonilla, L., and Hansen, P. J. (2013). Pregnancy rates of lactating cows after transfer of *in vitro* produced embryos using X-sorted sperm. *Theriogenology* **79**, 453–461. doi:10.1016/J.THERIOGENOLOGY.2012.10.017

Rath, D., and Johnson, L. A. (2008). Application and commercialization of flow cytometrically sex-sorted semen. *Reprod. Domest. Anim.* **43**, 338–346. doi:10.1111/J.1439-0531.2008.01182.X

Rath, D., Moench-Tegeder, G., Taylor, U., and Johnson, L. A. (2009). Improved quality of sex-sorted sperm: a prerequisite for wider commercial application. *Theriogenology* **71**, 22–29. doi:10.1016/J.THERIOGENOLOGY.2008.09.027

Rath, D., Barcikowski, S., de Graaf, S., Garrels, W., Grossfeld, R., Klein, S., Knabe, W., Knorr, C., Kues, W., Meyer, H., Michl, J., Moench-Tegeder, G., Rehbock, C., Taylor, U., and Washausen, S. (2013). Sex selection of sperm in farm animals: status report and developmental prospects. *Reproduction* **145**, R15–R30. doi:10.1530/REP-12-0151

Richter, K. S. (2008). The importance of growth factors for preimplantation embryo development and *in-vitro* culture. *Curr. Opin. Obstet. Gynecol.* **20**, 292–304. doi:10.1097/GCO.0B013E3282FE743B

Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248. doi:10.1002/MRD.1153

Rosenkrans, C. F., Jr, and First, N. L. (1994). Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. *J. Anim. Sci.* **72**, 434–437.

Rutledge, J. J. (2001). Use of embryo transfer and IVF to bypass effects of heat stress. *Theriogenology* **55**, 105–111. doi:10.1016/S0093-691X(00)00449-0

Sartori, R., Souza, A. H., Guenther, J. N., Caraviello, D. Z., Geiger, L. N., Schenk, J. L., and Wiltbank, M. C. (2004). Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. *Anim. Reprod.* **1**, 86–90.

Schenk, J. L., and Seidel, G. E., Jr (2007). Pregnancy rates in cattle with cryopreserved sexed spermatozoa: effects of laser intensity, staining conditions and catalase. *Soc. Reprod. Fertil. Suppl.* **64**, 165–177.

Schenk, J. L., Suh, T. K., Cran, D. G., and Seidel, G. E., Jr (1999). Cryopreservation of flow-sorted bovine spermatozoa. *Theriogenology* **52**, 1375–1391. doi:10.1016/S0093-691X(99)00224-1

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.

Seidel, G. E., Jr, and Garner, D. L. (2002). Current status of sexing mammalian spermatozoa. *Reproduction* **124**, 733–743. doi:10.1530/REP.0.1240733

Steeves, T. E., and Gardner, D. K. (1999). Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol. Reprod.* **61**, 731–740. doi:10.1095/BIOLREPROD61.3.731

Stewart, B. M., Block, J., Morelli, P., Navarette, A. E., Amstalden, M., Bonilla, L., Hansen, P. J., and Bilby, T. R. (2011). Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced *in vitro* with sex-sorted semen. *J. Dairy Sci.* **94**, 3437–3445. doi:10.3168/JDS.2010-4008

Stringfellow, D. A., Givens, M. D., and Society, I. E. T. (2010). 'Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the Use of Embryo Transfer Technology Emphasizing Sanitary Procedures.' (International Embryo Transfer Society: Savory, IL.)

Suh, T. K., Schenk, J. L., and Seidel, G. E., Jr (2005). High pressure flow cytometric sorting damages sperm. *Theriogenology* **64**, 1035–1048. doi:10.1016/J.THERIOGENOLOGY.2005.02.002

Tervit, H. R., Whittingham, D. G., and Rowson, L. E. (1972). Successful culture *in vitro* of sheep and cattle ova. *J. Reprod. Fertil.* **30**, 493–497. doi:10.1530/JRF.0.0300493

Thompson, J. G., Simpson, A. C., Pugh, P. A., Donnelly, P. E., and Tervit, H. R. (1990). Effect of oxygen concentration on *in-vitro* development of preimplantation sheep and cattle embryos. *J. Reprod. Fertil.* **89**, 573–578. doi:10.1530/JRF.0.0890573

Tsunoda, S., Kimura, N., and Fujii, J. (2013). Oxidative stress and redox regulation of gametogenesis, fertilization, and embryonic development. *Reprod. Med. Biol.* **13**, 71–79. doi:10.1007/S12522-013-0170-0

van Soom, A., Ysebaert, M. T., and de Kruif, A. (1997). Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in *in vitro*-produced bovine embryos. *Mol. Reprod. Dev.* **47**, 47–56. doi:10.1002/(SICI)1098-2795(199705)47:1<47::AID-MRD7>3.0.CO;2-Q

Van Winkle, L. J. (2001). Amino acid transport regulation and early embryo development. *Biol. Reprod.* **64**, 1–12. doi:10.1095/BIOLREPROD64.1.1

Visconti, P. E., Galantino-Homer, H., Moore, G. D., Bailey, J. L., Ning, X., Fornes, M., and Kopf, G. S. (1998). The molecular basis of sperm capacitation. *J. Androl.* **19**, 242–248.

Voelkel, S. A., and Hu, Y. X. (1992). Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology* **37**, 1117–1131. doi:10.1016/0093-691X(92)90109-5

Vredenburgh-Wilberg, W. L., and Parrish, J. J. (1995). Intracellular pH of bovine sperm increases during capacitation. *Mol. Reprod. Dev.* **40**, 490–502. doi:10.1002/MRD.1080400413

Wang, Q., and Sun, Q. Y. (2007). Evaluation of oocyte quality: morphological, cellular and molecular predictors. *Reprod. Fertil. Dev.* **19**, 1–12. doi:10.1071/RD06103

Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., and Lonergan, P. (2001). Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. *Mol. Reprod. Dev.* **60**, 47–55. doi:10.1002/MRD.1060

Wheeler, M. B., Rutledge, J. J., Fischer-Brown, A., VanEtten, T., Malusky, S., and Beebe, D. J. (2006). Application of sexed semen technology to *in vitro* embryo production in cattle. *Theriogenology* **65**, 219–227. doi:10.1016/J.THERIOGENOLOGY.2005.09.032

Wilson, R. D., Weigel, K. A., Fricke, P. M., Rutledge, J. J., Leibfried-Rutledge, M. L., Matthews, D. L., and Schutzkus, V. R. (2005). *In vitro* production of holstein embryos using sex-sorted sperm and oocytes from selected cull cows. *J. Dairy Sci.* **88**, 776–782. doi:10.3168/jds.S0022-0302(05)72741-7

Wilson, R. D., Fricke, P. M., Leibfried-Rutiedge, M. L., Rutledge, J. J., Penfield, C. M. S., and Weigel, K. A. (2006). *In vitro* production of bovine embryos using sex-sorted sperm. *Theriogenology* **65**, 1007–1015. doi:10.1016/J.THERIOGENOLOGY.2005.07.007

Xu, J., Chaubal, S. A., and Du, F. (2009). Optimizing IVF with sexed sperm in cattle. *Theriogenology* **71**, 39–47. doi:10.1016/J.THERIOGENOLOGY.2008.09.012

Yanagimachi, R. (2011). Mammalian sperm acrosome reaction: where does it begin before fertilization? *Biol. Reprod.* **85**, 4–5. doi:10.1095/BIOLREPROD.111.092601

Zhang, M., Lu, K. H., and Seidel, G. E. (2003). Development of bovine embryos after *in vitro* fertilization of oocytes with flow cytometrically sorted, stained and unsorted sperm from different bulls. *Theriogenology* **60**, 1657–1663. doi:10.1016/S0093-691X(03)00177-8