Zygote

cambridge.org/zyg

Sperm chromatin protamination influences embryo development in unsexed and sexed bull semen

Thiago Velasco Guimarães Silva[1], Priscila Di Paula Bessa Santana[2], Eduardo Baia de Souza[1], Ana Júlia Mota de Lima[1], Caroline de Araújo Santos[1], Nathália Nogueira da Costa Almeida[1], Vanessa Cunha de Brito[1], Arnaldo Algaranhar Gonçalves[1], Sebastião Tavares Rolim Filho[2], Marcela da Silva Cordeiro[3], Simone do Socorro Damasceno Santos[1], Moysés dos Santos Miranda[1] and Otávio Mitio Ohashi[1]

[1]Laboratory of *In Vitro* Fertilization, Institute of Biological Science, Federal University of Pará, Belém, Pará, Brazil; [2]Federal Rural University of Amazon, Belém, Pará, Brazil and [3]Federal Institute of Pará, Ananindeua, Pará, Brazil

Research Article

Cite this article: Silva TVG *et al*. (2021) Sperm chromatin protamination influences embryo development in unsexed and sexed bull semen. *Zygote*. **29**: 264–269. doi: 10.1017/S0967199420000775

Received: 14 October 2020
Revised: 20 November 2020
Accepted: 30 November 2020
First published online: 15 January 2021

Keywords:
Cattle; Chromomycin A3; *In vitro* embryo production; Protamine; Sexed semen

Author for correspondence:
Thiago Velasco Guimarães Silva. Federal University of Pará, Belém, Pará, Brazil.
Tel: +55 91988823358.
E-mail: velascotvgs@hotmail.com

Summary

Sex selection through sperm sorting offers advantages in regards selection pressure in high-producing livestock. However, the sex-sorting process results in sperm membrane and DNA damage that ultimately decrease fertility. We hypothesized that given the role of protamines in DNA packaging, protamine deficiency could account, at least partially, for the DNA damage observed following sperm sex sorting. To test this, we compared protamine status between unsexed and sexed spermatozoa from two bulls using the fluorochrome chromomycin A3 (CMA3) and flow cytometry. Then, we assessed embryo development following *in vitro* fertilization (IVF) using the same sperm treatments. Overall, sperm protamination was not different between sexed and unsexed semen. However, one of the two bulls displayed higher rates of protamine deficiency for both unsexed and sexed semen ($P < 0.05$). Moreover, unsexed semen from this bull yielded lower blastocyst ($P < 0.05$) and blastocyst hatching rates than unsexed sperm from the other bull. CMA3-positive staining was negatively correlated with cleavage (R^2 85.1, $P = 0.003$) and blastocyst hatching (R^2 87.6, $P = 0.006$) rates in unsexed semen. In conclusion, while the sex-sorting process had no effect on sperm protamine content, we observed a bull effect for sperm protamination, which correlated to embryo development rates following IVF.

Introduction

The use of sex-selected semen technology in the livestock industry offers many advantages for economic gains and increased selection pressure of high-producing animals (Butler *et al.*, 2014). However, its use has not become widespread, probably due to the high cost and decreased fertility of sex-sorted semen. The latter is due mainly to two factors, that is decreased number of spermatozoa per insemination dose and direct damage to sperm resulting from the sorting process (Frijters *et al.*, 2009; Carvalho *et al.*, 2013). During sorting, sperm are exposed to harsh conditions including the laser light, high pressure and electric charges that may compromise sperm quality and viability (Seidel, 2007; Carvalho *et al.*, 2010). There is some evidence that the sorting process may damage sperm DNA (Boe-Hansen *et al.*, 2005; Garcia-Herreros *et al.*, 2010; Zhao *et al.*, 2014) with the resulting decrease in embryo development rates when sexed semen was used for *in vitro* fertilization (IVF) (Morton *et al.*, 2007; Palma *et al.*, 2008). Therefore, it is plausible that the sorting process may also have an effect on sperm protamination and influence the fertility of sex-sorted semen.

Protamines are small cysteine-rich nuclear proteins that replace histones late in spermatogenesis and are responsible for the highly condensed nature and structural organization of chromatin in the sperm nucleus (Miller *et al.*, 2010; Cree *et al.*, 2011). The main role of protamines is preserving sperm DNA integrity, therefore defects in sperm protamination may compromise male fertility. In humans, a low protamine content was correlated with increased DNA fragmentation that, in turn, reduced sperm quality and fertility (Aoki *et al.*, 2005). In cattle (*Bos indicus*), sperm protamine content also displayed a positive correlation with DNA fragmentation (Fortes *et al.*, 2014) and a negative correlation with field fertility scores in breeding bulls (Dogan *et al.*, 2015). Additionally, heat stress conditions reduced bull sperm protamine content (Rahman *et al.*, 2011). However, while the influence of sperm protamination defects on IVF results has been widely studies in humans (Ni *et al.*, 2016), there are no studies in cattle relating sperm protamine deficiency to *in vitro* embryo production (IVEP).

CAMBRIDGE UNIVERSITY PRESS

Protamine content has been assessed by fluorescence microscopy imaging of chromomycin A_3 (CMA3)-stained sperm samples in both men (Marzeyeh Tavalaee *et al.*, 2009) and bulls (Simões *et al.*, 2009). Therefore, CMA3 identifies defective spermatozoa due to DNA packaging and indirectly evaluates protamine deficiency (Bianchi *et al.*, 1993). This method has been optimized using flow cytometry (Tavalaee *et al.*, 2010; Fathi *et al.*, 2011), allowing the assessment of thousands of cells and therefore providing a more statistically significant analysis. The results of sperm protamine deficiency assessment using flow cytometry have been correlated with sperm DNA fragmentation in both men (Tavalaee *et al.*, 2009; Fathi *et al.*, 2011) and bulls (Fortes *et al.*, 2014).

Therefore, the aims of this study were to evaluate whether the sex-sorting process induces defects in sperm protamination and, in turn, the potential consequences on IVEP results in cattle.

Materials and methods

Materials

All reagents used were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Experiment 1: Evaluation of protamine deficiency in sexed and unsexed semen

The rate of protamine deficiency was measured on sexed and unsexed sperm from each of two bulls (*Bos indicus*), performing four samples of frozen–thawed sperm, evaluated per each replicate. Protamine content was evaluated using CMA3 stain and flow cytometry as previously described for human sperm (Tavalaee *et al.*, 2010; Fathi *et al.*, 2011) with the modifications detailed below.

Purchased unsexed and sexed male sorted semen straws from two Nellore (*Bos indicus*) bulls, were thawed at 35°C and washed twice in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) by centrifugation for 10 min at 200 *g*. Each pellet was resuspended in PBS and split into three aliquots, corresponding to each of the three groups: (i) negative control (untreated and CMA3 unstained); (ii) positive control, treated with the disulfide bridging agent dithiothreitol (DTT; 5 µM) and stained with CMA3; and, (iii) experimental groups of unsexed or sexed sperm stained with CMA3. Negative and positive controls were used to define the parameters of fluorescence detection in the flow cytometer.

DTT is a reducing agent that causes the disruption of protein disulfide bonds and therefore can decondense the sperm nucleus *in vitro*, making it more accessible to CMA3 staining. DTT was added to positive control samples that were washed twice by centrifugation at 600 *g* for 5 min and incubated in an equal volume with a 4 M NaCl solution for 15 min at 38.5°C to inactivate the effect of DTT. Positive control and experimental groups were then resuspended in 0.25 mg/ml CMA3 (10 mM $MgCl_2.6H_2O$) and incubated at room temperature for 40 min in the dark. Finally, samples were washed once in PBS by centrifugation at 600 *g* for 5 min and loaded in the flow cytometer.

Briefly, CMA3 dye is a complex of several closely related glycosidic antibiotics derived from *Streptomyces griseus*. CMA3 competes with protamines for binding sites at the minor grooves of the DNA strand. Therefore, CMA3 has been considered a suitable tool to evaluate protamine deficiency (Carrell, 2012).

Analyses were performed in a flow cytometer (BD FACSCanto II, Franklin Lakes, NJ, USA) using the blue excitation laser (488/15 nm), fluorescence detection filter PE (568/42 nm) and BD FACSuite™ software for data acquisition. For each sexed and unsexed sperm three straws were analyzed, with each replicate consisting of 20,000 cells per group.

Experiment 2: Relationship between sperm protamination and in vitro embryo development

Oocyte collection and in vitro *maturation (IVM).* Cow ovaries were obtained at a local abattoir, washed in PBS and transported to the laboratory in 0.9% sodium chloride within 2 h of slaughter and the fluid from 2–8 mm antral follicles was aspirated. Only cumulus–oocyte complexes (COCs) with homogeneous cytoplasm, devoid of darkened vacuoles or granules, and presenting compact and refractive cumulus oophorus cells (Leibfried and First, 1979) were selected for maturation and washed in tissue culture medium 199 (TCM199) HEPES buffer with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 80 µg/ml pyruvate, 62.6 µg/ml penicillin and 50 µg/ml gentamicin.

Groups of 20–25 COCs were placed in 100-µl droplets of TCM199 with sodium bicarbonate and supplemented with 10% FBS (v/v), 0.5 µg/ml follicle stimulating hormone (Follitropin; Bioniche Animal Health, Belleville, Ontario, Canada), 50 µg/ml luteinizing hormone (LH; Lutropin; Bioniche Animal Health), 0.5 µg/ml EGF, 22 µg/ml pyruvate and 50 µg/ml gentamicin. Droplets were placed in 35-mm Petri dishes under sterile mineral oil for a 20 h incubation period under a 5% CO_2, 20% O_2 and 75% N_2 in humidified air atmosphere, at 38.5°C.

In vitro fertilization and embryo culture (IVC). IVF trials were performed with semen from sexed and unsexed sperm from the same two bulls used in Experiment 1, forming four experimental groups. One straw (250 µl) of semen from each bull was thawed in water at 35°C for 30 s. Thawed semen were carefully overlaid on an 800-µl Percoll column consisting of 400 µl 45% and 400 µl 90% Percoll, and centrifuged at 200 *g* for 7 min for washing. The pellet was recovered and washed again in 800 µl IVF-Tyrode's albumin lactate pyruvate (Parrish *et al.*, 1988) medium supplemented with 10 µg/ml heparin, 2 µM penicillamine, 1 µM hypotaurine, 0.25 µM epinephrine and 6 mg/ml BSA, by centrifugation at 200 *g* for 3 min to remove the Percoll residues.

Sperm concentration was determined using a Neubauer chamber and adjusted to 2×10^6 spermatozoa/ml. Sperm were co-incubated with groups of 20–25 COCs in 80-µl droplets of fertilization medium under sterile mineral oil for 24 h. Presumptive zygotes were then subjected to successive pipetting to remove the remaining cumulus oophorus cells and spermatozoa adhered to the zona pellucida, and then transferred to the IVC dish.

Groups of 15–20 presumptive zygotes were co-incubated over a monolayer of granulosa cells in 100-µl droplets of synthetic oviductal fluid medium supplemented with 6 mg/ml BSA, 10% FBS and 50 µg/ml gentamicin (Holm *et al.*, 1999), under sterile mineral oil. Incubation was performed in a 5% CO_2, 20% O_2 and 75% N_2 atmosphere in humidified air at 38.5°C for 7 days. Rates of cleavage and blastocyst formation were evaluated on days 2 and 7 of culture, respectively. On day 7, embryos were also classified by development kinetics into blastocysts (BL), expanded blastocysts (BX), and hatched blastocysts (BH), and by morphological quality into grades 1, 2 or 3 based on Stringfellow and Seidel (1998).

Statistical analysis

Rates of CMA3-positive sperm and embryo development were analyzed by two-way analysis of variance followed by Tukey's mean separation test. Results are reported as the means and their

https://doi.org/10.1017/S0967199420000775 Published online by Cambridge University Press

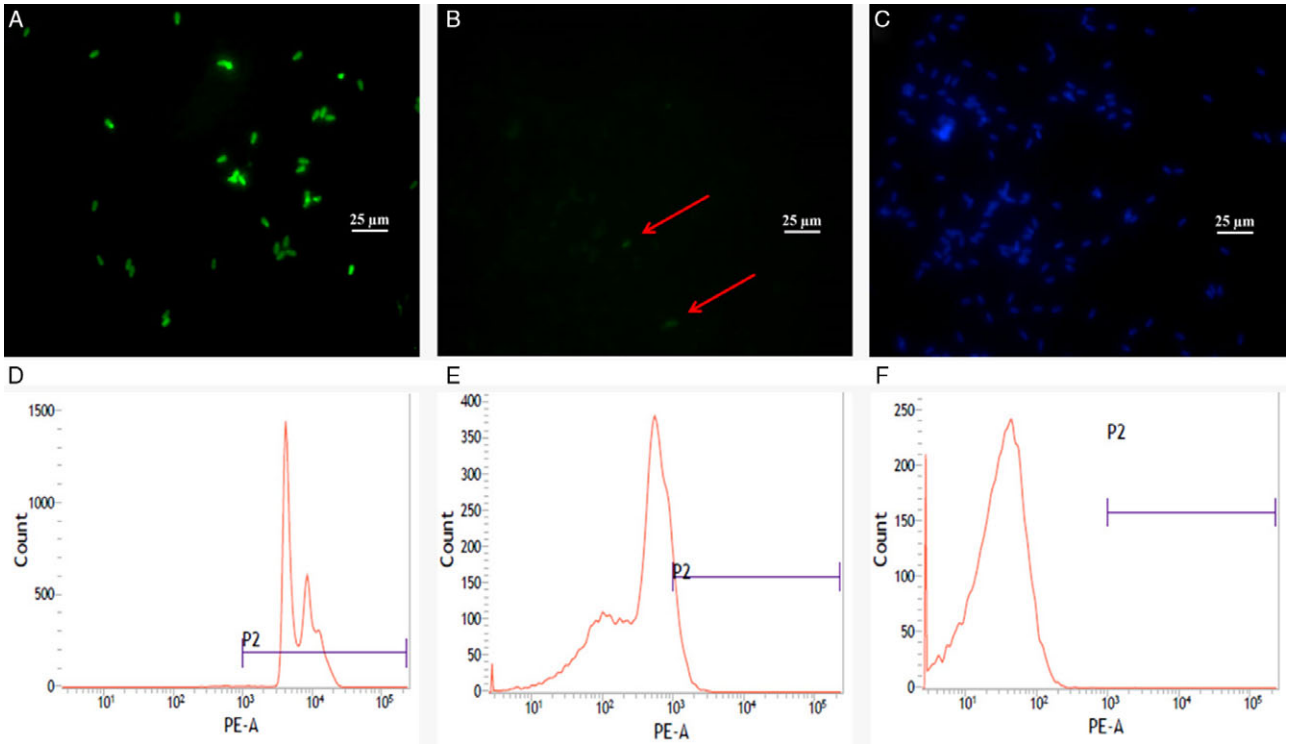

Figure 1. Flow cytometry analysis of CMA3-positive sperm cells. Fluorescence microscopy imaged samples (*A*–*C*) and histograms of flow cytometry analysis (*D*–*F*) represent frozen–thawed spermatozoa treated with DTT and stained with CMA3 (positive control; *A*, *D*), CMA3-stained test spermatozoa (*B*, *E*), and CMA3-unstained, with Hoechst 33342 only (negative control; *C*, *F*). Scale bars: 25 µm.

respective standard deviation (SD) values (mean ± SD). The association between the rates of CMA3-positive sperm and embryonic development was analyzed using a linear regression correlation test. All analyses were performed using SigmaPlot v.11.0 software (Systat Software, San Jose, CS, USA), using a significance level of 5% ($P < 0.05$).

Results

Experiment 1: Sperm protamination was not different between sexed and unsexed bull sperm

Flow cytometry was an accurate method for quantifying CMA-3 fluorescence, given that the percentage of CMA-3-positive staining increased from 0.02% in the negative control to 99.8% in the positive control. Therefore, negative and positive controls were used to gate the interval of fluorescence within which the experimental groups would be analyzed (Fig. 1).

Interestingly, bull 2 had a higher percentage of sperm with protamine deficiency than bull 1 for both unsexed (7.27 ± 2.0 versus 1.38 ± 0.1, respectively) and sexed (9.12 ± 0.8 versus 0.35 ± 0.1, respectively) sperm ($P < 0.05$). However, within subject, the sorting process had no effect on sperm protamination (Fig. 2).

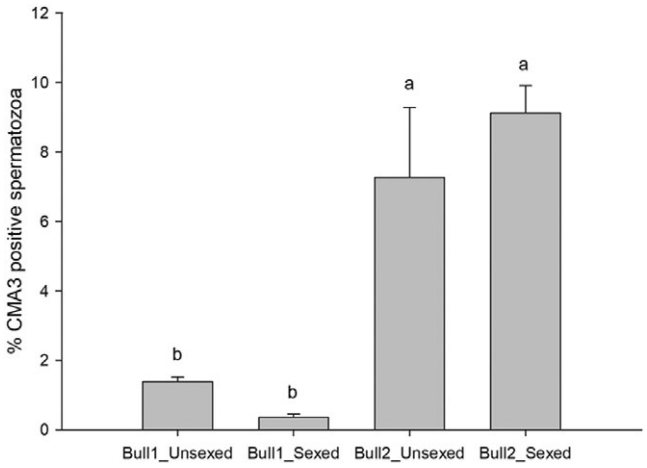

Figure 2. Percentage of CMA3-positive spermatozoa, evaluated by flow cytometry using unsexed and sex-sorted bull sperm. Results represent the mean ± standard deviation of three replicates, totalling 60,000 spermatozoa per group. [a,b]Different superscripts denote significant differences among groups ($P < 0.05$).

Experiment 2: Sperm protamination influences in vitro *embryo development*

We also assessed the effect of sperm origin, sexed or unsexed, on embryo development following IVF. As expected, for each bull, sexed spermatozoa yielded lower rates of embryo cleavage than unsexed spermatozoa ($P < 0.05$; Table 1). However, blastocyst rates did not differ ($P > 0.05$) between sexed and unsexed sperm for either bull. For 7-day embryos, there was no difference in measures morphological quality among groups.

Unsexed sperm from bull 1 yielded higher blastocyst and hatching rates than unsexed semen from bull 2 ($P < 0.05$). These results would suggest an effect of sperm protamination on blastocyst formation, considering the differences in CMA-3 positive staining levels detected between bulls (Experiment 1). Indeed a significant association was observed between CMA3-positive staining and cleavage rates (R^2 85.1, $P = 0.003$) as with blastocyst hatching rates

https://doi.org/10.1017/S0967199420000775 Published online by Cambridge University Press

Table 1. Rates of embryonic development following IVF with unsexed and sex-sorted spermatozoa from two different bulls

	Oocytes *n*	Cleavage*	Blastocyst*	Hatching†	Quality Grade 1†
Bull 1					
Non-sexed	105	82.9 ± 4.14^{a}	47.8 ± 4.71^{a}	48.2 ± 7.44^{a}	29.1 ± 10.1
Bull 1 sexed	100	67.2 ± 6.06^{b}	39.2 ± 5.71^{ab}	24.2 ± 9.54^{b}	28.7 ± 8.75
Bull 2					
Non-sexed	104	74.1 ± 3.86^{ab}	35.6 ± 2.42^{b}	17.9 ± 9.27^{bc}	29.5 ± 15.5
Bull 2 sexed	96	54.9 ± 6.06^{c}	32.3 ± 5.01^{b}	38.9 ± 9.62^{ac}	28.7 ± 8.75

The results represent the average of five replicates ± standard deviation.
^{a,b,c}Different superscripts denote a significant difference within columns ($P < 0.05$).
*Rates were calculated from the total number of oocytes in each group.
†The rates were calculated from the total number of blastocysts on day 7.

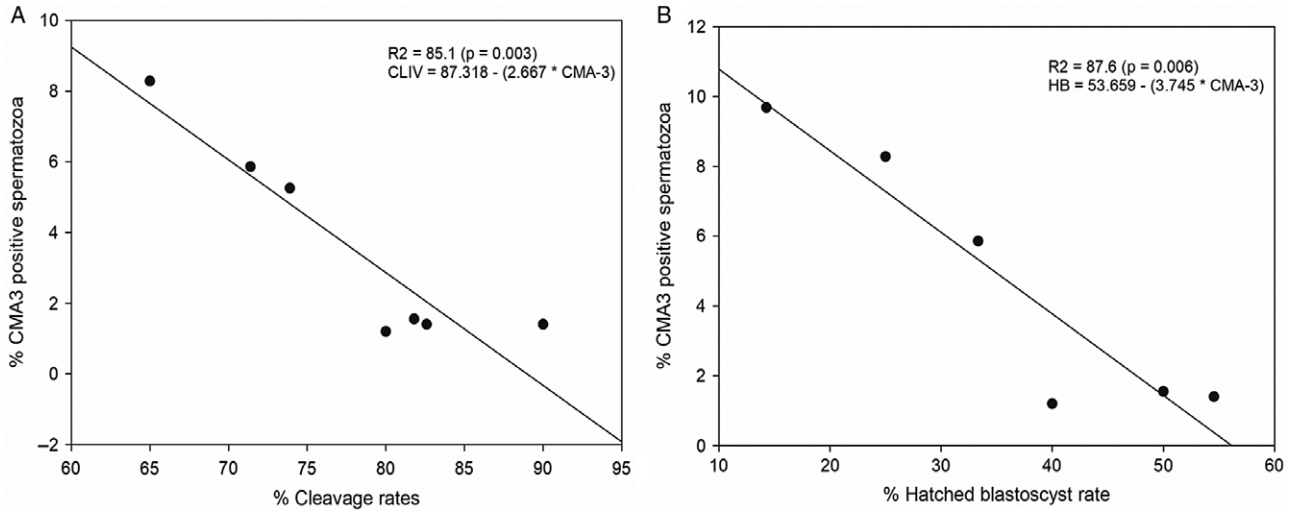

Figure 3. Association between sperm protamination and embryo development rates when using unsexed bull sperm. Sperm protamination was assessed by CMA-3 staining. Panels show the correlation rate of CMA3 staining with (*A*) cleavage or (*B*) blastocyst hatching rates following IVF with unsexed sperm from two bulls.

(R^2 87.6, $P = 0.006$) for unsexed semen from both bulls (Fig. 3). This inferred that these embryo development parameters are inversely correlated with protamine deficiency. In other words, protamine integrity is likely to be related to increased rates of early cleavage and blastocyst development. However, no association was found in sexed semen ($P > 0.05$), suggesting a negative influence of sex sorting on sperm quality.

Discussion

This study is the first to report the effect of sex sorting on bull sperm protamine content and, in turn, on rates and quality of embryo development following IVF. For this purpose, we compared unsexed and male sexed spermatozoa from two different bulls using CMA-3 and flow cytometry to assess sperm protamination. Reportedly, the sperm sexing process negatively affected all sperm membrane, cytoskeletal and DNA integrity (Xu *et al.*, 2009). Therefore, we hypothesized that DNA damage resulting from the sorting process could stem from direct effects on sperm chromatin packaging. However, we found no interference of the sexing process from sperm protamine content in either bull tested.

In humans, sperm protamine deficiency has been correlated with infertility (Iranpour *et al.*, 2000; Aoki and Carrell, 2004; Carrell *et al.*, 2007; Nanassy *et al.*, 2011). Notably, abnormal chromatin compaction resulting from protamine deficiency yielded DNA fragmentation, as well as methylation and epigenetic defects (Tavalaee *et al.*, 2009). The relationship between sperm protamine content and fertility was also evaluated in other species, including cattle (Simões *et al.*, 2009; Fortes *et al.*, 2014; Dogan *et al.*, 2015). Sperm protamine content in *Bos indicus* bulls correlated positively with DNA fragmentation index, as determined by sperm chromatin structure assay (Fortes *et al.*, 2014). Scrotal sac insulation, a model to study the effects of heat stress on bull fertility, reduced sperm protamine content (Rahman *et al.*, 2011). Moreover, field fertility scores of breeding bulls were negatively correlated with the percentage of sperm with reduced protamine content and fragmented DNA (Dogan *et al.*, 2015). These studies underscored the importance of sperm protamination and fertility in bull sperm.

In agreement with other groups, we observed individual differences in sperm protamine content, consistent with a bull effect (Simões *et al.*, 2009; Fortes *et al.*, 2014). Reportedly, the sorting process affected sperm quality, as well as fertility, in a bull-dependent manner (it varied according to individual) (Palma *et al.*, 2008; Xu *et al.*, 2009). In this study, for unsexed semen, there was a 5.89% difference in CMA3-positive sperm between the two

https://doi.org/10.1017/S0967199420000775 Published online by Cambridge University Press

bulls. This difference in protamination levels for unsexed semen was sufficient to affect embryo cleavage, blastocyst development and blastocyst hatching rates, which differed between the two bulls. Notably, the technique used to assess CMA-3 staining can also affect protamination level results. Using fluorescence microscopy to assess CMA-3 positive sperm, there was a 0.002% difference comparing fresh to frozen–thawed bull semen (Simões *et al.*, 2009) and a 0.15% difference between bulls yielding low or high *in vitro* cleavage and blastocyst development rates (Castro *et al.*, 2018). However, the range 7–14% CMA3-positive sperm, as assessed by flow cytometry, has been reported as normal in testis and epididymal sperm in bulls (Fortes *et al.*, 2014). This is considerably higher, not only compared with rates reported in previous studies using fluorescence microscopy, but also compared with the CMA3 rates observed in this study in which we used the same assessment technique. These differences may be due partially to the fact that protamination is completed only in the cauda epididymidis. In any case, we speculate that the ratio of protamine deficiency may be underestimated in cattle and more studies are needed to address this question.

It is worth noting that protamination or replacement of histones with protamines during sperm maturation is a crucial process that ensures epigenetic silencing in protamine-bound DNA regions and protection of the male genome up to fertilization (Carrell *et al.*, 2008). Therefore, abnormalities in protamine content might influence epigenetic signals transmitted by paternal DNA, interfering with embryonic development (Francis *et al.*, 2014; Rajabi *et al.*, 2016; Giacone *et al.*, 2019). Notably, while protamine content related to epigenetics and infertility has been widely studied in humans, information is only now being reported for other mammalian species (Ni *et al.*, 2016). In this study, the sex-sorting process had an effect on early embryo development, as assessed by cleavage rates following IVF. However, there was no effect on subsequent blastocyst formation rates. Reported results are conflicting. In some studies, no effects of sex sorting on IVEP were observed, although individual bull sperm quality, as assessed by motility and acrosome integrity, did affect IVF results (Carvalho *et al.*, 2010; Barceló-Fimbres *et al.*, 2011; Inaba *et al.*, 2016). However, other groups have reported decreased cleavage and embryo development rates with sex-sorted spermatozoa in cattle (Lu *et al.*, 2000; Sartori *et al.*, 2004; Bodmer *et al.*, 2005; Wilson *et al.*, 2006; Palma *et al.*, 2008). Notably, bovine male embryos tend to have faster developmental kinetics than female embryos *in vitro* (Beyhan *et al.*, 1999), and therefore we suspect that the use of male sexed spermatozoa in this study may have partially overcome any untoward effects related to the sexing process. Additional studies would be needed to test this hypothesis.

Interestingly, Puglisi *et al.* (2006) hypothesized that the sexing process may also select for a particularly study population of spermatozoa. Our data showed that sperm selection did not affect protamine content or blastocyst development following IVF. We hypothesized that oocyte-repair capabilities (Ménézo *et al.*, 2010; Fernández-Díez *et al.*, 2016) might aid in fixing any damage to sexed spermatozoa following fertilization. However, the entire fertilization process would be influenced by both oocyte quality and the extent of damage to spermatozoa, which is probably related to the bull effect, reflecting the different results in the literature.

For cattle, the number of replicates used here was in accordance with the current literature (Catteeuw *et al.*, 2017; Diógenes *et al.*, 2017; Botigelli *et al.*, 2017, 2018; Perrini *et al.*, 2018), although adding more animals could help to clarify the influence of the sex-sorting process on protamination, this was not possible as, firstly, we aimed to standardize the protocol to measure it. Therefore, future studies may clarify the influence of bull genotype on protamination of sexed semen. This study adds a reliable protocol for protamination analysis in sexed semen using flow cytometry, as showed by the accuracy and low SD. We hypothesize that while the sex-sorting process had no effect on bull sperm protamine content, protamination defects may interfere with embryo cleavage following IVEP. In conclusion, while the sex-sorting process had no effect on bull sperm protamine content, protamination defects may interfere with embryo cleavage following IVEP.

Acknowledgements. TVGS performed most of the experiments and wrote the manuscript. EBS, AAG and CAS contributed to the flow cytometry analysis. VCB and AJML contributed to the IVEP experiments. STRF and NNCA contributed to the statistical analysis. PPBS and MSC revised the manuscript. SSDS, MSM and OMO designed the study. All the authors read and accepted the manuscript.

Financial support. This study was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPQ) and Bubras Investiments and Participation.

Conflict of interest. Authors report no conflict of interest.

Ethical standards. Not applicable.

References

Aoki V and Carrell D (2004). Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. *Asian J Androl* **5**, 315–24.

Aoki VW, Liu L and Carrell DT (2005). Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Hum Reprod* **20**, 1298–306.

Barceló-Fimbres M, Campos-Chillón L F and Seidel Jr GE (2011). *In vitro* fertilization using non-sexed and sexed bovine sperm: sperm concentration, sorter pressure, and bull effects. *Reprod Domest Anim* **46**, 495–502.

Beyhan Z, Johnson LA and First NL (1999). Sexual dimorphism in IVM-IVF bovine embryos produced from X and Y chromosome-bearing spermatozoa sorted by high speed flow cytometry. *Theriogenology* **52**, 35–48.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U and Sakkas D (1993). Effect of Deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick-translation of murine and human mature spermatozoa. *Biol Reprod* **49**, 1083–8.

Bodmer M, Janett F, Hässig M, Daas N den, Reichert P and Thun R (2005). Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions. *Theriogenology* **64**, 1647–55.

Boe-Hansen GB, Morris ID, Ersbøll AK, Greve T and Christensen P (2005). DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology* **63**, 1789–802.

Botigelli RC, Schwarz KL, Zaffalon FG, Del Collado M Castro FC, Fernandes H and Leal CLV (2017). Influence of nitric oxide and phosphodiesterases during *in vitro* maturation of bovine oocytes on meiotic resumption and embryo production. *Zygote* **25**, 321–30.

Botigelli RC, Razzaa EM, Pioltinea EM, Fontesa PK, Schwarzb KRL, Leal CLV and Nogueira MFG (2018). Supplementing *in vitro* embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulus–oocyte complexes and embryos. *Reprod Biol* **1**, 66–75.

Butler ST, Hutchinson IA, Cromie AR and Shalloo L (2014). Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal*, **8**, 165–172.

Carrell DT (2012). Epigenetics of the male gamete. *Fertil Steril* **97**, 267–74.

https://doi.org/10.1017/S0967199420000775 Published online by Cambridge University Press

Carrell DT, Emery BR and Hammoud S (2007). Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update* **13**, 313–27.

Carrell DT, Emery BR and Hammoud S (2008). The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. *Int J Androl* **31**, 537–45.

Carvalho JO, Sartori R, Machado GM, Mourão GB and Dode MAN (2010). Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in *in vitro* embryo production. *Theriogenology* **74**, 1521–30.

Carvalho JO, Silva LP, Sartori R and Dode MAN (2013). Nanoscale differences in the shape and size of X and Y chromosome-bearing bovine sperm heads assessed by atomic force microscopy. *PLoS One* **8**, e59387.

Castro LS, Siqueira AFP, Hamilton TRS, Mendes CM, Visintin JA and Assumpção MEOA (2018). Effect of bovine sperm chromatin integrity evaluated using three different methods on *in vitro* fertility. *Theriogenology*, **107**, 142–8.

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM and Van Soom A (2017). *In vitro* production of bovine embryos derived from individual donors in the Corral® dish. *Acta Vet Scand* **59**, 41.

Cree LH, Balhorn R and Brewer LR (2011). Single molecule studies of DNA-protamine interactions. *Protein Pept Lett* **18**, 802–10.

Diógenes MN, Guimarães ALS, Leme LO and Dode MAN (2017). Bovine *in vitro* embryo production: the effects of fibroblast growth factor 10 (FGF10). *J Assist Reprod Genet* **34**, 383–90.

Dogan S, Vargovic P, Oliveira R, Belser LE, Kaya A, Moura A, Sutovsky P, Parrish J, Topper E and Memili E (2015). Sperm protamine-status correlates to the fertility of breeding bulls. *Biol Reprod* **92**, 92.

Fathi Z, Tavalaee M, Kiani A, Deemeh MR, Modaresi M and Nasr-Esfahani MH (2011). Flow cytometry: a novel approach for indirect assessment of protamine deficiency by CMA3 staining, taking into account the presence of M540 or apoptotic bodies. *Int J Fertil Steril* **5**, 128–33.

Fernández-Díez C, González-Rojo S, Lombó M and Herráez MP (2016). Impact of sperm DNA damage and oocyte-repairing capacity on trout development. *Reproduction* **152**, 57–67.

Fortes MRS, Satake N, Corbet DH, Corbet NJ, Burns BM, Moore SS and Boe-Hansen GB (2014). Sperm protamine deficiency correlates with sperm DNA damage in *Bos indicus* bulls. *Andrology* **2**, 370–8.

Francis S, Yelumalai S, Jones C and Coward K (2014). Aberrant protamine content in sperm and consequential implications for infertility treatment. *Hum Fertil* **17**, 80–9.

Frijters ACJ, Mullaart E, Roelofs RMG, van Hoorne RP, Moreno JF, Moreno O and Merton JS (2009). What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? *Theriogenology* **71**, 64–7.

Garcia-Herreros M, Carter TF, Villagómez DAF, MacAulay AD, Rath D, King WA and Lonergan P (2010). Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reprod Fertil Dev* **22**, 1272–8.

Giacone F, Cannarella R, Mongioì LM, Alamo A, Condorelli RA, Calogero AE and La Vignera S (2019). Epigenetics of male fertility: effects on assisted reproductive techniques. *World J Men Health* **37**, 148–56.

Holm P, Booth PJ, Schmidt MH, Greve T and Callesen H (1999). High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52**, 683–700.

Inaba Y, Abe R, Geshi M, Matoba S, Nagai T and Somfai T (2016). Sex sorting of spermatozoa affects developmental competence of *in vitro* fertilized oocytes in a bull-dependent manner. *J Reprod Dev* **62**, 451–6.

Iranpour FG, Nasr-Esfahani MH, Valojerdi MR and al-Taraihi TM (2000). Chromomycin A3 staining as a useful tool for evaluation of male fertility. *J Assist Reprod Genet* **17**, 60–6.

Leibfried L and First NL (1979). Characterization of bovine follicular oocytes and their ability to mature *in vitro*. *J Anim Sci* **48**, 76–86.

Lu KH, Cran DG and Seidel GE Jr (2000). *In vitro* fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology* **52**, 1393–405.

Ménézo Y, Dale B and Cohen M (2010). DNA damage and repair in human oocytes and embryos: a review. *Zygote* **18**, 357–65.

Miller D, Brinkworth M and Iles D (2010). Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* **139**, 287–301.

Morton KM, Herrmann D, Sieg B, Struckmann C, Maxwell WM, Rath D, Evans G, Lucas-Hahn A, Niemann H and Wrenzycki C (2007). Altered mRNA expression patterns in bovine blastocysts after fertilisation *in vitro* using flow-cytometrically sex-sorted sperm. *Mol Reprod Dev* **74**, 931–40.

Nanassy L, Liu L, Griffin J and Carrell DT (2011). The clinical utility of the protamine 1/protamine 2 ratio in sperm. *Protein Pept Lett* **18**, 772–7.

Ni K, Spiess A-N, Schuppe H-C and Steger K (2016). The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: a systematic review and meta-analysis. *Andrology* **4**, 789–99.

Palma GA, Olivier NS, Neumüller C and Sinowatz F (2008). Effects of sex-sorted spermatozoa on the efficiency of *in vitro* fertilization and ultrastructure of *in vitro* produced bovine blastocysts. *Anat Histol Embryol* **37**, 67–73.

Parrish JJ, Susko-Parrish J, Winer M and First NL (1988). Capacitation of bovine sperm by heparin. *Biol Reprod* **38**, 1171–80.

Perrini C, Esposti P, Cremonesi F and Consiglio AL (2018). Secretome derived from different cell lines in bovine embryo production *in vitro*. *Reprod Fertil Dev* **30**, 658–71.

Puglisi R, Vanni R, Galli A, Balduzzi D, Parati K, Bongioni G, Crotti G, Duchi R, Galli C, Lazzari G and Aleandri R (2006). *In vitro* fertilisation with frozen–thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. *Reproduction* **132**, 519–26.

Rahman MB, Vandaele L, Rijsselaere T, Maes D, Hoogewijs M, Frijters A, Noordman J, Granados A, Dernelle E, Shamsuddin M, Parrish JJ and Van Soom A (2011). Scrotal insulation and its relationship to abnormal morphology, chromatin protamination and nuclear shape of spermatozoa in Holstein-Friesian and Belgian Blue bulls. *Theriogenology* **76**, 1246–57.

Rajabi H, Mohseni-Kouchesfehani H, Mohammadi-Sangcheshmeh A, Farifteh-Nobijari F and Salehi M (2016). Pronuclear epigenetic modification of protamine deficient human sperm following injection into mouse oocytes. *Syst Biol Reprod Med* **62**, 125–32.

Sartori R, Souza AH, Guenther JN, Caraviello DZ, Geiger LN, Schenk JL and Wiltbank MC (2004). Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. *Anim Reprod* **1**, 86–90.

Seidel GE (2007). Overview of sexing sperm. *Theriogenology* **68**, 443–6.

Simões R, Feitosa WB, Mendes CM, Marques MG, Nicacio AC, de Barros FR, Visintin JA and Assumpção ME (2009). Use of chromomycin A3 staining in bovine sperm cells for detection of protamine deficiency. *Biotech Histochem* **84**, 79–83.

Stringfellow DA and Seidel SM (1998). *Manual of the International Embryo Transfer Society* (3rd edn). International Embryo Transfer Society, IETS.

Tavalaee M, Razavi S and Nasr-Esfahani MH (2009). Influence of sperm chromatin anomalies on assisted reproductive technology outcome. *Fertil Steril* **91**, 1119–26.

Tavalaee M, Kiani A, Arbabian M, Deemeh MR and Esfahani MHN (2010). Flow cytometry: a new approach for indirect assessment of sperm protamine deficiency. *Int J Fertil Steril* **3**, 184.

Wilson RD, Fricke PM, Leibfried-Rutledge ML, Rutledge JJ, Penfield CMS and Weigel KA (2006). *In vitro* production of bovine embryos using sex-sorted sperm. *Theriogenology* **65**, 1007–15.

Xu J, Chaubal SA and Du F (2009). Optimizing IVF with sexed sperm in cattle. *Theriogenology* **71**, 39–47.

Zhao XM, Ren JJ, Zhao SJ, Cui LS, Hao HS, Wang HY, Du WH, Qin T, Liu Y, Wang D and Zhu HB (2014). Apoptosis-Like events and *in vitro* fertilization capacity of sex-sorted bovine sperm. *Reprod Domest Anim* **49**, 543–9.