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Effect of Percoll volume, duration and force of centrifugation, on in vitro production and sex ratio of bovine embryos

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Abstract

The objective was to evaluate the effect of Percoll volume, and duration and force of centrifugation on sperm quality characteristics, embryo development, and sex ratio of in vitro-produced (IVP) bovine embryos. Frozen–thawed semen from four bulls were submitted to three Percoll procedures: T1—4 mL of Percoll, centrifuged for 20 min at 700 g; T2—800 μL of Percoll, centrifuged for 20 min at 700 g; and T3—800 μL of Percoll, centrifuged for 5 min at 5000 g. Sperm total motility, morphology and integrity of the sperm acrosome, membrane and chromatin were determined before and after Percoll treatment, and semen was used for in vitro fertilization (IVF) of in vitro-matured oocytes. All Percoll methods increased the proportion of motile sperm ($P < 0.05$). There were no significant effects of treatment for any sperm characteristic; however, for every end point, there were significant differences among bulls. Similarly, rates of cleavage and blastocyst formation were not affected by the Percoll procedure ($P > 0.05$), but were affected by sire ($P < 0.05$). Sex ratio was similar among treatments for Bulls 2 and 3, whereas semen from Bull 1 processed by T1 yielded a greater percentage of male embryos. However, when only treatments were considered, independent of bulls, the proportion of male:female embryos did not differ significantly from an expected 1:1 ratio. In conclusion, decreasing Percoll volume, reducing duration of centrifugation, and using a higher force of centrifugation did not significantly affect sperm quality, embryo development, or sex ratio of in vitro-produced bovine embryos.
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Keywords: In vitro embryo production; Percoll centrifugation; Sex ratio; Sperm quality; Cattle

1. Introduction

Sperm selection methods are routinely applied to prepare semen for in vitro fertilization (IVF) in various species. These procedures are used to improve sperm quality characteristics and to remove seminal plasma/cryoprotectant, as well as other background material and debris [1]. Several methods are available for sperm preparation for IVF, including swim up, Percoll gradient, and wash by centrifugation. Although there were no differences among these methods in rates of fertilization [2] or blastocyst production [3], Percoll gradient is the most widely used in bovine IVF laboratories [4–6].

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With increasing utilization of sexed sperm, changes in Percoll protocols have been proposed [7], because sex-sorted samples had reductions in concentration, post-thaw motility [8], and rate of blastocyst production relative to unsorted semen [9]. In essence, the proposed modifications were a lower volume of Percoll and centrifugation at a greater force, but of shorter duration [7]. If these changes could be applied routinely for sperm preparation, independent of whether semen samples were sex sorted, this would be of great interest for IVF laboratories, as it would reduce cost and preparation time. However, it is not known whether these changes would affect sperm quality, in vitro production of bovine embryos, and sex ratio when unsorted semen is used.

It has been reported that not only motility [10] was enhanced after Percoll selection, but also the percentage of cells with normal morphology [11], an intact membrane [12], and an intact acrosome [4,13]. Conversely, the force of centrifugation used with a Percoll gradient might affect motility and membrane integrity of sperm from bulls [14] and rams [15]. Perhaps changes in Percoll volumes and force of centrifugation can influence acrosome and membrane integrity of sperm, as well as motility, with effects on sperm–egg interaction, fertilization, and ultimately embryo production.

It is well established that the X and Y chromosome have morphological differences, e.g. size and DNA content [16]. The difference in DNA mass between X and Y chromosome-bearing sperm causes a difference in weight and density, which permits their separation in a discontinuous and/or continuous gradient. In that regards, Percoll density gradient centrifugation has been used in humans and cattle to separate sperm cells carrying an X or Y chromosome [17–20]. Furthermore, changes in volume of Percoll, as well as duration of centrifugation, can favor lighter or heavier sperm, thereby altering the ratio of X to Y bearing sperm [19]. Therefore, if alterations in Percoll procedures are contemplated, it is essential to determine whether the changes will affect the sex ratio of the embryos produced.

The objective of the present study was to evaluate the effects of centrifugation force and duration, as well as Percoll volume, on sperm quality characteristics, embryo development, and sex ratio of IVP bovine embryos.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO, USA). All media used for in vitro embryo production, maturation medium, fertilization medium, sperm washing medium (sp-TALP), gradient Percoll 90% and synthetic oviduct fluid (SOF) medium were supplied by Nutricell® (Campinas, SP, Brazil).

2.2. Sperm quality parameters assessment

All sperm samples were evaluated, before and after Percoll treatments, for concentration, total motility, morphology, and integrity of the plasma membrane, acrosome, and chromatin.

2.2.1. Assessment of motility, morphology and sperm concentration

The percentage of total motile sperm was determined subjectively, on a drop of semen placed in a pre-warmed glass-slide covered with a coverslip, and examined under a bright-field microscope at 400× magnification. Sperm morphology was evaluated according to Barth and Oko [20], using phase contrast microscopy (1000× magnification). Specifically, head defects (underdevelopment, narrow and narrow at the base, abnormal contour and pyriform shape), nuclear vacuoles (pouch formation and crater defects), acrosome defects (knobbed, folded, tapered, detached), midpiece defects (tail stump, corkscrew, broken and denuded), proximal droplets, tail defects (broken, coiled, bent tail and abaxial attachment), and loose heads were considered. A total of 200 cells were counted and the results were expressed as percentages.

Sperm concentration was determined in a hemocytometer in a 1:200 dilution. The results are presented as sperm cell/mL. The recovery rate was calculated according to the following formula:

$$\text{Recovered sperm}(\%) = \frac{(\text{Concentration}_{\text{final}} \times \text{Volume}_{\text{final}})}{(\text{Concentration}_{\text{initial}} \times \text{Volume}_{\text{initial}})} \times 100$$

2.2.2. Assessment of plasma membrane, acrosome and cromatine integrity

Sperm membrane integrity was assessed using 6-carboxy-fluorescein diacetate (FDA; propidium iodide [(PI), Molecular Probe®, Eugene, OR, USA)], as described by Harrison and Vickers [21]. An aliquot of semen (10 μL) was added to the stain solution (40 μL) and incubated for 10 min. The stain solution was composed of buffered formal saline (96 mL of 0.9% saline solution and 4 mL of formal 40%), sodium citrate (3%), PI (0.75 mM), and FDA solution (0.46 mg/mL in

dimethyl sulfoxide). An aliquot (5 μL) of stained suspension was placed on a slide, covered with a coverslip, and observed under an epifluorescence microscope (Axiophot Zeiss: barrier filter 494/517 nm excitation/emission and 536/617 nm excitation/emission). For each slide, a total of 200 sperm were counted and classified as having an intact or damaged plasma membrane. Those cells stained green with FDA were considered as having an intact membrane, whereas those stained red with IP were deemed to have a damaged membrane.

Acrosome status was examined using fluorescent probe isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and PI, as described by Klinc and Rath [22]. An aliquot (10 μL) of a thawed semen sample was diluted with staining solution (30 μL) and incubated for 10 min. The stain solution consisted of buffered formal saline, sodium citrate (3%), PI (0.75 mM), and FITC-PNA solution (1 mg/mL in PBS). An aliquot (5 μL) of stained suspension was placed on a slide and covered with a coverslip. At least 200 sperm were examined under a phase contrast and epifluorescence microscope (Axiophot Zeiss: barrier filter 494/518 nm excitation/emission). Sperm labeled in red with PI were considered damaged, whereas those without the red staining were considered alive. Living cells were classified as acrosome-reacted, if the acrosome had uniform FITC-PNA green fluorescence, or as acrosome-intact, if no fluorescence was visible.

Chromatin integrity was determined using the acridine orange staining procedure. Three smears were prepared for each sample, air-dried and fixed overnight in freshly prepared Carnoy solution (methanol:acetic acid, 3:1). Then, the slides were again air-dried and incubated in a solution containing 80 mmol/L citric acid and 15 mmol/L Na2HPO4 (pH 2.5) at 75 °C for 5 min to test chromatin stability. Subsequently, slides were stained for 5 min with a solution containing acridine orange stain (0.2 mg/mL), citric acid (0.1 M) and di-sodium phosphate (0.3 M). The slides were analyzed the same day using an epifluorecense microscope (Axiophot Zeiss: barrier filter 490/530 nm excitation/emission). For all samples, 200 cells were examined in each slide at 1000× magnification. Sperm with normal DNA content had green fluorescence, whereas sperm with abnormal DNA emitted fluorescence in a spectrum varying from yellow-green to red.

2.3. Embryo production

2.3.1. Oocyte recovery and in vitro maturation (IVM)

Ovaries from crossbred cows (*Bos indicus* × *Bos taurus*) were collected immediately after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 μg/mL) at 35 °C. Cumulus oocyte complexes (COC) were aspirated from 2 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 mL conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and at least three layers of cumulus cells were used. The selected COCs were washed and transferred, in groups of 30–35, to a 200 μL drop of maturation medium under silicone oil, and incubated for 22 h at 39° C in 5% of CO_2 in air.

2.3.2. Sperm selection procedures

Frozen–thawed sperm cells from each bull were selected by centrifugation on a Percoll discontinuous gradient (45–90%). The 45% Percoll solution was prepared with 1 mL of 90% Percoll and 1 mL of sp-TALP. The volume of Percoll gradient, duration, and velocity of centrifugation were determined according to treatment. For the first treatment (T1), semen samples were layered on Percoll gradient consisting of 2 mL of 45% Percoll gradient and 2 mL of 90% Percoll gradient, in a 15-mL centrifuge tube, and centrifuged at 700 × *g* for 20 min at 30 °C in a refrigerated centrifuge. The second treatment (T2), consisted of centrifugation of the semen samples in a total volume of 800 μL, comprised 400 μL of 45% Percoll gradient and 400 μL of 90% Percoll gradient, placed in a 2-mL microtube, and centrifuged for 20 min at 700 × *g* at 30 °C. In treatment 3 (T3), semen samples were layered in a total volume of 800 μL, with 400 μL of 45% Percoll gradient and 400 μL of 90% Percoll gradient, placed in a 2-mL microtube, and centrifuged at 5000 × *g* for 5 min. After centrifugation, the supernant was discarded and the pellet was centrifuged for 5 min in sp-TALP at 700 × *g* (T1 and T2) or 5000 × *g* (T3). Then, the resultant pellet was re-suspended with 80 μL of sp-TALP and used for semen analysis and/or IVF.

2.3.3. In vitro fertilization (IVF) and embryo culture (IVC)

Following maturation, COCs were transferred to a 200 μL drop of fertilization medium. Frozen semen from each bull, after being subjected to the various Percoll treatments, was counted in a hemocytometer and added into the fertilization drop (final concentration, 1×10^6 spermatozoa/mL). Sperm and oocytes were co-incubated for 20 h at 39 °C with 5% of CO_2 in air (the day of in vitro insemination was defined as Day 0). After co-incubation, presumptive zygotes were

washed and transferred to 200 μL drops of SOF medium supplemented with 5% fetal calf serum (FCS), and cultured at 39 °C and 5% of CO_2 in air for 7 days. Embryos were evaluated on Day 2 for cleavage, and on Days 6 and 7 for blastocyst rate. On Day 7, only grades 1 and 2 blastocysts were frozen individually and stored at −80 °C until they were subjected to embryo sexing.

2.4. Embryo sexing procedure

Embryo sex identifications were determined by PCR, using two pairs of primers. The first pair was specific to a region of the Y chromosome, whereas the second one was specific to a bovine autosomal gene. The primer sequences and fragment size for each gene are shown (Table 1). The Y and X chromosome control solution was bovine genomic DNA which originated from a male and a female. Initially, embryos were exposed for 5 min at 50 °C to a lysis solution containing 1 × PCR buffer and 15 μg proteinase K (Invitrogen®) in a final volume of 10 μL; thereafter, proteinase K was inactivated at 95 °C for 5 min. The PCR was performed by adding to each sample tube a PCR mix containing 20 nM of each pair of primers, 200 μM of each dNTP, PCR buffer 1X and 1U Platinum Taq DNA Polimerase® (Invitrogen®), in a final volume of 30 μL. The PCR program used 40 cycles of 94 °C for 20 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a final extension of 72 °C for 30 min. Amplified PCR products were eletrophoresed in 2.0% agarose gel stained with ethidium bromide (10 mg/mL), and visualized under UV illumination. When two amplicons were detected, the embryo was considered male, whereas detection of only one amplicon was considered female.

2.5. Experimental design

Frozen semen from four bulls (1, 2, 3, and 4) was used. Only Bull 1 had known in vitro fertility; he had been used for several years as the reference bull for in vitro embryo production in our laboratory. In each replicate, three straws of semen from each bull were thawed and pooled into a centrifuge tube; samples were taken for sperm evaluation and then semen was distributed equally to each Percoll treatment. After Percoll centrifugation and re-suspension, samples were removed for semen analysis and then semen was used for IVF. A total of 1628 COCs were used for in vitro maturation. All sperm quality tests and IVF experiments were repeated three times in independent replicates.

2.6. Statistical analysis

The effect of treatment, sire, and their interaction on sperm characteristics was analyzed by two-way ANOVA and Tukey's test, when values had a normal distribution. Otherwise, they were submitted to Friedman's test. Cleavage and blastocyst rates, expressed as percentages, were also examined by two-way ANOVA and Tukey's test (Prophet program, version 5.0; 1997); the effects of bull, treatment, and their interaction were evaluated. A chi-square analysis (Prophet program, Version 5.0, 1997) was used to compare sex ratios of Day 7 embryos with the expected 1:1 ratio for various sperm separation treatments and bulls. Comparisons among the three bulls (one was excluded due to very limited numbers of embryos produced) and the three treatments for these ratios were also performed by chi-square analysis. For all statistical analyses, the level of significance was $P < 0.05$.

3. Results

Semen characteristics before and after Percoll selection are shown (Table 2). For all sperm quality end points, there were no significant differences among Percoll treatments. However, there were significant differences before versus after Percoll treatments; all treatments improved total motility. A higher proportion of cells with intact membrane and intact acrosome were observed after Percoll centrifugation for T2 and T1, respectively. Conversely, morphological abnormalities and chromatin integrity had the least changes after Percoll treatments.

The recovery rate after Percoll gradient was similar ($P > 0.05$) for all treatments (mean ± SEM, 19.2 ± 2.7, 17.0 ± 2.9, and 17.3 ± 2.7% for T1, T2, and T3, respectively).

Table 1
Sequences of the primers used for sexing bovine embryos (S: sense; A: antisense) and size of the amplified fragments.

Gene	Primer sequence	Amplicon size (bp)	Reference
bSRY S	5′-CCTCCCCTTCAAACGCCCGGAATCATT -3′	210	Bondiolli et al. [40]
bSRY A	5′-GGCCATAGTCAGGATCTT-3′		
Autosomal S	5′-CCCATCACCATCTTCCAGG-3′	280	Ellis et al. [41]
Autosomal A	5′-AGTGAGCTTCCCGTTCAGC-3′		

Table 2
Mean ± SEM total motility and percentages of bovine sperm with normal morphology, intact membrane, intact acrosome, and intact chromatin before and after passage through a Percoll gradient.

Treatment	Total motility (%)	Normal morphology (%)	Membrane integrity (%)	Live with intact acrossome (%)	Intact chromatin (%)
T0	41.3 ± 3.6a	83.8 ± 2.3a	44.7 ± 2.0a	42.8 ± 2.9a	97.1 ± 0.5a
T1	76.3 ± 3.1b	86.6 ± 2.8a,b	56.1 ± 3.2a,b	57.3 ± 3.3b	98.0 ± 0.4a,b
T2	77.9 ± 3.8b	89.2 ± 2.1b	57.6 ± 4.7b	47.9 ± 5.2a,b	98.2 ± 0.4b
T3	74.6 ± 4.0b	88.6 ± 2.4b	52.3 ± 3.9a,b	46.8 ± 4.9a,b	98.0 ± 0.5a,b

Within a column, means without a common letter differed ($P < 0.05$). T0—Assessment before passage through Percoll; T1—700 g/20 min in centrifuge tubes using 2 mL of Percoll 45% and 2 mL of Percoll 90%; T2—700 g/20 min in microtubes using 400 μL of Percoll 45% and 400 μL of Percoll 90%, T3 – 5000 g/5 min in microtubes using 400 μL of Percoll 45% and 400 μL of Percoll 90%, difference ($P < 0.05$).

Table 3
Mean ± SEM characteristics of bovine sperm (from four bulls) following Percoll centrifugation.

Bull	Total motility (%)	Normal morphology (%)	Intact membrane (%)	Alive with intact acrosome (%)	Intact chromatin (%)
1	83.9 ± 2.6a	85.6 ± 1.5a	64.6 ± 4.2a	62.2 ± 4.6a	98.9 ± 0.3a
2	80.8 ± 4.2a	94.3 ± 0.4b	55.5 ± 2.9a,b	55.5 ± 4.0a,b	99.4 ± 0.2a
3	55.4 ± 4.6b	77.8 ± 2.4a	47.9 ± 5.6b	39.0 ± 4.5b	96.3 ± 0.4b
4	60.4 ± 5.1b	94.7 ± 0.8b	53.4 ± 3.6a,b	45.9 ± 4.2a,b	97.6 ± 0.3b

Within a column, means without a common letter differed ($P < 0.05$).

Although there was no significant interaction between treatments and sires and no significant differences among Percoll treatments for any of the sperm characteristics studied, there were significant bull-related differences for all end points (Table 3). When sperm quality was evaluated among bulls, independent of Percoll treatment, Bulls 1 and 2 were similar for most characteristics, with higher motility and higher percentage of cells with intact chromatin than Bulls 3 and 4. In contrast, Bull 3 had the lowest values for all characteristics studied (Table 3).

Cleavage and blastocyst rates for each bull in each treatment are shown (Table 4); there were no significant differences among treatments. Although

Table 4
Number and mean ± SEM (%) of embryos that underwent cleavage and blastocyst production after IVF using sperm from four bulls subjected to three Percoll protocols.

Bull 1	No.	Cleavage	Blastocysts (Day 6)	Blastocysts (Day 7)
T1	276	241 (87 ± 1.5)	70 (28 ± 4.3)	130 (48 ± 2.1)
T2	123	100 (82 ± 4.9)	35 (29 ± 6.3)	54 (45 ± 10.7)
T3	113	97 (86 ± 4.2)	30 (27 ± 6.5)	49 (44 ± 6.6)
Bull 2				
T1	113	95 (84 ± 2.7)	17 (16 ± 4.7)	33 (31 ± 11.3)
T2	106	91 (86 ± 1.4)	18 (17 ± 3.8)	42 (40 ± 11.1)
T3	121	108 (89 ± 6.0)	34 (30 ± 13.7)	55 (46 ± 14.5)
Bull 3				
T1	120	75 (61 ± 12.7)	9 (7.0 ± 3.3)	12 (10 ± 3.8)
T2	119	73 (60 ± 11.5)	9 (7.0 ± 2.2)	20 (16 ± 6.0)
T3	103	76 (74 ± 8.0)	16 (14 ± 6.6)	27 (26 ± 9.3)
Bull 4				
T1	145	70 (46 ± 9.8)	2 (1.0 ± 0.6)	3 (2.0 ± 1.7)
T2	152	65 (42 ± 5.3)	1 (0.6 ± 0.6)	2 (1.2 ± 0.6)
T3	137	59 (43 ± 2.7)	0	0

T1—700 g/20 min in centrifuge tubes using a 2 mL of Percoll 45% and 2 mL of Percoll 90%; T2—700 g/20 min in microtubes using a 400 μL of Percoll 45% and 400 μL of Percoll 90%; T3—5000 g/5 min in microtubes using a 400 μL of Percoll 45% and 400 μL of Percoll 90%.

Table 5
Number and mean ± SEM (%) of embryos that underwent cleavage and blastocyst production after IVF using sperm from three bulls.

Bull	No.	Cleavage	Blastocysts (Day 6)	Blastocysts (Day 7)
1	512	438 (86 ± 1.6)a	135 (28 ± 2.9)a	233 (47 ± 2.6)a
2	340	294 (86 ± 2.1)a	69 (21 ± 4.9)a,b	130 (39 ± 6.6)a
3	342	222 (65 ± 5.9)b	34 (10 ± 2.5)b	59 (17 ± 4.2)b

Within a column, means without a common letter differed ($P < 0.05$).

there was no significant interaction between Percoll treatment and sire, there was a significant effect of the sire on rates of cleavage and blastocyst formation. Bulls 1 and 2 had higher ($P > 0.05$) rates of cleavage and blastocyst formation than Bull 3 (Table 5; Bull 4 was excluded, due to very limited numbers of embryos produced).

From the 427 Day-7 embryos produced, 360 (84%) were successfully sexed by PCR. The sex ratios obtained for Bulls 2 and 3 in all treatments were not different from the expected 1:1 ratio (Table 6). However, there was a shift in sex ratio (increased proportion of male embryos) for Bull 1 when T1 was used (Table 6). Moreover, when only Percoll treatments were considered (independent of the sire), there were no significant deviations (from the 1:1 ratio) in the proportions of male and female embryos (Table 6).

Table 6
Sex ratio of bovine blastocysts at Day 7 after IVF using sperm from three bulls and three treatments (T1, T2 and T3).

	Sexed embryos	Male:female (%)	P[a]
Bull 1			
T1	80	49:31 (61:39)	0.04
T2	52	24:28 (46:54)	0.58
T3	48	21:27 (44:56)	0.39
Bull 2			
T1	30	12:18 (40:60)	0.27
T2	40	19:21 (48:52)	0.75
T3	54	22:32 (41:59)	0.17
Bull 3			
T1	12	6:6 (50:50)	0.99
T2	19	7:12 (37:63)	0.25
T3	25	13:12 (52:48)	0.84
Total			
T1	122	67:55 (55:45)	0.28
T2	111	50:61 (45:55)	0.30
T3	127	56:71 (44:56)	0.18

T1—700 g/20 min in centrifuge tubes using a 2 mL of Percoll 45% and 2 ml of Percoll 90%; T2—700 g/20 min in microtubes using a 400 μL of Percoll 45% and 400 μL of Percoll 90%; T3—5000 g/5 min in microtubes using a 400 μL of Percoll 45% and 400 μL of Percoll 90%.

[a] Sex ratio compared to expected ratio of 1:1.

4. Discussion

A Percoll gradient is the most widely used method to prepare bull sperm for IVF. Although the protocol has undergone slight changes over the years, with the recent widespread use of sex-sorted sperm in commercial IVF, more substantial modifications have been proposed. Since it is not desirable for an IVF laboratory to have different protocols for sexed and non-sexed semen samples, a method that can be used with both types of samples should be chosen. In the present study, we compared various protocols of Percoll preparation to determine the effects on sperm quality end points, embryo production, and embryo sex ratio.

Many groups have reported that Percoll gradient is the preferred method to prepare sperm for IVF, because it yields the best quality sperm sample [4,10,13,23]. Nevertheless, centrifugation is a potentially sperm-damaging step in sperm processing [24] and Percoll passage imposes mechanical contact with the cells. Therefore to ensure that the force of centrifugation and volume of Percoll would not affect bovine sperm characteristics, we compared sperm quality end points before and after various Percoll treatments.

As expected, the proportion of sperm with total motility increased after Percoll passage in all treatments; this was the end point with the greatest magnitude of post-treatment improvement. The effect of Percoll in selecting sperm with higher motility has been well documented [4,10,12,25–27]. It is noteworthy that there were no significant differences among treatments in the magnitude of the improvement.

Although the shorter duration of centrifugation used in T3 could have reduced the percentage of cells recovered after Percoll centrifugation, there were no significant differences among treatments. Perhaps the highest centrifugal force used in this treatment compensated for the reduced duration of centrifugation.

There were no significant differences among treatments for all sperm end points evaluated. It has been reported that centrifugation might damage sperm from rams [15], mice [28], and humans [29,30]. Additionally it has also been reported that passage through a Percoll

gradient can alter the plasma membrane and cause a premature acrosome reaction [4], due to membrane destabilization. However, these alterations were not detected in the present study; Percoll volumes, force and duration of centrifugation did not significantly affect sperm quality. That bovine and equine sperm were somewhat less sensitive to centrifugation than sperm from other mammals [31,32] may have accounted for the apparent lack of damage.

In contrast to treatment, there was a significant bull effect for all end points analyzed. High variability among individual bulls was expected, since they did not have the same semen quality. Furthermore, clear difference in semen quality among bulls persisted after Percoll selection. For most sperm end points, Bulls 1 and 2 were similar, but they were different from Bulls 3 and 4, which were also similar.

We also assessed embryo production following the use of all sperm preparation protocols. Similar to sperm quality end points, rates of cleavage and blastocyst formation were not significantly affected by treatment. However, there were differences among bulls in embryo production; Bulls 1 and 2 had higher rates of cleavage and blastocyst formation than Bull 3. These results were consistent with semen quality, as Bulls 1 and 2 were similar for sperm motility, as well as percentages of normal sperm, and those with an intact membrane, acrosome, and chromatin. Bull 3 had lower fertility than the other two, based on sperm quality and embryo development. For unknown reasons, although Bull 4 was similar to Bull 3 for all sperm characteristics studied, it had very poor embryo development.

Individual variation among bulls in the outcome of bovine IVF has been well documented [6,26,33–35] and is considered a limiting factor in commercial IVF. One of the reasons for the wide variation in efficiency of bovine IVP could be the ability, on an individual bull basis, of sperm to retain their ability to initiate and support development when they are submitted to IVF procedures [27]. Semen processing before IVF may change sperm characteristics, such that sires with high in vivo fertility may have low in vitro fertility [6,35]. Unfortunately, the underlying causes and mechanisms are not well characterized. Furthermore, based on the present study, bulls with similar sperm characteristics can have a very different ability to produced embryos in vitro. Perhaps sperm from certain bulls are more sensitive to Percoll passage; this could induce capacitation and a premature acrosome reaction [36], thereby affecting embryo production. However, in the present study, there were no significant differences among bulls in membrane or acrosome integrity. Although sperm quality characteristics of Bull 4 were similar to the other bulls, few embryos were produced. Apparent structural normality of sperm may be an indication of its quality, but this alone is not an assurance of in vitro fertility. In that regard, many other factors, e.g. an abnormal paternal genome, can influence fertilization and embryo development. In addition, the biochemical composition of the plasma membrane and RNA repertoire that the sperm carries to the oocyte with the genomic DNA, may affect in vitro fertility [37].

Percoll density gradient centrifugation has been used to separate human and bovine sperm carrying X or Y chromosomes [17–19]. Changes in volume of Percoll, as well as duration of centrifugation, can favor lighter or heavier sperm, thereby altering the ratio of X to Y bearing sperm [19]. Therefore, we determined whether changes in the Percoll protocol affected the sex ratio. In the present study, for the majority of the bulls and treatments, sex ratios were not different from the expected 1:1 ratio. These results were consistent with other studies that used Percoll to select sperm for IVF [5,38,39]. However, a shift toward male embryos was observed for Bull 1, but only when sperm passed through a larger Percoll volume. When the final volume of Percoll gradient was reduced to 800 μL, independent of time and force of centrifugation, the sex ratio for this bull, was unaffected. Why this occurred in only one of the four bulls remains to be elucidated, but it was probably related to individual variation. Perhaps the higher proportion of male embryos of Bull 1 was due to the lighter Y-bearing sperm, which have a smaller mass, taking longer to migrate in the column, resulting in a more prolonged contact with Percoll; this could induce some alterations, such that when the sperm was placed in IVF medium, it would be hyperactivated and capacitated faster and, consequently, would be more likely to fertilize oocytes faster than X-bearing sperm. In smaller volumes of Percoll, neither the sperm carrying the X or Y chromosome would take longer to pass though the column, thereby eliminating any advantage at fertilization.

When we compared sex ratio among treatments, independent of sires, the proportion of male embryos was higher for T1 than for the other treatments. However, this difference was probably caused by the effect of bull, since it was only observed for Bull 1 and no differences among treatments were detected for the other sires.

In conclusion, although only four bulls were used in this study, we concluded that decreasing Percoll volume, reducing the duration of centrifugation, and

using a higher centrifugation force had no negative effect on sperm quality, embryo development, or sex ratio. Therefore, prior to IVF, it is possible to routinely use a Percoll protocol for sperm processing that reduces both costs of materials and preparation time.

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