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The sex ratio of *in vitro* produced bovine embryos is affected by the method of sperm preparation

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Abstract

With the *in vitro* production (IVP) of bovine embryos, a deviation in the expected sex ratio has been reported; the percentage of male embryos is > 50%. The objective of this study was to compare the effects of sperm preparation methods on the sex ratio of IVP bovine embryos. Cumulus oocyte complexes (COCs), recovered from abattoir-derived ovaries, were randomly allocated into two groups after *in vitro* maturation (IVM) and inseminated with sperm prepared with either a swim-up or Percoll gradient. From Days 1 to 8, presumptive zygotes were cultured in SOFaa medium with 5% fetal calf serum (FCS). Embryos (n = 2044) were sexed by PCR. Only the swim-up method resulted in > 50% male embryos ($P < 0.05$) on both Days 5 and 8 of culture (59.5 and 58.4%, respectively). Moreover, the swim-up method resulted in a greater percentage of male embryos than Percoll ($P < 0.05$) on every day analyzed (Day 2, 55.7 vs. 44.1%; Day 3, 53.8 vs. 42.6%; Day 5, 59.5 vs. 45.3%; and Day 8, 58.4 vs. 46.4%). In conclusion, the swim-up method altered the sex ratio of IVP bovine embryos by increasing the percentage of male embryos.

Keywords: sex ratio, swim-up, Percoll, IVF, bovine.

Introduction

With the *in vitro* production (IVP) of bovine embryos, a deviation in the expected sex ratio has been reported; the percentage of male embryos is > 50% (King *et al*., 1991; Avery *et al*., 1992; Marquant-Le-Guienne *et al*., 1992; Carvalho *et al*., 1996; Pegoraro *et al*., 1998). Though a prevalence of males could be beneficial for beef production, it would be undesirable for production of dairy cattle. Although the causes of this difference remain unclear, the following have been postulated: developmental speed according to the sex of the embryos (Carvalho *et al*., 1996); culture conditions (Kochhar *et al*., 2001); higher sensitivity of female embryos to manipulation (King *et al*., 1992); expression of Y chromosome genes (Gutiérrez-Adán *et al*., 1997); duration of maturation period (Park *et al*., 2005; Agung *et al*., 2006); and method of sperm selection for IVF (Blottner *et al*., 1993; Cesari *et al*., 2006).

To date, the influence of sperm preparation methods on the sex ratio of IVP embryos has not been extensively studied. In general, methods of sperm separation rely on certain different features of X and Y spermatozoa, including: mass, motility, surface charge, DNA content, and surface antigen structure (McEvoy, 1992). The two most frequently methods used for sperm preparation in bovine IVF are the swim-up and Percoll gradient methods (Saeki *et al*., 1990; 1991; Shamsuddin and Rodriguez-Martinez, 1994; Lechniak, 1995; Hossepian de Lima *et al*., 2000). The swim-up method is based on the innate tendency of highly motile sperm to migrate upward, to the top of medium, whereas less motile and dead sperm remain in the lower fraction (Parrish *et al*., 1988). It was speculated that Y-bearing sperm were faster than X-bearing sperm (Sarkar *et al*., 1984; McEvoy, 1992), which could favor male sperm selection when using the swim-up method. Although this was later confirmed in human sperm (Claassens *et al*., 1989), it has not yet been investigated in bovine sperm. As the method of sperm selection for IVF is easy to manipulate, it provides substantial impetus for investigation in this area. In mammals, X-bearing sperm has more mass than their Y-bearing counterparts (Roberts, 1972). Consequently, the Percoll gradient method should favor the separation of X-bearing spermatozoa, which would have a higher density than Y-bearing spermatozoa when centrifuged and would be concentrated in the lower fraction of the gradient. This was reported with human (Kaneko *et al*., 1983; Mohri *et al*., 1986) and bovine sperm (Roberts, 1972; Suzuki, 1989; Hossepian de Lima *et al.*, 2000). However, other authors reported no differences in sedimentation speed of X- versus Y-bearing sperm for bovine (Iwasaki *et al*., 1988) and murine (Yoshizawa *et al*., 1995) semen.

Although the Percoll gradient and the swim-up methods have frequently been used for sperm selection in bovine IVF, their effects on the sex of the embryos produced are controversial (Iwasaki *et al*., 1988; Blottner *et al*., 1993; Pegoraro *et al*., 1998; Cesari *et al*., 2006). Moreover, no contemporaneous comparison has

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been conducted to compare the influence of these two methods on the sex of the embryos at different developmental stages. Our objective was to compare the effect of these methods on the male:female ratio of bovine embryos produced *in vitro* on Day 2, 3, 5, and 8 of culture by comparing each treatment with the expected sexual ratio of 1:1 and comparing the male ratio among the treatments.

Materials and Methods

Collection and in vitro maturation of cumulus-enclosed oocytes

Abattoir-derived ovaries were collected and transported to the laboratory in 0.9% NaCl solution at 22°C. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm follicles; only oocytes with an even granular cytoplasm, three or more uniform layers of cumulus cells, and without visible cytoplasmic degeneration in the morphologic evaluation were used in this experiment. Unless otherwise specified, all chemical reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Oocytes were matured in TCM-199/25 mM hepes (Gibco BRL, Gaithersburg, MD, USA) with 10% FBS (Gibco BRL), 0.25 mg/ml sodium pyruvate, 0.01 UI/ml rFSH (Gonal F-75, Serono, UK), 5 µg/ml LH, 0.05 mg/ml streptomycin sulfate, and 0.065 mg/ml potassium penicillin G. Groups of approximately 40 COCs were cultured in 400 µl of this medium for 22 to 24 h, at 38.5°C in an atmosphere of 5% of CO_2 in air with maximum humidity. After maturation, the COCs were transferred to the fertilization medium IVF-TALP (without PHE but containing 10 µg/ml heparin; Parrish *et al*., 1988) in groups of 25 to 50 oocytes per 400 µl of medium. The COCs were immediately inseminated with sperm prepared by one of the two methods described below. The day of the insemination was considered Day 0.

Sperm selection and in vitro fertilization

Frozen-thawed bovine semen from a pool consisting of a combination of single ejaculates from two bulls was used throughout this experiment. The semen was cryopreserved in 0.5-ml straws each containing 30 x 10^6 sperm. Straws were thawed for 30 s in a water bath at 37°C immediately before use. For each day that IVF was done, *in vitro* matured COCs were randomly allocated into two treatments. For Treatment 1, COCs were inseminated with sperm prepared by the swim-up method (Parrish *et al*., 1986) and for Treatment 2, COCs were inseminated with sperm prepared by the Percoll gradient method (Saeki *et al*., 1990).

The swim-up method was performed by layering 0.1-ml aliquots of semen under 1-ml aliquots of the medium Sp-TALP (Parrish *et al*., 1988) in three 15-ml conical tubes. After 40 min at 38.5°C, the top 0.85 ml of medium from each tube, which contained selected and "washed" spermatozoa, was removed and pooled in a 15-ml centrifuge tube that was centrifuged at 200 x g for 10 min. The supernatant was discarded, the sperm pellet diluted with 0.2 ml of Sp-TALP, and the insemination dose was calculated with a Neubauer chamber.

The Percoll gradient method consisted of 0.2 ml of semen layered over 2 ml of 45% Percoll and 2 ml 90% Percoll in a 15-ml conic tube. The gradient and sperm were centrifuged at 700 x g for 30 min. The sperm pellet was isolated and resuspended in 4 ml of Sp-TALP and centrifuged at 200 x g for 10 min. The supernatant was discarded, the sperm pellet diluted with 0.2 ml of Sp-TALP, and the insemination dose was calculated with a Neubauer chamber.

A single straw of semen was thawed each day that IVF was done, and its contents were divided between the two treatments, which were carried out simultaneously. For each sperm preparation method, sperm (concentration = 1 x 10^6 spermatozoa/ml) was added to the fertilization medium containing oocytes. The gametes were incubated for 18 to 22 h at 385°C in an atmosphere of 5% of CO_2 in air with maximum humidity.

Embryo culture

After the IVF period, presumptive zygotes were vortexed for 2 min (to remove cumulus cells and adhered spermatozoa), washed three times in SOFaa medium, and then transferred in groups of 35 to 40 to 400-µl drops of SOFaa medium (107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM $CaCl_2$, 0.49 mM $MgCl_2$, 25.05 mM $NaHCO_3$, 3.3 mM sodium lactate, 0.3 mM sodium pyruvate, 1.3 µg/ ml phenol red, 50 µg/ml gentomicin sulfate, 0.146 mg/ml L-glutamine, and MEM and BME amino acids; Takahashi and First, 1992) with 5% FBS and covered with mineral oil. The zygotes were incubated for 1 to 7 d (Days 2 to 8) at 38.5°C in an atmosphere of 5% of CO_2 in air with maximum humidity. At the end of this period, the embryos of each treatment were morphologically evaluated, classified according to their stage of development, and prepared for sexing. Only excellent, good, or regular quality embryos were used (IETS Grades 1 and 2).

Embryo sexing

After morphological evaluation, embryos were placed in 0.25% pronase solution (type XIV) for removal of the zona pellucida (to prevent contamination with DNA of spermatozoa that had remained in the zona pellucida) and were subsequently washed four times in PBS solution. Each embryo was lysed by transferring it to a 0.2-ml microcentrifuge tube containing 20 µl of lysis buffer (150 µg/ml proteinase K, Gibco, BRL, 15 mM Tris-HCl pH 8.9, 50 mM KCl, 2 mM MgCl2,

and 0.1% Triton X-100) and incubated for 60 min at 37°C. The tubes containing the embryos were incubated for 15 min at 95°C (to inactivate the proteinase K) and stored at -20°C pending sexing.

The embryos were sexed by the polymerase chain reaction (PCR) method, using two primer pairs simultaneously in the same tube. The first pair (primers 1+2) recognized a specific autosomal bovine sequence, the satellite DNA 1,715 that is amplified in bovine females as well as males (Ellis and Harpold, 1986). The second pair (primers 3+4) recognized a specific repetitive sequence of the bovine Y-chromosome, which is amplified only in bovine males (Ellis and Harpold, 1986; 1988; Ellis *et al*., 1988; Bondioli *et al*., 1989). The length of the PCR product for the bovine specific primers is 216 bp whereas the length of the PCR product using Y-specific primers is 175 bp.

Prior to PCR, the tubes containing the samples (embryo lysates) were thawed at room temperature. The PCR reactions were carried out in a total volume of 30 µl, utilizing 10 µl of each sample as a template. All of the amplifications were carried out in a PCR mixture consisting of 10 mM Tris-HCl with a pH of 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 U/sample Taq DNA polymerase (Gibco BRL), 20 nM primers 1+2, and 20 nM primers 3+4. The samples were amplified in a thermocycler (GeneAmp 2400, Perkin Elmer) using the following program: 94°C for 4 min, followed by 40 cycles consisting of denaturation (95°C for 1 min), annealing (56°C for 30 s), and polymerization (72°C for 1 min). After the last cycle, all samples were incubated for 10 min at 72°C. In each experiment, a negative control (sample without DNA), a positive-female control (0.1 ng bovine female genomic DNA), and a positive-male control (0.1 ng bovine male genomic DNA) were included as quality controls.

Analysis of PCR products

The PCR products (15 µl) were separated by electrophoresis on a 2.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Samples that contained only a 216 bp product were considered female embryos whereas those containing both the 216 bp product as well as a 175 bp (specific to the Y chromosome) were considered male embryos.

Experimental design

Experiment 1, a completely randomized design, was conducted to determine the sex ratio of embryos from each treatment in the developmental stages in which they are usually transferred to recipients. All presumptive zygotes, obtained from 15 separate IVF sessions, were cultured over 7 d (to Day 8). The blastocysts obtained were morphologically classified as: early blastocyst (Bi), blastocyst (Bl), expanded blastocyst (Bx), hatching blastocyst (Bh), and hatched blastocyst (Be). According to their developmental stages, they were distributed in three groups: S (slow: Bi and Bl), I (intermediate: Bx), and F (fast: Bh and Be).

Experiment 2, also a completely randomized design, investigated the sex ratio of early developmental-stage embryos from each treatment, thus avoiding the possible effects of culture conditions on the sex ratio. The presumptive zygotes, obtained from 17 separate IVF sessions, were cultured for 1, 2, or 4 d (to Days 2, 3, or 5, respectively). On each day, the embryos were classified as 2-cell, 4-cell, 8-cell, 16-cell, early morulae (Mo), or compact morulae (Mc). To overcome concerns regarding cytoplasmic fragmentation, about 40% of the embryos were stained with Hoechst 33342, and the number of nuclei was counted. After this, the embryos were sexed.

In Experiment 2, a control of the spontaneous parthenogenic index of the system was conducted, as reported by Lechniak *et al*. (1998a; b). On each day of IVF, after the maturation period, a group of 25 to 50 oocytes was randomly separated and transferred to 400 µl of the fertilization medium IVF-TALP where they remained for 18 to 22 h in the absence of spermatozoa. Thereafter, the oocytes were vortexed (to remove cumulus cells), washed, and transferred for culture into 400 µl of SOFaa medium under mineral oil, using the same conditions described in Experiment 1. Parthenogenetically-activated oocytes were cultured for the same duration as embryos (until Day 5) with cleavage evaluated every 24 h. On Day 5, the parthenotes obtained were stained with Hoechst 33342, and the number of nuclei was determined with an epifluorescent inverted microscope (Jenalunar SH 250, Karl Zeiss, Germany) with a 365-nm exciter filter and 420-nm barrier filter to discard the possibility of fragmentation.

Statistical analysis

A chi-square analysis was performed to compare sex ratios between different sperm separation treatments and to compare sex ratios to an expected ratio of 50:50. The level of significance was $P < 0.05$.

Results

Experiment 1: embryo sexing on Day 8

From a total of 700 blastocysts, 687 (98.1%) had PCR amplification for bovine autosomal primers. From these, 361 (52.5%) had amplification of the Y-specific sequence. The results of sex determination of Day 8 blastocysts are presented in Table 1. The swim-up method resulted in a percentage of males significantly higher than expected (50%) in the intermediate and fast blastocyst groups and also in the total number of blastocysts. Furthermore, the swim-up method produced more males than the Percoll gradient in intermediate blastocysts and in the total number of blastocysts ($P < 0.05$).

Table 1. Sex ratio (on Day 8) of IVP bovine blastocysts according to the method used to select sperm (Experiment 1)

	Male: Female ratio (% of males)	
Group[1]	Swim-up	Percoll
Slow (Bi + Bl)	59:54 (52.2)	58:78 (42.6)
Intermediate (Bx)	91:61 (59.9)**[a]	65:75 (46.4)[b]
Fast (Bh + Be)	56:32 (63.6)**	32:26 (55.2)
Total	206:147 (58.4)***[c]	155:179 (46.4)[d]

Sex ratio differs from the expected 50%: ** (P < 0.02); *** (P < 0.01).
Within a row, values without a common superscript differ: [a, b] (P < 0.05); [c, d] (P < 0.01).
[1]Blastocysts were morphologically classified as: early blastocyst (Bi), blastocyst (Bl), expanded blastocyst (Bx), hatching blastocyst (Bh), and hatched blastocyst (Be). According to their developmental stages, they were distributed in three groups: slow (Bi and Bl), intermediate (Bx), and F (fast: Bh and Be).

Experiment 2: embryo sexing on Days 2, 3, and 5

From a total of 1407 embryos submitted to PCR, 1357 (96.4%) had amplification of bovine autosomal primers (95% to 2-cells embryos: 269/283). Among these, 677 (49.9%) also had amplification of the Y-specific sequence (Table 2). Regarding total embryos, the swim-up method yielded a percentage of male embryos higher than the expected ratio of 50:50 on Day 5 and higher than the Percoll gradient on Days 2, 3, and 5 (P < 0.05). On every day of evaluation, a proportion of males higher than 50% (P < 0.05) was observed at the most developed stage with the swim-up but not with Percoll gradient method. Conversely, a deviation of sex ratio towards females was observed with Percoll at the least advanced stages on Days 2, 3, and 5 (P < 0.05).

Table 2. Sex ratio of IVP bovine embryos on Days 2, 3, and 5 according to the method used to select sperm for IVF (Experiment 2).

Day of culture		Sex ratio (% males)	
	Developmental stage	Swim-up	Percoll
2	2-cell	31:36 (46.3)	27:44 (38.0)*
	4-cell	41:31 (56.9)	32:42 (43.2)
	8-cell	46:27 (63.0)*	35:33 (51.5)
	Total	118:94 (55.7)[a]	94:119 (44.1)[b]
3	2-cell	24:41 (36.9)*	20:46 (30.3)***
	4-cell	30:37 (44.8)	27:43 (38.6)
	8-cell	44:28 (60.3)*	32:37 (46.4)
	16-cell	55:26 (67.9)***	41:36 (53.2)
	Total	154:132 (53.8)[c]	120:162 (42.6)[d]
5	16-cell	30:32 (48.4)	22:41 (34.9)**
	Early morulae	41:24 (63.1)*	29:31 (48.3)
	Compact morulae	39:19 (67.2)***	30:26 (53.6)
	Total	110:75 (59.5)**[c]	81:98 (45.3)[d]

Sex ratio differs from the expected 50%: *(P < 0.05); ** (P < 0.02); *** (P < 0.01).
Within a row, values without a common superscript differ: [a, b] (P < 0.05); [c, d] (P < 0.01).

From a total of 586 oocytes used for the control of parthenogenesis, 6.7, 3.6, and 0.9% cleaved spontaneously up to the 2-, 4-, and 8-cell stages, respectively (parthenogenesis rate of 11.1%, 65/586). Parthenotes had delayed development in comparison to fertilized embryos; none exceeded the 8-cell stage. These results are presented in Table 3.

Discussion

Although it was not a primary objective to determine which method of sperm preparation produced better results with *in vitro* maturation in Experiment 1, the swim-up method was superior to the Percoll gradient regarding cleavage rates (P < 0.01) and *in vitro* development to the blastocyst phase (P < 0.001). These results were published and discussed previously (Rheingantz *et al*., 2003).

The parthenogenetic rate in the present study was in agreement with average rates previously reported (Lechniak *et al*., 1998a; b) and apparently contributed to the higher percentage of females in the least-developed stages, mainly in 2-cell embryos (Table 2). If the percentage of parthenotes was taken into account at

each stage, it would be possible to subtract 6.7, 3.6, and 0.9% of the female embryos at the 2-cell, 4-cell, and 8-cell stages, respectively (Table 3). In each treatment, this would result in a percent of males similar to that observed in slow, intermediate, and fast embryos on Day 8 if possible parthenotes were not present among sexed embryos (Table 1). The high percentage of females among the slowest embryos (2- and 4-cell) observed on Day 3 in both treatments (Table 2) could also be explained by parthenogenesis. Furthermore, the delayed development of the parthenotes would favor their increase in number from Days 2 to 3 and account for the large number of 2- and 4-cell embryos at Day 3 that underwent degeneration whereas those with at least 8 cells had a better chance to further develop into a morulae or blastocyst (as in Experiment 1).

Table 3. Estimation of the spontaneous parthenogenic index of the system (Experiment 2).

Repetition	Number of oocytes*	2-cell**	4-cell**	8-cell**	Total (%)
1	32	6	2	-	8 (25.0)
2	27	4	1	-	5 (18.5)
3	37	1	1	1	3 (8.1)
4	40	1	1	-	2 (5.0)
5	45	2	-	-	2 (4.4)
6	40	1	3	-	4 (10.0)
7	50	2	2	1	5 (10.0)
8	40	2	2	1	5 (12.5)
9	45	3	1	-	4 (8.9)
10	55	4	3	1	8 (14.5)
11	25	2	-	-	2 (8.0)
12	25	2	1	-	3 (12.0)
13	25	3	1	-	4 (16.0)
14	25	1	1	1	3 (12.0)
15	25	1	1	-	2 (8.0)
16	25	2	1	-	3 (12.0)
17	25	2	-	-	2 (8.0)
Total (%)	586 (100.0)	39 (6.7)	21 (3.6)	5 (0.9)	65 (11.1)

* Oocytes were cultured to Day 5 and parthenotes obtained were stained with Hoechst 33342, and the number of nuclei was determined with an epifluorescent microscope.
** Cell number = nuclei number.

For sperm selected by the swim-up method, the proportion of males exceeded 50% on Days 5 and 8 when there were no more parthenotes and embryos with reduced viability had degenerated. On these days, the increased proportion of males was present at nearly all developmental stages. In humans, despite the fact that Check *et al*. (1994) did not observe a difference in the proportion of males:females, it was noticed that children born from IVF of sperm selected by the swim-up method presented the F corpuscle, indicating that this method could have favored the selection of sperm carrying the Y chromosome, mainly after a ascendant migration period of 30 to 45 min (Claassens *et al*., 1989). In other studies, the use of swim-up method resulted in a proportion of male bovine blastocysts ranging from 61 to 67 % (Yadav *et al*., 1993; Pegoraro *et al*., 1998; Gutiérrez-Adán *et al*., 1999a; b) after IVF, but no other method of sperm selection was included to compare results. From these observations and our results, it seems that the swim-up method favors the selection of a higher proportion of sperm carrying the Y chromosome. Otherwise, it could be speculated that sperm preparation methods would result in physiological differences in terms of viability or functional capacity of spermatozoa rather than altering the proportion of sperm carrying the X or the Y chromosome. In this sense, Cesari *et al*. (2006) showed that Percoll-selected bovine spermatozoa had higher motility and better acrosome exocytosis than swim-up-selected swim-up spermatozoa, but no differences in sex ratio of hatched embryos were observed.

In general, the Percoll gradient did not significantly alter the sex ratio, which is consistent with the results obtained by Iwasaki *et al*. (1988) and Grisart *et al*. (1995). However, Suzuki (1989) and Blottner *et al*. (1993) reported that the Percoll method increased the number of female bovine embryos. In the present work, > 50% females was observed only in the more delayed developmental stages (slow). During the first days of culture (Days 2 and 3), part of this deviation can be explained by the presence of parthenotes, especially among embryos with the greatest delays in development. However, on Day 5, the high percentage of females present in the slower embryos from the Percoll method could not be attributed to parthenogenesis (16-cell parthenotes were not

observed), but were probably due to the slower speed of development of female embryos. The fact that the proportion of males was lower among the more delayed stages and higher among the more developed for both methods and on all days suggested the higher speed of development of male embryos, as previously reported in various mammalian species (Tsunoda *et al*., 1985; Avery *et al*., 1989; Cassar *et al*., 1994; Bernardi and Delouis, 1996). For bovine embryos, Yadav *et al*. (1993) reported that 75 and 46% of male and female embryos, respectively, completed their first cleavage 30 h after insemination. Similarly, the *in vitro* development of female human embryos is delayed approximately 4.5 h (relative to male embryos) from Day 2 of development. On Days 2 and 3, the majority of the most-developed embryos (81%) was male, and their average cell number was greater than that of females, mainly on Day 2 (Ray *et al*., 1995). This difference was maintained until the blastocyst stage, suggesting that it started in the very early stages of development, at fertilization or during the first two cleavages (Ray *et al*., 1995).

There are also indications that culture conditions could account for the disparity between sexes in speed of development (Marquant-le-Guienne and Humblot, 1998; Kochhar *et al*., 2001; Lonergan *et al*., 2001). Components of the culture media could favor sex differences based on developmental speed, survival, or both, due to metabolic differences (Yadav *et al*., 1993; Grisart *et al*., 1995). In that regard, glucose, which seems to increase the developmental speed of male embryos (Peippo and Bredbacka, 1996; Bredbacka and Bredbacka, 1996), is frequently present in embryo culture media (Takahashi and First, 1992). However, it was noteworthy that glucose was not used in culture media in the present study. However, since embryos of both treatments were cultured under identical conditions, there is no evidence that culture conditions could have differently affected the sex ratio of Percoll-gradient- or swim-up-produced embryos in the present experiments.

In conclusion, the swim-up method of sperm separation created a deviation in the sex ratio, resulting in a significantly higher proportion of male embryos among the most-developed embryos. Moreover, the swim-up method resulted in a higher rate of male embryos than the Percoll gradient method across all embryos produced. In contrast, the Percoll gradient method did not alter the sex ratio across all embryos because it only occurred among the belated embryos of Experiment 2.

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