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Sperm Morphology and Preparation Method Affect Bovine Embryonic Development

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ABSTRACT: This study was conducted to evaluate the effect of sperm separation methods of semen samples collected from bulls subjected to scrotal insulation on embryonic development after in vitro fertilization (IVF) and to determine whether IVF results would be affected by various heparin concentrations. Morphologically abnormal semen samples were obtained and cryopreserved from Holstein bulls following scrotal insulation for 48 hours. Standard protocols using the Percoll gradient (90%/45%) method and the swim-up method were used to separate spermatozoa fractions in experiment I. The pellet (A_p) and the 45% layer (B_p) were isolated from the Percoll separation, while for the swim-up separation, the supernatant (A_s) and the interphase (B_s) were isolated. The overall blastocyst rate for our laboratory control semen was 23.1 ± 2.1% for Percoll separations (A_p and B_p) and 18.2 ± 2.0% for swim-up (A_s and B_s) separations. This rate was higher ($P < .01$) than the rate observed for the semen from the bull that had the greatest response to scrotal insult 5 days prior to the insult, when it was 9.2 ± 2.1% for the Percoll separation and 20.7 ± 2.3% for the swim-up separation, while semen from 27 days after scrotal insulation (D +27) resulted in no blastocyst formation for the Percoll separation and a 4.2 ± 2.1% rate for the swim-up separation. In experiment II, semen was sampled from the bulls that responded in the greatest and least degrees to scrotal insult 5 days before scrotal insulation (D −5) and on days 23 (D +23) and 34 (D +34) after scrotal insulation. These samples were exposed to IVF mediums with 3 different heparin concentrations (0.1, 1.0, and 10 μg/mL). There was a significant difference ($P < .05$) in developmental scores between the D −5 (1.08 ± 0.08), D +23 (0.9 ± 0.08), and D +34 (0.8 ± 0.08) samples, but no differences were observed in blastocyst formation based on the number of cleaved embryos. Increasing the heparin concentration resulted in higher ($P < .01$) embryonic developmental scores. In conclusion, when semen samples with high percentages of abnormal spermatozoa are used for IVF, semen separation preparation methods affect results. Our results show that the separation methods used under these conditions were inadequate in their ability to provide potentially competent sperm for IVF. However, selecting appropriate sperm separation procedures could improve in the IVF embryonic development of semen from bulls used in artificial insemination. Also, an increase in the heparin concentration was able to partially overcome deficiencies, which suggests that morphologically abnormal spermatozoa undergo capacitation despite possible structural changes to the plasma membrane.

Key words: Percoll, swim-up, abnormal spermatozoa, thermal insult, in vitro fertilization.

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The probability of successful development of a bovine embryo is partially predetermined during the earliest stages of development by virtue of the genetic and epigenetic contributions acquired from the male and female gametes. Moreover, Saacke et al (2000) suggested that both fertilization failure and failure in subsequent bovine embryonic development are of seminal origin. The factor with the highest predictive index for success with in vitro fertilization (IVF) is sperm quality, which includes motility, morphology, and the percentage of sperm with intact acrosomes (Zhang et al, 1998). Only viable spermatozoa are able to interact with the oocyte and initiate fertilization. While Barth and Oko (1989) documented that the percentage of bovine spermatozoa with normal morphology in a semen sample is generally related to its viability, there is convincing evidence that other factors associated with lowered sperm quality may result in reduced embryonic quality or failure of the embryo to induce maternal recognition of pregnancy (Kot and Handel, 1987; Saacke et al, 1998, 2000; Thundathil et al, 1999).

Kot and Handel (1987) suggested that the interaction between abnormal murine spermatozoa and the oocyte is different from the interaction between normal spermatozoa and the oocyte, resulting in decreased penetration or fertilization and, subsequently, increased embryonic death. Further in vivo bovine studies by Saacke et al (1998) demonstrated that severe and moderately misshapen sperm heads were excluded from participating in fertilization, but subtly misshapen sperm heads appear as accessory sperm on fertilized ova, regardless of their proportion in the inseminate. However, since spermatozoa with the pyriform defect apparently have normal acro-

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somes and normal motility, they could be expected to penetrate the zona pellucida and enter the ooplasm under IVF conditions. Moreover, Thundathil et al (1999) reported that with bovine IVF, the percentage of pyriform spermatozoa that penetrate the zona pellucida and gain access to the ooplasm was lower than that of normal-shaped sperm. They found that most pyriform-shaped spermatozoa that bound to the zona pellucida also managed to penetrate it, suggesting that pyriform-shaped spermatozoa are primarily discriminated against during zona binding.

Interference with the normal scrotal thermoregulation of bulls resulted in decreased percentages of sperm with normal head and tail morphology and reduced pregnancy rates when they were used for natural mating (Lunstra and Coulter, 1997). Furthermore, Saacke et al (1994, 2000) defined compensable seminal traits as those deficiencies in semen that preclude the availability of sperm for fertilization in vivo but stated that the effect can be minimized by increasing the sperm dosage. This is opposed to the uncompensable seminal deficiencies that cannot be overcome by increased sperm dosages and that most likely impair embryonic development.

For IVF purposes, the removal of extender components, including cryoprotectant, from cryopreserved bovine semen is necessary to obtain a high percentage of normal, viable spermatozoa. Methodological factors such as sperm preparation could easily influence IVF results within the same bull. Several methods for sperm selection have been described, but the most widely used are swim-up and Percoll gradient separation techniques (Rodriguez-Martinez et al, 1997; Palomo et al, 1999). Sapienza et al (1993) and Dode et al (2002) reported no differences between the quality of the swim-up–separated and Percoll-separated spermatozoa. However, other researchers have reported a lower recovery but a better quality of spermatozoa using swim-up, whereas, according to these researchers, Percoll separation resulted in a higher recovery rate and percentage of acrosome-intact spermatozoa (Parrish et al, 1995; Somfai et al, 2002). Moreover, IVF results were not affected when high concentrations of spermatozoa were used, which is contrary to when low concentrations were used (Ward et al, 2003). Furthermore, spermatozoa must undergo capacitation in order to bind to the zona and undergo the acrosome reaction. The exact cellular mechanism by which spermatozoa undergo capacitation is still largely unknown, but several studies have shown that the addition of heparin to the IVF medium is an effective method for inducing capacitation in vitro of bovine sperm and that heparin concentrations can have an effect on IVF results (Parrish et al, 1988, 1995; Saeki et al, 1995).

The effects of sperm preparation procedures and heparin concentrations on bovine IVF are not well established when abnormal sperm are used. Our hypothesis was that a short-term thermal insult caused by scrotal insulation would induce abnormal spermatozoa production that would, in turn, provide semen to test the effects of semen preparation methods and heparin addition when using IVF on early bovine embryonic development. Therefore, the objectives of this study were to 1) evaluate the effect of sperm separation methods on semen samples collected from bulls that were subjected to scrotal insulation (to induce abnormal sperm morphology) on embryonic development after IVF, and 2) determine whether morphologically abnormal and normal bovine spermatozoa would be differentially affected by variation in heparin concentrations.

Methods

Thermal Treatment of Bulls and Semen Collection

Six Holstein bulls, selected for their ability to produce semen with greater than 70% morphologically normal sperm and at least 70% progressive motility, were subjected to a 48-hour scrotal insulation period to obtain semen samples with high percentages of morphologically abnormal spermatozoa (Vogler et al, 1993). Briefly, for each of the bulls (aged 13–24 months) housed at the State University's (Blacksburg, Va) Dairy Cattle Center and handled by routine procedures to emphasize caution and highlight well-being, ejaculates were collected twice weekly and pooled from 2 ejaculates each day beginning 16 days prior to insulation (D −16) and continuing for 34 days after scrotal insult. Preinsulation collections (D −8, D −5, and D −1) served as within-bull control samples, while semen collected on D +2 through D +9 postinsulation corresponded to ejaculated sperm that were presumed to be in the epididymis and rete testis during the time of scrotal insulation. Spermatozoa collected between D +13 and D +34 were presumed to be spermatozoa that were undergoing spermiogenesis at the time of scrotal insulation and were presumed to be abnormal (Vogler et al, 1993).

Semen Cryopreservation

On each semen collection day, the volume of each ejaculate was determined. The 2 ejaculates from each bull were pooled and diluted with egg yolk–citrate extender. Ejaculates were prepared for cryopreservation at a concentration of 50×10^6 sperm per milliliter with egg yolk–citrate-glycerol (Robbins et al, 1976).

Sperm Morphology

The collected semen samples were evaluated for morphological abnormalities according to the methods of Barth and Oko (1989). Abnormalities were classified by defects in the shape of the sperm head (pyriform, tapered, and asymmetrical) and completely distorted head shapes, plus nuclear vacuolization (apical vacuoles, diadems, and random vacuoles), which were all classified as primary abnormalities that, if produced, appeared in a specific chronological order (Vogler et al, 1993). Thus, the scrotal insulation provided a means of varying the quality of the

sperm population within bulls as well as across bulls (Vogler et al, 1993). The bulls were then ranked according to the severity of their response to scrotal insulation on the basis of the morphological assessment of the semen samples collected. Semen samples from 3 of these bulls were used in these studies.

Semen Separation Procedures

The standard protocols for the Percoll gradient (90%/45%) separation (Pertoft et al, 1978) and the swim-up method (Parrish et al, 1986) were used to separate fractions for IVF. Both the pellet (A_p) and the interphase layer between the 45%/90% gradient (B_p) were isolated from the Percoll gradient; from the swim-up method, both the supernatant (A_s) and the interphase (B_s) were isolated and used for IVF. In short, a Percoll gradient was prepared in a 15-mL conical tube with 2 mL of 90% Percoll (1:9 [vol/vol] mixture of Percoll and 10× synthetic oviductal fluid buffered with HEPES; SOF HEPES; Sigma Chemical Co, St Louis, Mo) added to the tube, which was layered with 2 mL of 45% Percoll (1:1 [vol/vol] mixture of 90% Percoll and 1× SOF HEPES). Frozen-thawed semen was layered on the Percoll gradient and centrifuged at 700 × *g* for 30 minutes. Both the A_p and B_p fractions were then washed in SOF HEPES by centrifugation at 500 × *g* for 10 minutes at room temperature. For the swim-up method, each of 4 tubes was filled with 1.0 mL of SOF HEPES medium, and a 1.0-mL volume of frozen-thawed semen was layered under the medium. After a 1-hour incubation at 38.7°C, the upper 0.8 mL of the medium was collected as the A_s fraction, and the lower portion (0.8 mL) was collected as the B_s fraction. Both fractions were washed twice by centrifugation at room temperature (700 × *g* for 10 minutes).

IVF and Embryonic Culture

For each replicate, slaughterhouse-collected oocytes (n = 400) were matured in tissue culture medium 199 (TCM 199; Gibco, Grand Island, NY) supplemented with 10% bovine calf serum (Gibco), 0.01 U/mL luteinizing hormone (Sigma), and 0.01 U/mL follicle-stimulating hormone (Sigma) for a 24-hour period (Thompson et al, 1998).

IVF was performed with frozen-thawed semen samples from the different bulls according to their responses to scrotal insulation, which were based on the morphological assessment of each sample after collection. Matured oocytes in a quantity sufficient to perform 3 replicates per day were then washed in SOF HEPES medium and placed in a 47-μL SOF-IVF drop (10 oocytes per drop) supplemented with a heparin concentration of 10 μg/mL, and a 3-μL sample of each separation fraction was added to the appropriate drop for a final concentration of 1×10^6 spermatozoa per milliliter (Parrish et al, 1995). Each treatment was replicated on each of 3 days.

After 18 hours of sperm–oocyte incubation at 38.7°C in an atmosphere of 5% CO_2, the presumptive embryos were denuded by vortexing for 5 minutes in 1.0 mL of SOF HEPES medium. Embryos were washed and placed in a 30-μL SOF–in vitro culture (SOF-IVC) drop and cultured at 38.7°C in an atmosphere of 5% CO_2, 10% O_2, and 85% N_2 (Thompson et al, 1998). Subsequent development was recorded on day 8 (IVF = day 0), and a developmental score (0 = degenerate, 1 = 2-cell embryo through 5 = blastocyst stage) was assigned to each embryo.

Sperm Morphology Evaluation

In addition to the morphological evaluation conducted at the time of collection and freezing, a morphological assessment was conducted after the IVF procedures in 2 of the replicates for each sample, with care being taken to keep the individual fractions (A_p, B_p, A_s, and B_s) separated. Wet smears were prepared for analysis by placing a drop from each fraction onto a microscope slide and immobilizing the spermatozoa with a drop of 40 mM sodium fluoride and then covering the drop with a coverslip (Mitchell et al, 1985). Morphology was evaluated on 200 cells using differential interference contrast microscopy at 1000× magnification under oil. The percentage of morphologically normal spermatozoa and the percentage of specific primary abnormalities that characterize a disturbance in spermatogenesis due to scrotal insulation were recorded for each sample. Spermatozoa with multiple abnormalities were counted only once, but each abnormality of a particular spermatozoa was classified separately (Barth and Oko, 1989) and included pyriform, tapered, vacuolated, decapitated, and severely misshapen spermatozoa (Table 1). Data for asymmetrical shape and diadem defects made up less than 10% of the total and are not listed in the table.

Experiment I: Separation Protocols

Semen Samples—Three types of frozen-thawed semen samples were used for IVF. The first treatment was semen collected from a bull, predetermined to be effective for IVF in our laboratory (control). A second sample was obtained from one of the more extreme responders to scrotal insulation (D −5 prior to the 48-hour scrotal insulation). A third semen sample was taken from the same bull on day 27 after scrotal insulation (D +27). The D +27 sample was selected because it had the smallest overall percentage of normal spermatozoa, being reduced from about 70% to about 20%. This sampling protocol permitted an internal and external control for IVF.

Experiment II: Heparin Effectiveness

Semen from the bulls that displayed the greatest and least responses to scrotal insulation was chosen to evaluate differences in sperm morphology. For each of these bulls, 3 semen samples were used for IVF: a preinsult sample collected 5 days before insulation (D −5) and 2 postinsult samples, one collected 23 days later (D +23), when the percentages of pyriform, tapered, vacuolated, and severely abnormal spermatozoa were observed to be the greatest for bull I (the bull that displayed the greatest response), and the other collected 34 days after insult (D +34) at the end of the collection period (when sperm morphology had returned to near-normal levels). The bull that displayed the least response (bull II) to scrotal insulation showed no differences in sperm morphology during the same time period. Within each treatment group, semen samples separated using Percoll procedures (A_p) (Pertoft et al, 1978) were exposed to 3 different heparin concentrations (0.1, 1.0, and 10 μg/mL) that had been added to the SOF-IVF medium. Slaughterhouse-collected oocytes were randomly assigned to each heparin concentration within each treatment group (n = 30) after a 24-hour maturation period, and this was replicated on 3 different days. After an 18-hour sperm–oocyte incubation, the presumptive zygotes were cultured as in experiment I; on day 8 (IVF = day 0), a developmental score

Table 1. *The percentages of primary abnormalities present in each semen sample fraction after morphological evaluation in experiment I**

Sample†	Separation Fractions‡	Normal§	Pyriform	Tapered Heads	Apical Vacuoles	Random Vacuoles	Severely Abnormal‖	Decapitated
Control	A_p	76	3[a]¶	17	5	0	2	2.0[b,c]
	A_s	75	3[a]	20	3	2	0	0[a]
	B_p	64	4[a]	25	7	3	2	1.5[a,b,c]
	B_s	74	5[a]	28	2	1	1	0[a]
D −5	A_p	69	5[a]	14	2	9	3	1.5[a,b,c]
(preinsulation)	A_s	69	11[a,b]	13	1	8	3	1.0[a,b]
	B_p	61	8[a]	20	2	11	3	0.5[a,b]
	B_s	53	15[b]	33	0	3	0	0[a]
D +27	A_p	22	43[c]	17	12	19	10	17.5[d]
(postinsulation)	A_s	16	55[d]	8	3	10	16	0.5[a,b]
	B_p	10	57[d]	19	9	12	10	3.0[c]
	B_s	27	49[c,d]	25	2	6	4	3.0[c]
	SE	6.4	2.5	3.4	3.1	3.1	2.0	0.5

* Multiple abnormalities were recorded per single sperm cell when present.
† Control indicates laboratory control semen; D −5, semen collected 5 days prior to scrotal insulation; and D +27, semen collected 27 days after the 48 hour scrotal insulation.
‡ Percoll: pellet (A_p) and the 45% layer (B_p); swim-up: supernatant (A_s) and the interphase (B_s).
§ Morphologically normal-shaped heads.
‖ Severely abnormal spermatozoa, head shape completely distorted.
¶ Means without common superscripts within category differ at $P < .05$.

(0 = degenerate, 1 = 2-cell embryo through 5 = blastocyst stage) was assigned to each embryo.

Statistical Analyses

Data from both experiments were analyzed by 1-way analysis of variance using the PROC GLM and/or PROC MIXED procedure of Statistical Analysis Systems (1999) and chi-square analysis. Cleavage and developmental rates were determined for both experiments. The effects included in the model for experiment I were semen sample collection day, separation method, separation fractions, repetitions, and interactions among semen sample, separation fraction, and separation method. Replicate effects were not significant ($P > .05$) and were deleted from the final models. Pearson simple correlations were conducted between the development of sperm abnormalities and the percentages of abnormalities in each sample using the PROC CORR procedure of SAS (Statistical Analysis Systems, 1999). In experiment II, the model included the main effects of bull, semen sample collection day, heparin concentration, and interactions among bull and heparin concentration, bull and semen sample collection day, and semen sample collection day and heparin concentration. Replicate effects were not significant and were deleted from the final models.

Results

Experiment I: Separate Protocols

The percentages of individual sperm abnormalities are presented in Table 1. Overall, 72 ± 3.2% of the spermatozoa from the control bull were normal, which was not different from the D −5 normal sperm population (63 ± 3.2%), but both were higher ($P < .01$) than the 18.5 ± 3.2% normal population from the D +27 collections. The percentage of pyriform sperm heads was affected by sample collection ($P < .01$), and the control was less than (3.8 ± 1.2%; $P < .01$) the D −5 semen, which was intermediate (9.5 ± 1.2%) and different ($P < .01$) from the D +27 semen (50.6 ± 1.2%). The significant interactions between samples collected relative to separation method and separation fraction ($P < .01$) for pyriform heads are shown in Table 1. There was a significant interaction ($P < .01$) for sample collection relative to the separation fraction for tapered head abnormalities. Generally, the A fractions had lower ($P < .05$) percentages of tapered heads (control, 18.0 ± 2.4%; D −5, 13.3 ± 2.4%; and D +27, 12.5 ± 2.4%) than the B fractions (26.0 ± 2.4%, 26.0 ± 2.4%, and 21.8 ± 2.4%, respectively). However, the control A and D +27 B fractions were not different from each other ($P > .05$). Apical vacuole defects were lowest for D −5 (0.9 ± 1.6%) and were different ($P < .01$) from both the control (4.0 ± 1.6%) and the D +27 (6.3 ± 1.6%) samples. The significant 3-way interaction for decapitated heads is shown in Table 1. The D +27 A_p fraction had the greatest percentage of decapitated heads.

Cleavage rates were significantly ($P < .01$) different among semen samples and were significantly affected ($P < .01$) by the method of separation. For the control semen, the cleavage rate was 64 ± 2.1%, which was higher ($P < .01$) than the 51 ± 2.3% that was observed for the D −5 semen sample and the 31 ± 2.2% cleavage that was observed for the D +27 semen sample, which, in turn, was the lowest and different ($P < .01$) from the D −5 sample. The cleavage rates for semen from the control bull were highest (75 ± 3%) for the swim-up method and 53 ± 3% for the Percoll separation ($P < .01$) compared

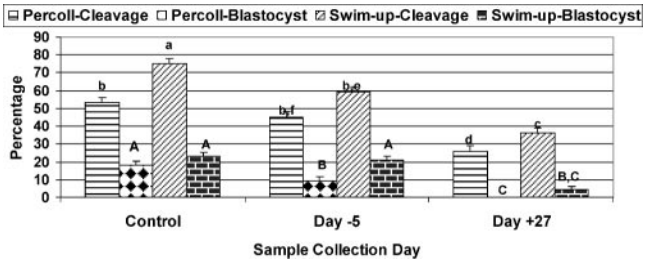

Figure 1. Cleavage and blastocyst rates (±SE) following in vitro fertilization (IVF) relative to the separation procedure (Percoll or swim-up) for control, day 5 before scrotal insulation (D −5), and day 27 after scrotal insulation (D +27) semen samples following a 48-hour scrotal insulation in experiment I. Means of 3 replicates without common superscripts differ at $P < .05$ (lowercase for cleavage rates, uppercase for blastocyst rates).

to 59 ± 3 and 45 ± 3% for the D −5 semen collections (Figure 1). Cleavage rates were lowest and different ($P < .01$) for Percoll (26 ± 3%) and swim-up separation (35 ± 3%) procedures for D +27. Cleavage rates were affected ($P < .01$) by separation fractions (A and B). The control bull semen fraction A (A_p and A_s) was higher ($P < .01$) than the D −5 fraction A (79 ± 3% vs 55.1 ± 3.2%), which was not different from the control fraction B (49.1 ± 2.9%) and the D −5 fraction B (48.6 ± 3.2%). These fractions had greater cleavage rates ($P < .01$) than the D +27 fractions A (33.5 ± 3.0%) and B (28.3 ± 3.1%), which were not different from each other.

The cleavage rates (Table 2) were significantly lower ($P < .01$) between the A_p and the B_p fractions for control bull semen and semen from the D −5 sample, but not for semen from the D +27 sample, while the there was no difference in the cleavage rates between the A_s and B_s fractions. However, within each fraction, A_s and B_s, the cleavage rates were significantly different ($P < .01$) only among all 3 semen samples for A_s fractions and decreased from 81% for the control A_s fractions to 38% for the D +27 samples, while for the B_s fractions, cleavage rates decreased from 67% (control and D −5) to 34% at D +27.

The overall embryonic developmental scores were significantly different ($P < .01$) among the 3 semen samples, with the control having the highest development at 1.8 ± 0.06, and it was different from D −5 samples (1.5 ± 0.06), which was greater than D +27 samples (0.6 ± 0.06). Additionally, there was a significant difference ($P < .01$; Table 3) in the developmental score between preparation methods, Percoll vs swim-up. Notably, the swim-up separation provided more competent sperm than did the Percoll separation. A significant ($P < .01$) interaction was found between the control, D −5, and D +27 semen samples and their separation fractions (A_p, B_p, A_s, and B_s; Table 3) for embryonic development. Moreover, among the different fractions, the developmental score for the control was lower ($P < .01$) for both the B_p and B_s fractions when compared to the A_p and A_s fractions, while for semen from the D −5 samples, only the B_p developmental score was lower ($P < .01$) than the others. The developmental score for semen from the D +27 sample was lowest for the B_p fraction but was not different ($P > .05$) from the A_p, A_s, or B_s fractions. The developmental scores from the A fractions (A_p and A_s) decreased significantly ($P < .01$) from the control bull semen to the D −5 sample and then was further decreased to the D +27 sample. However, the control bull and D −5 B_s fractions resulted in greater development ($P < .01$) than all other B fraction semen samples.

The overall blastocyst rates for the control semen for the Percoll and swim-up separations were not different ($P > .05$), nor were they different from the D −5 swim-up–separated semen samples (Figure 1). But the blastocyst rates were lower ($P < .05$) for the D −5 Percoll preparations, while for semen from the D +27 sample, there was no blastocyst formation for the Percoll separation; however, there was a 4.2% rate for the swim-up separation (Figure 1). There was a significant interaction for semen samples relative to the semen separation fraction ($P < .01$). The control bull semen separation fraction A used for IVF resulted in the highest blastocyst development (Figure 2). The D −5 fractions were not different from each other ($P > .05$), but the A fractions resulted in a higher blastocyst rate ($P < .05$) than the control fraction B. Blastocyst rates for both D +27 samples were lowest ($P < .05$).

Table 2. *Mean cleavage rate (±SE) for sperm separation fractions following IVF for semen obtained from a control bull and for semen collected before and after 48 hours of scrotal insulation in experiment I**

Semen Sample	Percoll, % (Total No. Oocytes)		Swim-up, % (Total No. Oocytes)	
	A_p†	B_p	A_s	B_s
Control	77^{a}‡ ± 4 (140)	$29^{b,e}$ ± 4 (121)	81^{a} ± 5 (104)	$69^{a,c}$ ± 4 (130)
D −5	57^{d} ± 5 (104)	$33^{b,e}$ ± 4 (118)	54^{d} ± 5 (101)	$64^{c,d}$ ± 5 (92)
D +27	$29^{b,e}$ ± 4 (134)	23^{e} ± 4 (109)	38^{b} ± 4 (108)	$34^{b,e}$ ± 5 (107)

* IVF indicates in vitro fertilization.
† Percoll: pellet (A_p) and the 45% layer (B_p); swim-up: supernatant (A_s) and the interphase (B_s).
‡ Mean rates of 3 replicates without common superscripts differ at $P < .05$.

Table 3. *Developmental scores (±SE) for sperm separation fractions following IVF for semen obtained from a control bull and semen collected before and after 48 hours of scrotal insulation in experiment I**

Semen Sample	Percoll		Swim-up	
	A_p†	B_p	A_s	B_s
Control	2.4 ± 0.1[a]‡	0.7 ± 0.1[c,d]	2.4 ± 0.2[a]	1.7 ± 0.1[b]
D −5	1.5 ± 0.2[b]	0.8 ± 0.1[c]	1.7 ± 0.2[b]	1.8 ± 0.2[b]
D +27	0.5 ± 0.1[c,d]	0.4 ± 0.1[d]	0.7 ± 0.1[c]	0.7 ± 0.1[c]
Mean	1.1 ± 0.1[A]§		1.5 ± 0.1[B]	

* IVF indicates in vitro fertilization.
† Percoll: pellet (A_p) and the 45% layer (B_p); swim-up: supernatant (A_s) and the interphase (B_s).
‡ Scores with different superscripts differ at $P < .05$.
§ Means with different superscripts differ at $P < .01$.

A positive correlation was found between the percentage of morphologically normal sperm heads and the embryonic developmental score ($r = 0.32$, $P < .01$), while a negative correlation was found between the developmental score and the percentage of pyriform sperm ($r = -0.31$, $P < .01$), diadems ($r = -0.20$, $P < .01$), apical vacuoles ($r = -0.18$, $P < .01$), random vacuoles ($r = -0.30$, $P < .01$), decapitated sperm ($r = -0.21$, $P < .01$), and severely abnormal sperm ($r = -0.29$, $P < .01$) in the semen sample.

Experiment II: Heparin Effectiveness

Descriptive data on the percentages of individual abnormalities for experiment II are shown in Table 4.

The cleavage rate was significantly ($P < .01$) affected by the bull from which the semen sample was taken, the semen sample collection day, and the heparin concentration. A lower cleavage rate ($P < .01$) was found for bull I (23 ± 2%; highest responding bull) than for bull II (33 ± 2%; lowest responding bull), while the cleavage rates for the D +23 and D +34 samples were lower ($P < .01$) than for the semen from the D −5 sample (Figure 3). Furthermore, the overall cleavage rate was higher when heparin concentrations of 10 μg/mL (42 ± 2.2%) were used than when heparin concentrations of 0.1 μg/mL (17 ± 0.7%) and 1.0 μg/mL (24.8 ± 2.3%; $P < .01$) were used. There was no interaction between the bull from which the semen sample was taken, the semen sample collection day, and the heparin concentration; however, the interaction between the bull from which the semen sample was taken and the heparin concentration approached significance ($P < .08$; Figure 4). For bull I, the cleavage rate doubled when the heparin concentrations increased from 0.1 to 1.0 μg/mL and almost tripled with a 100-fold increase in the heparin concentration from 0.1 to 10 μg/mL. For bull II, there was no difference ($P > .05$) in the cleavage rates when the heparin concentrations were increased from 0.1 to 1.0 μg/mL, but cleavage rates doubled when the heparin concentration was increased to 10 μg/mL.

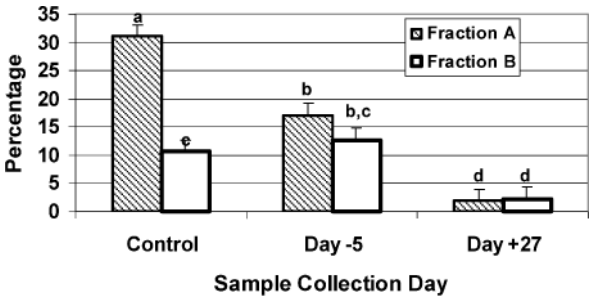

Figure 2. Blastocyst rates (±SE) following in vitro fertilization (IVF) according to separation fraction (A or B) for control, day 5 before scrotal insulation (D −5), and day 27 after scrotal insulation (D +27) semen samples following the 48-hour scrotal insulation in experiment I. Means of 3 replicates without common superscripts differ at $P < .05$.

In contrast to the cleavage rates, the embryonic developmental scores between the 2 bulls were not different, but there was a significant difference ($P < .05$) between the developmental score for semen from the D −5 sample when compared to the D +23 and D +34 samples (Figure 3). The increasing heparin concentrations resulted in no difference in the developmental score from 0.1 μg/mL (1.4 ± 0.06) to 1.0 μg/mL (1.5 ± 0.06), with a significant increase ($P < .01$) at 10 μg/mL (2.1 ± 0.06) that corresponded with the increase in overall cleavage rate from 18% to 42% as the heparin concentration increased from 0.1 to 10 μg/mL. There was no significant interaction ($P > .05$) between the bulls from which the semen samples had been taken, the semen sample collection days, and the heparin concentrations when developmental scores were tested. The rate of blastocyst formation based on the percentage of cleaved embryos was not different ($P > .05$) for the main effects of the bull from which the semen sample was taken, the semen sample collection, and the heparin concentration.

Discussion

Cleavage With Sperm Separation

The objective of the current study was to determine the effects that various sperm preparation procedures and

Table 4. *Descriptive data on the percentages of abnormalities present in semen samples collected from bull I (high abnormal sperm producer) and bull II (no relative change in abnormal sperm production) when both bulls were exposed to 48 hours of scrotal insulation in experiment II*

	Semen Sample*	Pyriform	Diadem	Apical Vacuoles	Random Vacuoles	Decapitated	Severely Abnormal†
Bull I	D −5	7	0	8	3	2	1
(responder)	D +23	55	30	40	8	20	22
	D +34	12	0	8	2	9	30
Bull II	D −5	0	0	0	0	2	4
(nonresponder)	D +23	3	0	8	3	3	4
	D +34	0	0	0	0	0	4

* D −5 indicates semen collected 5 days prior to scrotal insulation; D +23, semen collected 23 days after 48 hours of scrotal insulation; D +34, semen collected 34 days after 48 hours of scrotal insulation.

† Severely abnormal spermatozoa, head shape completely distorted.

heparin concentrations had when bovine IVF was performed with abnormal sperm. Our hypothesis was that a short-term thermal insult caused by scrotal insulation would induce sufficient abnormal spermatozoa production to provide semen that would, in turn, allow us to determine what effects our objective would have on early bovine embryonic development. The decreased cleavage rates with high proportions of abnormal sperm in the ejaculate in experiments I and II were similar to what has been reported by Eid et al (1994), who showed that when low-fertility bulls were used for IVF, the cleavage rates were low, and pronuclear formation was delayed.

Separation fraction (A vs B) effects were greatest on cleavage only in the control bull (Figure 1), suggesting that there are bull differences under routine conditions and that deleterious impacts occurred due to thermal insult. Other studies conducted with cattle have shown that the donor of the semen influences the outcome of both IVF and IVC (Parrish et al, 1986; Shi et al, 1990; Shamsuddin et al, 1993; Rodriquez-Martinez et al, 1997; Ward et al, 2003). Our higher cleavage rate for the swim-up method compared to the Percoll separation was in agreement with the findings of Parrish et al (1995). They associated the higher rate with a difference in the ability of spermatozoa to penetrate ova in vitro after separation by swim-up procedures when compared to Percoll separation. Moreover, hyperactivation of spermatozoa permits an enhanced penetration of the zona pellucida (Stauss et al, 1995).

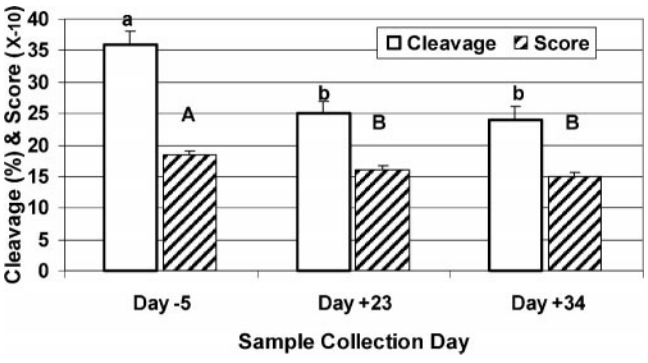

Figure 3. Overall percentage of embryonic cleavage and developmental scores (10× higher) (±SE) according to the semen collection date (D −5, D +23, and D +34 following a 48-hour scrotal insult) after Percoll separation of semen from a bull that responded to scrotal insulation (bull I) and a bull that did not respond to scrotal insult (bull II) when the semen was subjected to various heparin concentrations in experiment II. Means within an effect with different superscripts differ at $P < .05$ (lowercase for cleavage rates, uppercase for developmental score).

Development With Sperm Separation

We found no difference in the development rates when only the normal (A_s and A_p) fractions of each separation method were compared, which supports the findings of Parrish et al (1995). But for the B_s and B_p separation fractions, lower developmental scores and developmental rates were detected for the Percoll separation, while the scores and rates for the swim-up method were not different. Fractionation with the separation procedures showed effective separation with the Percoll separation technique in the control and D −5 semen samples in contrast to no change in cleavage for the swim-up separation and the D +27 Percoll separation. Developmental data mimic these changes (Table 3). Somfai et al (2002) reported that both the swim-up and Percoll methods improved the proportion of live spermatozoa used for IVF but that the developmental rate was higher with sperm from a Percoll sep-

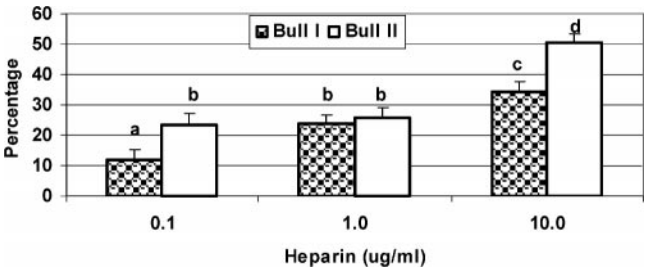

Figure 4. Overall cleavage rates (±SE) for the different heparin concentrations included in the fertilization medium following Percoll separation of semen from a bull that responded to scrotal insulation (bull I) and a bull that did not respond to scrotal insult (bull II) for experiment II. Means of 3 replicates without common superscripts differ at $P < .05$.

aration than with sperm from a swim-up separation. These results imply that Percoll separation is more efficient for partitioning high-quality, live, acrosome-intact spermatozoa from damaged or dead spermatozoa and reflect the decrease in the developmental rate for the B_p fraction from control bull and D −5 semen. In support, Brandeis and Manuel (1993) found that the swim-up method separated a higher percentage of motile spermatozoa than a Percoll separation but that the Percoll method yielded a higher percentage of spermatozoa with intact acrosomes. Furthermore, Somfai et al (2002) indicated that the results with Percoll separation were more uniform and resulted in decreased variances between semen samples of different quality. In contrast, we found a significant decrease in developmental rates as the percentages of morphologically abnormal sperm per sample increased (D +27) for both Percoll and swim-up separations, regardless of whether A or B fractions were used. The differences may be due to the type of semen samples used. Somfai et al (2002) used semen samples of high quality, whereas we evaluated semen of high quality as well as semen with high proportions of abnormalities. The fraction differences were evident only with control bull semen and the D −5 Percoll separations and were lost with the other samples (Table 3). This suggests that a decrease in the efficiency of both sperm separation methods will bring about an increase the percentage of high-quality spermatozoa for IVF procedures. In support, Chen et al (1995) demonstrated that the Percoll gradient was effective in removing debris and other contaminants but that it resulted in a low rate of sperm recovery for low-quality semen samples. They reported that the percentage of immotile sperm after a Percoll separation was higher than for the swim-up method when both methods were used to prepare abnormal semen samples for IVF. Moreover, Ng et al (1992) found that the swim-up method was superior to the Percoll method for the selection of normal spermatozoa, which corresponds with our results (Figure 1). The percentage of dead spermatozoa with swollen or damaged acrosomes was higher after swim-up procedures than after Percoll separations (Somfai et al, 2002), showing the inability of the swim-up method to separate gross abnormalities from the A fraction. Moreover, Correa and Zavos (1996) reported that the percentage of intact sperm recovered from either preparation method was lower than the percentage of motile sperm. This provides evidence that spermatozoa with primary abnormalities have enough motility to participate in the swim-up process and therefore be present in the recovery fraction without clear evidence that there was any potential to presumable participation during IVF.

With the marked decrease in the developmental rates (Table 3) and blastocyst formation rates (Figure 1), the actual participation of abnormal spermatozoa (D +27) in IVF is questionable, and fertilization may have been initiated by the small number of normal spermatozoa present. There is evidence that grossly misshapen spermatozoa can become involved in the fertilization process once they gain access to the oolemma (Burruel et al, 1996). According to Ward et al (2003), IVF results were not affected when high concentrations of presumable normal spermatozoa were used, but when low concentrations of normal spermatozoa were used, the effect was more prominent. However, Thundathil et al (1999) reported that abnormal spermatozoa that penetrate the oocyte can be involved in the fertilization process, but the resulting zygotes may be less competent, thus explaining the reductions observed in the embryonic developmental score in the current study. Moreover, spermatozoa with the pyriform defect apparently have normal acrosomes and normal motility and would be expected to penetrate the zona pellucida and enter the ooplasm. However, Thundathil et al (1999) reported that the percentage of pyriform spermatozoa that bound to and penetrated the zona and subsequently gained access to the ooplasm was lower than that of normal-shaped sperm, which is supported by the negative correlation found in this study ($r = -0.31$), as evidenced by developmental scores.

Heparin

Heparin was tested to assess its impact on cleavage and early embryonic development. The overall cleavage rates more than doubled (18%–42%) when the heparin concentration was increased from 0.1 to 10 μg/mL. The same effect was observed within each bull (Figure 4), despite a lower cleavage and developmental score for the samples with high percentages of abnormal spermatozoa present (Figure 3). The nonresponding bull (bull II) had no response to heparin concentrations until given the high dose, while the responding bull (bull I) had a linear increase in cleavage from heparin concentrations of 0.1–10 μg/mL. With regard to fertilization, these results suggest that increasing heparin levels provided a mechanism by which to partially overcome the deleterious effect of morphologically abnormal spermatozoa that were induced by thermal insulation. Saeki et al (1995) examined the fertilization rates of individual bulls at different heparin concentrations of 1.0, 10, and 100 μg/mL and found that the maximum fertilization rate occurred at 10 μg/mL. The percentage of oocytes fertilized by sperm was heparin dose-dependent and had a maximum response when heparin concentrations of 10 μg/mL were added to the fertilization medium (Parrish et al, 1988). Chamberland et al (2000) concluded that a heparin concentration of 10 μg/mL in the IVF medium was necessary to induce the physiological changes associated with capacitation and the subsequent increase in sperm motility. Spermatozoa must undergo capacitation in order to bind to the zona

and undergo the acrosome reaction. Mendes et al (2003) reported that the addition of heparin to the fertilization media improved the cleavage rate and embryonic development, regardless of which sperm separation method was used.

Our results indicate the possibility that the normal physiological and biochemical events associated with cryopreservation and capacitation are hindered in abnormal spermatozoa and therefore decrease the effectiveness of heparin as a capacitation agent. In support, Kot and Handel (1987) suggested that the interaction between abnormal spermatozoa and the oocyte may be different from that between normal spermatozoa and the oocyte, resulting in decreased penetration or fertilization and subsequent impairment of embryonic development. Although differences in cleavage rates existed between bulls and the intermediate heparin level appeared to equalize cleavage between the bulls (Figure 4), subsequent development did not differ, which suggests that the detrimental effects of scrotal insulation on sperm quality are delayed. Moreover, the failure of normal embryonic development correlates with the results of experiment I, in which the cleavage rate was lower when a semen sample with a high percentage of abnormal spermatozoa was used for IVF, and may be related to uncompensable seminal traits (Saacke et al, 1994) that cannot be minimized by increased sperm concentrations or increased heparin concentrations. However, this effect seems to be eliminated after initial cleavage. Thus, the effect of abnormal spermatozoa on IVF was manifested either prior to or during the early stages of embryonic development, as indicated by the differences in cleavage rates, but not during blastocyst formation. Also, an increase in the heparin concentration could partially overcome deficiencies, which suggests that morphologically abnormal spermatozoa undergo capacitation despite possible structural changes to the plasma membrane.

Interference with the normal scrotal thermoregulation of bulls (Lunstra and Coulter, 1997) caused an increase in the percentage of morphologically abnormal spermatozoa and resulted in deleterious effects on embryonic development. In addition, aberrant spermatogenesis may affect the normal development of the structure of the sperm plasma membrane, making it more susceptible to structural changes. Fraser et al (1995) suggested that the functional integrity of the sperm plasma membrane influences the physiological status of the spermatozoa and that the destabilization of the plasma membrane leads to premature capacitation.

In conclusion, in contrast to semen with high populations of normal spermatozoa when semen samples with a high percentage of abnormal spermatozoa are used for IVF, the outcome of swim-up and/or Percoll separation methods might be insufficient to allow successful embryonic development. Our data show that both separation methods for abnormal sperm populations were inadequate in their ability to provide potentially competent sperm for IVF. Furthermore, the types of abnormalities induced by scrotal insulation could be partially overcome by an increase in the heparin concentration, which indicates that morphologically abnormal spermatozoa may undergo capacitation despite possible structural changes to the plasma membrane.

References

Barth AD, Oko RJ, eds. *Abnormal Morphology of Bovine Spermatozoa.* Iowa City: Iowa State University Press; 1989:136–143.

Brandeis VT, Manuel MT. Effect of four methods of sperm preparation on the motile concentration, morphology, and acrosome status of recovered sperm from normal semen samples. *J Assist Reprod Genet.* 1993;10:409–416.

Burruel VR, Yanagimachi R, Whitten WK. Normal mice develop from oocytes injected with spermatozoa with grossly misshapen heads. *Biol Reprod.* 1996;55:709–714.

Chamberland A, Fournier V, Tardif S, Sirard MA, Sullivan R, Bailey JL. The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. *Theriogenology.* 2000;55: 823–835.

Chen S, Ho H, Chen H, Chao K, Lin H, Huang S, Lee T, Yang Y. Discontinuous Percoll gradient and swim-up for sperm preparation on normal and abnormal semen samples. *J Assist Reprod Genet.* 1995; 12:698–703.

Correa JR, Zavos PM. Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. *Theriogenology.* 1996;46:1225–1232.

Dode MAN, Rodovalho NC, Ueno VG, Fernandes CE. The effect of sperm preparation and co-incubation time on in vitro fertilization of *Bos indicus* oocytes. *Anim Reprod Sci.* 2002;69:15–23.

Eid LN, Lorton SP, Parrish JJ. Paternal influence on S-phase in the first cycle of bovine embryo. *Biol Reprod.* 1994;51:1232–1237.

Fraser LR, Abeydeera LR, Niwa K. Ca^{2+}-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlorotetracycline analysis. *Mol Reprod Dev.* 1995;40:233–241.

Kot MC, Handel MA. Binding of morphologically abnormal sperm to mouse egg zonae pellucidae in vitro. *Gamete Res.* 1987;18:57–66.

Lunstra DD, Coulter GH. Relationship between scrotal infrared temperature patterns and natural-mating fertility in beef bulls. *J Anim Sci.* 1997;75:767–774.

Mendes JOB, Burns PD, DeLa Torres-Sanchez JF, Seidel GE. Effect of heparin on cleavage rates and embryo production with four bovine sperm preparation protocols. *Theriogenology.* 2003;60:331–340.

Mitchell JR, Senger PL, Rosenberger JL. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. *J Anim Sci.* 1985;61:956–967.

Ng FL, Lui DY, Baker HW. Comparison of Percoll, mini Percoll and swim-up methods for sperm preparation from abnormal semen samples. *Hum Reprod.* 1992;7:261–266.

Palomo MJ, Izquierdo D, Mogas T, Paramio MT. Effect of semen preparation on IVF of prepubertal goat oocytes. *Theriogenology.* 1999;51: 927–940.

Parrish JJ, Krogenaes A, Susko-Parrish JL. Effects of bovine sperm by either swim-up or Percoll method on success of in vitro fertilization

and early embryonic development. *Theriogenology*. 1995;44:859–869.

Parrish JJ, Susko-Parrish JL, Liebfried-Rudledge ML, Crister ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology*. 1986;25:591–600.

Parrish JJ, Susko-Parrish JL, Minner MA, First NL. Capacitation of bovine sperm by heparin. *Biol Reprod.* 1988;38:1171–1180.

Pertoft H, Laurent T, Laas C, Kagedl L. Density gradients prepared from colloidal silica particles coated by polyvinylpirrolidone (Percoll). *Ann Biochem.* 1978;88:271–282.

Robbins RK, Saacke RG, Chandler PT. Influence of freeze rate, thaw rate, and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *J Anim Sci.* 1976;42:145–154.

Rodriquez-Martinez H, Larrson B, Pertoft H. Evaluation of sperm damage and techniques for sperm clean-up. *Reprod Fertil Dev*. 1997;9:297–308.

Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim Reprod Sci.* 2000;60–61:663–677.

Saacke RG, DeJarnette JM, Bame JH, Karabinus DS, Whitman SS. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? *Theriogenology*. 1998;50:117–128.

Saacke RG, Nadir S, Dalton JC, Bame JH, DeJarnette JM, Degelos S, Nebel RL. Accessory sperm evaluation and bull fertility—an update. In: *Proceedings of the 15th Technical Conference on Artificial Insemination and Reproduction.* Columbia, Mo; National Association of Animal Breeders; 1994:57–68.

Saeki K, Hoshi Y, Nagai M. Effects of heparin, sperm concentration and bull variation on in vitro fertilization of bovine oocytes in a protein-free medium. *Theriogenology*. 1995;43:751–759.

Sapienza F, Verheyen G, Tournaye H, Janssens R, Pletinex I, Derde M, van Steirteghem A. An auto-controlled study in in vitro fertilization reveals the benefit of Percoll centrifugation to swim-up in the preparation of poor quality semen. *Hum Reprod.* 1993;8:1856–1862.

Shamsuddin M, Rodriguez H, Larsson B. Fertility capacity of bovine spermatozoa selected after swim-up in hyaluronic acid-containing medium. *Reprod Fertil Dev*. 1993;5:307–315.

Shi DS, Lu KH, Gordon I. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. *Theriogenology*. 1990;33:324.

Somfai T, Bodó S, Nagy S, Papp ÁB, Iváncsisc J, Baranyai B, Gócza E, Kovócs A. 2002. Effect of swim-up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. *Reprod Domest Anim.* 2002;37:285–290.

Statistical Analysis Systems. *SAS User's Guide: Statistics.* 8th ed. Cary, NC: Statistical Analysis Systems Institute Inc; 1999.

Stauss CR, Votta TJ, Suarez SS. Sperm motility hyperactivation facilitates penetration of hamster zona pellucida. *Biol Reprod.* 1995;53:1280–1285.

Thompson JG, Allen NW, McGowan LT, Bell ACS, Lambert MG, Tervit HR. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. *Theriogenology.* 1998;49:1239–1249.

Thundathil J, Palasz AT, Mapletoft RJ, Barth AD. An investigation of the fertilization characteristics of pyriform-shaped bovine spermatozoa. *Anim Reprod Sci.* 1999;57:35–50.

Vogler CJ, Bame JH, DeJarnette JM, McGilliard ML, Saacke RG. Effects of elevated testicular temperature on morphology characteristics of ejaculated spermatozoa in the bovine. *Theriogenology*. 1993;40:1207–1219.

Ward F, Rizos D, Boland MP, Lonergan P. Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress. *Theriogenology*. 2003; 59:1575–1584.

Zhang BR, Larson B, Lundeheim N, Roderiguez-Martinez H. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int J Androl.* 1998;21:207–216.