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Effects of scrotal insulation in Nellore bulls (*Bos taurus indicus*) on seminal quality and its relationship with in vitro fertilizing ability

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Abstract

The objectives of this study were to assess the effects of induced testicular degeneration in *Bos taurus indicus* (Nellore) bulls on changes in seminal characteristics and fertilizing ability of sperm. Four Nellore bulls (30–36-month-old, 500–550 kg) with good seminal quality (>80% motile and morphologically normal sperm) had scrotal insulation applied for 5 d. Semen was collected by electroejaculation and cryopreserved at the pre-insulation moment, and 7, 14, and 21 d after insulation was removed. Gross motility, vigor of sperm movement (1–5), acrosome integrity, sperm morphology (phase-contrast microscopy), nuclear vacuoles and abnormal chromatin (Feulgen-stain) were determined after sperm preparations for in vitro fertilization (IVF). Prior to IVF, sperm were separated using a Percoll gradient (45% and 90%). Normal sperm decreased ($P < 0.05$) 14 and 21 d after insulation was removed. On 14 and 21 d, the incidence of head defects (9.7 ± 0.6 and 17.0 ± 0.8, respectively; mean ± S.E.M.) was higher ($P < 0.05$) in agreement with the incidence of nuclear vacuoles (14.0 ± 5.0 and 12.3 ± 2.3) and abnormal chromatin (24.4 ± 7.2 and 30.8 ± 2.8). Although the frequency of cleaved oocytes decreased only on 21 d ($P < 0.05$), blastocyst rates were lower ($P < 0.05$) than pre-insulation on 14 and 21 d. In regression analyses, only nuclear vacuoles, head defects and intact acrosome accounted for differences in cleavage ($R^2 = 0.38$, 0.48, and 0.30, respectively) and blastocyst rates ($R^2 = 0.35$, 0.37, and 0.44). Abnormal chromatin was associated only with blastocyst rates ($R^2 = 0.35$). In conclusion, blastocyst rate was more sensitive than cleavage rate and the assessment of nuclear integrity is recommended to predict the fertilizing ability of bull sperm.

Keywords: Bovine semen; Sperm morphology; Fertilization; Abnormal sperm chromatin

1. Introduction

Historically, the evaluation of seminal quality has focused on characteristics or attributes associated with variations in fertility. Regardless of the classification system, morphological abnormalities related to the structure and shape of the sperm head played a major role in reproductive efficiency [1,2].

Scrotal insulation has frequently been used to study the dynamics of sperm defects in the bovine spermogram; based on the severity and duration of the thermal insult and at the interval between insulation and semen collection. Scrotal insulation increased the testicular temperature and metabolism; in the absence of increased blood flow, the testes, which are normally operating on the brink of hypoxia, become hypoxic [3]. Anaerobic metabolism

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of the testes resulted in deep changes in the nuclear structural configuration of secondary spermatocytes and spermatids. In particular, it caused variations in nuclear shape and chromatin decondensation that were associated with reduced semen quality and fertilizing ability [2]. In other studies, sperm with a high incidence of specific abnormalities were collected and fertility was determined [3–5].

In general, the response to testicular heat stress in *B. taurus indicus* bulls was similar to that in *Bos taurus taurus* bulls, despite the anatomic-vascular differences in the former, which favored testicular thermoregulation [6]. Nevertheless, there have been few studies using scrotal insulation in *B. taurus indicus* in relation to the fertilizing ability of sperm with defective chromatin. In *B. taurus taurus* bulls with a higher prevalence of nuclear sperm abnormalities, Pilip et al. [7] reported that approximately 20% of the oocytes were fertilized. In contrast, Thundathil et al. [8] found no differences in cleavage, morula and blastocyst rates for semen with approximately 60% nuclear vacuoles, versus control sperm with few defects. Recently, Walters et al. [5] have not detected a substantial decrease in embryonic development rates for sperm from *B. taurus taurus* bulls submitted to scrotal insulation, despite reductions in cleavage rates. Thus, many aspects involving gamete function still need to be clarified, especially the structural integrity of the sperm nucleus and chromatin abnormalities after testicular degeneration, and their effects on fertilization and embryonic development.

The objectives of the present study were to assess the effects of induced testicular degeneration in *B. taurus indicus* (Nellore) bulls on the changes in seminal characteristics and fertilizing ability of sperm.

2. Materials and methods

2.1. Bulls and experimental testicular degeneration

Nellore bulls ($n = 4$), 30–36-month-old, approximately 500–550 kg and with good seminal quality (>80% motile and morphologically normal sperm) were used. The bulls were maintained in semi-confinement at EMBRAPA/CNPGC, National Center for Beef Cattle Research, Campo Grande, MS Brazil (20°26′34″S and 54°38′47″W). The scrotum was insulated for 5 d; it was wrapped with a wool bag (kept in place with tape) and covered with nylon plastic (approximately 2 mm thick). A single ejaculate was collected by eletroejaculation on four occasions: just prior to scrotal insulation (pre-insulation), 7, 14, and 21 d after insulation was removed. Semen was diluted in TRIS (hydroxymethyl aminomethane 0.2 M; ACS 252859; Sigma–Aldrich Chemical Co., St. Louis, MO, USA) solution with egg yolk–citrate extender containing 7% glycerol (v/v), loaded in 0.5-mL straws (final concentration, 50×10^6 cells/mL) and cooled for 4 h. Loaded straws were kept in liquid nitrogen vapor (3–5 cm above the surface) for 14 min, and then plunged into liquid nitrogen [9].

2.2. Seminal evaluation

The seminal characteristics were evaluated after Percoll gradient preparation; the percentage of motile sperm and vigor of sperm movement (scale, 1–5) were evaluated using a single straw/bull/period. Those variables were assessed subjectively, on a drop placed on a warmed glass slide, covered with a coverslip and examined (bright-field microscopy at 100 × magnification).

Sperm morphology (200 cells) was evaluated in formal saline fixed samples using a phase-contrast microscope at 1000 × magnification. Morphology was classified considering the percentage of normal sperm, head defects (underdevelopment, narrow and narrow at base, abnormal contour and pyriform shape), acrosome defects (knobbed, folded, tapered, detached, and vacuoles), midpiece defects (tail stump, corkscrew, broken and denuded), proximal droplet, tail defects (broken, coiled, bent tail and abaxial attachment), and loose heads.

Nuclear vacuoles (pouch formation and crater defects) and abnormal chromatin condensation (colorless, clumpy and fragmented nucleus) were determined through the Feulgen-stain procedure, under a phase-contrast microscope at 1000 × magnification in 500 sperm/sample [10,11]. Briefly, smears of thawed semen were prepared and fixed in 10% neutral formal saline buffered solution for 15 min and hydrolyzed in 5N HCl for 30 min. The slides were washed and placed in 0.5% period acid solution for 10 min, washed again and submerged in Schiff's reagent for 60 min. The preparations were then washed in running water for 10 min.

To assess acrosomal integrity, 20 μL of semen was mixed with Trypan-blue (0.25% in NaCl 0.81% and pH 6.9–7.2; Sigma T 8154) and used to prepare the smears. The slides were air-dried at room temperature for 20 min, fixed in methanol for 15 min, stained overnight with Giemsa (Sigma GS 500), rinsed in distilled water for 2 min, dried at room temperature, and then covered with a coverslip. The sperm was evaluated using a bright-field microscope (1000×); a total of 200 sper-

matozoa/slide were analyzed. Only cells that were alive and had an intact acrosome were considered viable.

2.3. In vitro fertilization (IVF) and embryo culture

Ovaries from crossbreed cows (*B. taurus indicus* × *B. taurus taurus*) were collected just after slaughter and transported to the laboratory in saline buffered solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 μg/mL) at 30 °C. Cumulus oocyte complexes (COCs) were aspirated from 2- to 8-mm diameter follicles with an 18-gauge needle and pooled in a 15 mL conical tube. After sedimentation, the COCs were recovered and selected using a steremicroscope. Only COCs with a homogenous cytoplasm and at least three layers of cumulus cells were used. The selected COCs were washed three times and transferred to a drop of maturation medium covered with silicone oil, and incubated for 22 h at 39 °C in 5% of CO_2 in air. The maturation medium consisted of TCM-199 (Invitrogen, Carsbad, CA, USA) supplemented with 10% FCS (v/v), 24 IU/mL of LH (Sigma L 9773), 10 μg/mL of FSH (Sigma F 8001) and antibiotics (100 IU/mL of penicillin and 50 μg/mL of streptomycin), in an atmosphere of 5% CO_2 in air at 39 °C.

For IVF, matured COCs were separated in groups of 25–30, washed and transferred to a 200 μL drop of fertilization medium under silicone oil. The fertilization medium was TALP [12], supplemented with penicillamine (21.1 μM), hypotaurine (10.4 μM), epinephrine (1 μM), and heparin (10 μg/mL). Frozen semen from each bull/period was thawed and used for IVF. Sperm cells were obtained after treatment with a 45% and 90% Percoll gradient (Amersham Biosciences AB, Uppsala, Sweden) as previously described [12]. They were added into the fertilization drop in a final concentration of 1×10^6 sperm/mL. Sperm from all bulls and all periods and oocytes were co-incubated for 22 h at 39 °C with 5% CO_2 in air. After co-incubation, presumptive zygotes were washed and transferred to an embryo culture medium. Culture was carried out in SOFaaci [13], supplemented with 2.77 mM of myo-inositol and 5% FCS in a humidified atmosphere of 5% CO_2 in air at 39 °C at 2 and 7 d after insemination, the embryos were evaluated in relation to cleavage and blastocyst formation, respectively. Three replicates were performed in the study of IVF results (25–30 COCs/bull/period).

2.4. Statistical analysis

Prior to statistical analysis, seminal characteristics were submitted to simple rank transformation. The effect of experimental periods on seminal characteristics and cleavage and blastocyst rates were calculated using the general linear model (repeated measures, adjusted for the bull effect, LSD test). Seminal characteristics, cleavage and blastocyst rates were determined both for the periods (general analysis) and for individual bull (bull effect), considering three replicates (straws/bull/period).

The model for curves estimation of the regression multiple analysis was used to determine the relation between seminal variables (independent) and IVF data (dependent) with linear and quadratic adjustment, using the mean obtained by each replicate/bull/period. For all analyses, $P < 0.05$ was considered significant.

3. Results

3.1. Effect of scrotal insulation on seminal characteristics

Seminal characteristics after selection by Percoll gradient are shown (Table 1). Morphologically normal sperm decreased ($P < 0.05$) starting 14 d after insulation was removed. For sperm abnormalities, the effect of the experimental period was significant on 14 and 21 d. Head and chromatin abnormalities were the most affected. However, the percentages of sperm cells with nuclear vacuoles on 14 and 21 d, and chromatin damage, were greater than the percentage of sperm with abnormal head morphology during the same periods. In addition, acrosome (21 d) and midpiece defects (14 and 21 d) were higher. Proximal droplet and tail defects were not significant between periods, and loose heads decreased on 14 and 21 d. The patterns deemed as nuclear vacuoles (pouch and crater defects) and abnormal chromatin Feulgen-stain are shown (Fig. 1). These forms ranged from the presence of a crater in the central region of the sperm head to the complete fragmentation of the chromatin. Due to low quality (<10% motility and Vigor 1), semen collected from Bull B on 21 d was not cryopreserved (excluded from the experiment).

3.2. Effect on cleavage and blastocyst rates

The results of general and individual analyses are shown (Table 2). The frequency of cleaved oocytes was similar for pre-insulation, 7 and 14 d, but lower on 21 d ($P < 0.05$). However, there was a decrease ($P < 0.05$) in blastocyt rates on 14 d (in relation to the pre-insulation and 7 d) and on 21 d (in relation to 7 d). When bulls were analyzed individually, the pattern of

Table 1
Mean (±S.E.M.) effect of interval before and after scrotal insulation on characteristics of semen collected from Nellore (*Bos taurus indicus*) and subjected to treatment with a Percoll gradient

Seminal variable	Interval relative to insulation (d)			
	Pre-insulation	7	14	21
Motility (%)	68.8 ± 4.2^{a}	73.3 ± 2.8^{a}	70.0 ± 5.5^{a}	61.1 ± 7.1^{a}
Intact acrosome[A]	49.9 ± 3.9^{a}	67.1 ± 2.4^{b}	68.6 ± 2.9^{b}	72.8 ± 4.5^{b}
Vigor (1–5)	4.2 ± 0.2^{a}	3.8 ± 0.2^{a}	4.5 ± 0.1^{a}	4.1 ± 0.3^{a}
Sperm defect (%)				
None (normal)	63.8 ± 4.6^{a}	71.2 ± 4.9^{a}	35.8 ± 6.7^{b}	16.4 ± 5.7^{c}
Head defect	4.1 ± 0.9^{a}	2.3 ± 0.6^{a}	9.7 ± 0.6^{b}	17.0 ± 0.8^{c}
Nuclear vacuoles[B]	1.7 ± 0.2^{a}	0.7 ± 0.2^{a}	14.0 ± 5.0^{b}	12.3 ± 2.3^{b}
Abnormal chromatin[B]	4.7 ± 2.3^{a}	6.8 ± 2.3^{a}	24.4 ± 7.2^{b}	30.8 ± 2.8^{b}
Acrosome	3.0 ± 0.3^{a}	3.1 ± 0.2^{a}	1.5 ± 0.7^{b}	8.3 ± 1.2^{c}
Midpiece	3.0 ± 0.2^{a}	0.8 ± 0.2^{a}	2.9 ± 0.2^{b}	3.6 ± 0.3^{b}
Proximal droplet	$0,8 \pm 0.1^{a}$	0.4 ± 0.1^{a}	2.8 ± 1.0^{a}	2.6 ± 0.7^{a}
Loose head	12.3 ± 0.6^{a}	8.4 ± 0.7^{a}	4.3 ± 0.6^{b}	4.5 ± 0.9^{b}
Tail	6.6 ± 0.5^{a}	6.1 ± 3.0^{a}	4.6 ± 1.7^{a}	4.5 ± 1.5^{a}

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).
[A] Trypan-blue Giemsa stain.
[B] Feulgen-stain.

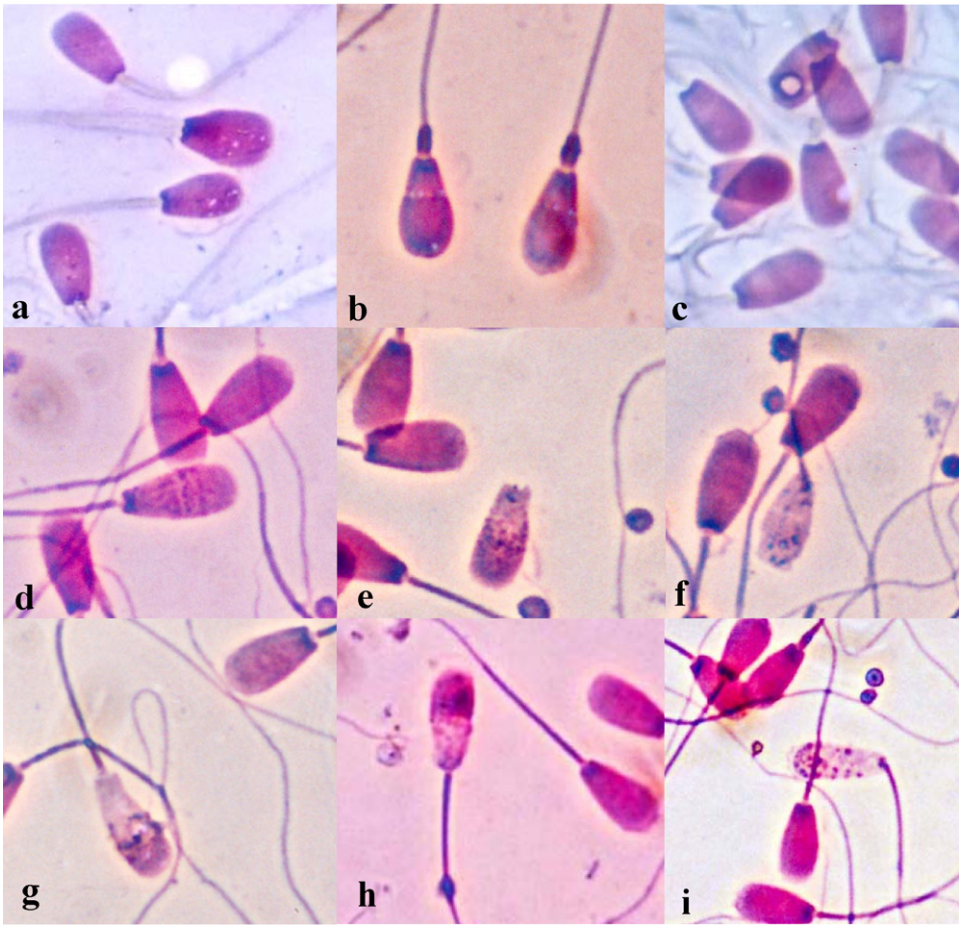

Fig. 1. Representative images of different profiles of nuclear vacuoles (a–c), and abnormal chromatin condensation (d–i) of sperm from Nellore (*Bos taurus indicus*) bulls following scrotal insulation (21 d interval). Fuelgen-stain. Phase-contrast microscopy (1000×).

Table 2
Effect of interval before and after scrotal insulation of Nellore (*Bos taurus indicus*) bulls on mean (±S.D.) rates of cleavage and blastocyst production

Bull	Interval relative to insulation (d)			
	Pre-insulation	7	14	21
Cleavage				
A	$62.1 \pm 13,2^{a}$	54.4 ± 13.8^{a}	53.0 ± 13.1^{a}	32.5 ± 3.2^{b}
B	60.0 ± 6.4^{a}	67.3 ± 4.8^{a}	55.5 ± 4.1^{a}	#
C	52.3 ± 5.2^{a}	53.5 ± 3.6^{a}	59.7 ± 6.7^{a}	29.8 ± 1.8^{b}
D	72.0 ± 10.4^{a}	54.1 ± 6.7^{b}	45.3 ± 13.0^{b}	44.6 ± 8.3^{b}
Total	58.5 ± 4.8^{a}	58.5 ± 6.3^{a}	56.0 ± 4.5^{a}	35.7 ± 5.8^{b}
Blastocysts				
A	18.3 ± 3.6^{a}	14.0 ± 2.5^{a}	0.0^{b}	0.0^{b}
B	19.9 ± 5.0^{a}	22.2 ± 7.8^{a}	0.0^{b}	#
C	15.7 ± 4.8^{a}	16.8 ± 5.9^{a}	7.2 ± 2.7^{b}	2.2 ± 1.0^{b}
D	18.5 ± 6.4^{a}	3.8 ± 0.9^{b}	3.3 ± 0.4^{b}	5.5 ± 0.7^{b}
Total	18.0 ± 3.0^{a}	14.4 ± 3.3^{a}	5.6 ± 3.3^{b}	2.6 ± 1.9^{b}

[a,b]Within a row, means without a common superscript differ ($P < 0.05$); #sample not considered.

both frequencies changed. Cleavage rates were decreased ($P < 0.05$), only on 21 d for Bulls A and C. Bull D was lower in all periods after scrotal insulation. Blastocyst rates for Bulls A, B, and C were lower ($P < 0.05$) on 14 and 21 d when compared with pre-insulation. However, for Bull D, the frequencies of blastocyst formation were lower ($P < 0.05$) on 7, 14, and 21 d.

3.3. Relationship between seminal characteristics and cleavage and blastocyst rates

The outcome of curve estimation for multiple regression analysis is shown (Table 3). Of all variables studied, only nuclear vacuoles, head defect and intact acrosome accounted for differences in cleavage and nuclear vacuoles, head defect, abnormal chromatin and intact acrosome in blastocyst frequencies. These variables were individually considered with cleavage and blastocysts rates (Fig. 2).

Table 3
Curve estimation (multiple regression analysis) among seminal variables and percentage of cleavage and blastocysts obtained by IVF after Percoll gradient treatment of sperm from Nellore (*Bos taurus indicus*) bulls subjected to scrotal insulation

Percentage	Seminal variables	R^2	Slope	P
Cleavage	Head defect[a]	0.48	−0.5530	0.0007
	Nuclear vacuoles[a,b]	0.38	−0.5287	0.0084
	Intact acrosome[c]	0.30	−0.8344	0.0421
Blastocysts	Intact acrosome[a,c]	0.44	−0.0681	0.0019
	Head defect[a]	0.37	−0.3660	0.0120
	Nuclear vacuoles[a,b]	0.35	−0.4204	0.0162
	Abnormal chromatin[a,b]	0.35	−0.3578	0.0179

[a] Linear adjust.
[b] Feulgen-stain.
[c] Trypan-blue Giemsa stain.

Cleavage rates were found in all the studied periods, even with a high proportion of nuclear vacuoles and abnormal chromatin (i.e. 14 and 21 d). On 14 d, Bull B and Bull C had the highest abnormal chromatin rate with similar cleavage rates. Bulls C and D had lower rates of blastocysts in relation to the elevation of abnormal chromatin levels. Intact acrosome proportions did not follow the same tendency among the bulls. Bull A had an elevation on 14 d, whereas Bulls C and D had the highest proportion on 21 d, in contrast to other studied variables.

4. Discussion

In the present study, there was a relationship between sperm cell characteristics and in vitro fertility of semen from *B. taurus indicus* bulls following testicular degeneration induced by scrotal insulation. This is a classic method for studies regarding sperm quality variation, but its relationship with fertilizing ability has not been emphasized in Nellore bulls. Although the zona pellucida is selective for binding spermatozoa with normal morphology, some spermatozoa with abnormal morphology are able to bind to and penetrate the zona pellucida [14]; this could include nuclear defects and chromatin abnormal condensation. However, exclusion of spermatozoa with abnormal head shapes by the fertilization process was related to the severity of the deformity; the more severe the deformation from the normal shape, the greater the chances of exclusion [8,15].

The effects of insulation on sperm morphology are well known. This method resulted in an abnormal increase of DNA condensation and nuclear vacuoles. After induction of degeneration, semen quality varied according to the stage of development and maturation of the germ cells at the moment of the insult [10]. Therefore, changes in the spermiogram depended upon the time elapsed since the testicular insult. The percentages of morphologic defects were similar to those reported in other studies of *B. taurus indicus* bulls [6,16]. However, in the present study, we collected samples only at specific intervals after insulation was removed (i.e. 7, 14, and 21 d), in which we expected to have a higher percentage of specific sperm abnormalities. For collection on 7 d, there were no significant changes in seminal characteristics, probably because at

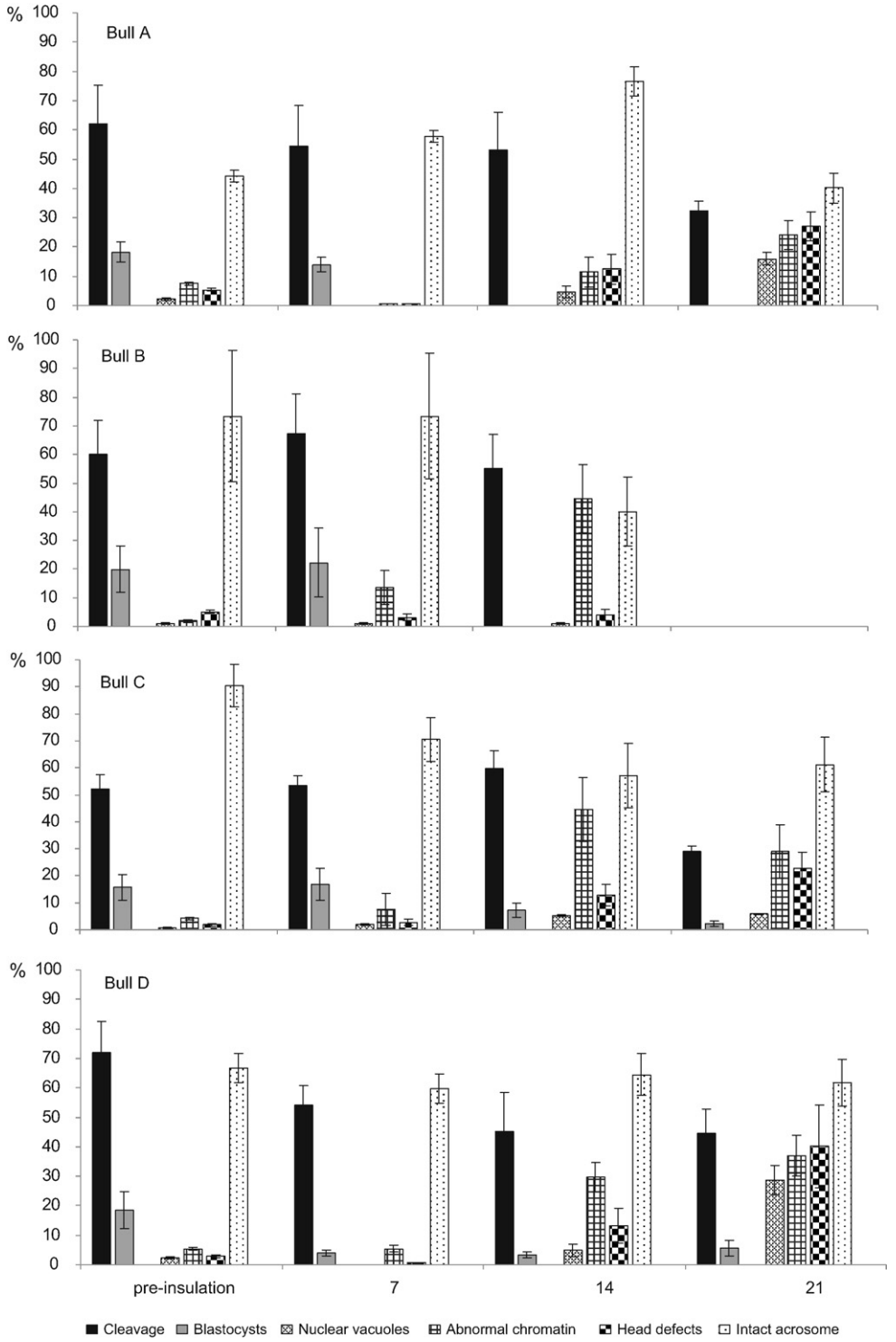

Fig. 2. Individual analysis (bull effect) of the mean (±S.D.) percentage of cleavage, blastocysts and seminal variables before and after scrotal insulation in Nellore bulls.

the time of the thermal insult, the sperm collected were outside the testis (tail of the epididymis and ampulla of ductus deferens [17]). This way, the head and nuclear structures can be more compact and resistant although the epididymal ambient is also altered by heat stress [4]. Perhaps the Feulgen-stain was not sensitive enough to specifically detect abnormal chromatin condensation.

In the subsequent periods (14 and 21 d), significant changes occurred, especially for sperm head, nuclear vacuoles and chromatin abnormalities. An increased incidence of those defects at that time was consistent with alterations in spermatid nucleus condensation during the acrosome and Golgi phases of spemiogenesis [10]. The present results were consistent with Karabinus et al. [4], who reported reduction in the nuclear stability 12 d after scrotal insulation. Modifications in head format and shape are related to nuclear chromatin compactation; they indicate variation in spermatid nuclear remodeling, which is the type of germ cell most sensitive to heat stress [3]. In previous studies with *B. taurus indicus* bulls [6,16], increased head abnormalities occurred approximately 28 d after the thermal insult; however, we did not collect or evaluate semen at that time.

The higher percentage of cells with abnormal chromatin in relation to the percentage of cells with head abnormalities on 14 and 21 d suggests that when morphology is evaluated under phase-contrast microscopy, specific defects of nucleus and chromatin abnormalities can be underestimated, as cells with a morphologically normal head may have nuclear structure alterations. Changes in chromatin condensation occurred due to a high sensitivity of the disulphide bonds and were detected as early as 3 and 9 d after scrotal insulation [18]. Since abnormal chromatin was more evident on 14 and 21 d in the present study, the damage occurred during spermatogenesis and not in epididymal sperm. The pattern of nuclear abnormalities found by Feulgen-stain appeared similar to that previously reported [19,20]. In this staining method, sperm are exposed to acid hydrolysis that removes purine bases, unmasking aldehyde groups present in DNA. If there is chromatin fragmentation or decondensation, those chemical groups react with basic fuschin (pararosaniline) and turn reddish or magenta [21,22]. In phase-contrast microscopy, those damaged regions became whitish, similar to *cracks* distributed in various regions of the head, which represents chromatin lesions. In the present study, nuclear vacuoles and abnormal chromatin were associated more specifically 21 d after scrotal insulation, with variation among the bulls. However, both defects have been considered independent in terms of origin and pathogenesis. Nuclear vacuoles may be found in the nucleus posterior to the apical ridge or throughout the sperm head; abnormal chromatin condensation is not very common and head sperm displays clumping of the DNA [10]. Thus, the abnormal chromatin is not evident through traditional methods, but Feulgen-stain techniques reveal morphological abnormalities significant to fertility variations.

Cleavage rates were found in all the studied periods, even with a high proportion of nuclear vacuoles and abnormal chromatin (i.e. 14 and 21 d). On 14 d, Bull B and Bull C had the highest abnormal chromatin rate with similar cleavage rates. Bulls C and D had lower rates of blastocysts in relation to the elevation of abnormal chromatin levels. Intact acrosome proportions did not follow the same tendency among the bulls. Bull A had an elevation on 14 d, whereas Bulls C and D had the highest proportion on 21 d, in contrast to other studied variables. The main functions of sperm during fertilization are to contribute to a set of haploid chromosomes and to active the oocyte, initiating a cascade of biochemical events that culminate in the development of a new embryo [23]. Bovine oocytes ready to be fertilized are retained at metaphase II. The signal to complete the second meiotic division, formation of pronuclei and preparation for embryonic development, is only activated after the sperm penetration in the ooplasma [24,25]. The sequence of events leading to the zygote formation can be observed in vitro assays. Although the use of in vitro fertilization events as a comparative and predictive method for bull's fertility remains questionable, it does play a valuable role in evaluating sperm cell attributes, as it provides a multifactor prediction in a controlled experimental, homogeneous and highly repeated environment [26].

In the present study, the sperm population after Percoll gradient selection consistently had a satisfactory percentage of acrosome integrity in all experimental periods, compatible with good rates of acrosome reaction and fertilization. Conversely, the individual effect was important mainly in Bull A, where the proportions were elevated on 14 d. The reasons for the differences are not clear yet. Percoll gradient efficiently removes dead sperm, those with damaged acrosome, specifically in the frozen–thawed semen with low post-thaw motility and low rate of intact acrosome [27,28]. Thus, a great part of spermatozoa population was probably retained in Percoll, where uncompensable defects of head morphology, nucleus and chromatin were optimized. The sperm with a high percentage of head and chromatin abnormalities could have intact acrosomes and produce a satisfactory rate of cleavage,

emphasizing the limitation of using IVF to estimate in vivo fertility.

Multiple regression analysis was used to determine the relation between seminal characteristics and fertility; it allows the inclusion of predictive variables to derive a model that explains fertility variation. Nuclear vacuoles, head defects, and intact acrosomes were the independent variables that best accounted for variation in rates of cleavage and also the abnormal chromatin for blastocyst formation. Although nuclear, head and chromatin defects significantly increased on 14 and 21 d, cleavage rate only decreased on 21 d; therefore, these did not preclude the ability of the sperm to support cleavage. Perhaps mechanisms responsible for nuclear remodeling and subsequent male pronuclear formation are preserved, at least until the initial embryo divisions [29]. In relation to nuclear and head defects, this possibility has already been proven [7,8,14], however, it is not clear for abnormal chromatin.

The effect of abnormal chromatin could become evident 30 h post-insemination, with reduced kinetics of embryo division in bulls with low fertility [30]. Similar observations were not made in the present study, since cleavage was evaluated 48 h post-insemination. However, the rate of blastocyst formation was more closely associated with variations in the nuclear and chromatin defects, for individuals (bull effect), as well as for the group (period effect). In addition, cleavage rates were more homogenous in relation to the magnitude of the chromatin defects. These results disagreed with those reported by Walters et al. [5], who observed higher blastocyst rates for bull semen with 50–60% nuclear vacuoles (strong individual bull effect). In the present study, with the exception of Bull D, all bulls had a decreased blastocyst formation rate 14 d after insulation, when chromatin abnormalities were at least 15% and nuclear vacuoles were 20%. For Bull D, the blastocyst rate was already decreased on 7 d. The reduction in blastocyst rates supported the notion that sperm nuclear abnormalities affect embryo development by modifying transcriptional activity of the regulatory genes of cell cycle, which would lead to a delay in the first meiotic divisions [31–33]. Similarly, Kawarsky et al. [34] and Thundathil et al. [8,14] reported that the percentage of blastocyst formation, rather than the percentage of cleavage, was the most important factor to determine differences in semen quality, especially for semen with a high percentage of head defects and nuclear vacuoles. The results of the present study confirmed those findings, since the effects on blastocyst rate were more evident than those on cleavage rate. However, other components of the sperm viability, not detected in standard laboratory tests, could be present in sperm cells classified as having abnormal chromatin, and therefore could also be associated with subfertility.

In conclusion, the efficiency of in vitro embryo production was closely related to sperm cell characteristics, especially nuclear morphology, as evaluated by chromatin abnormalities. Therefore, in routine semen evaluation, the inclusion of functional tests and methods to determine nuclear integrity would be very useful to predict fertilizing ability of bulls, particularly prior to their use in assisted reproductive programs.

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