Scholarly Research Exchange

SRX Veterinary Science • Volume 2010 • Article ID 324529 • doi:10.3814/2010/324529

Research Article

Sire Effect and Sperm Apoptosis on Bovine Embryonic Cleavage and Subsequent In Vitro Embryo Development

Antonio Chaveiro,[1,2] Pedro Santos,[1] Isabel Carvalhais,[1,2] Ricardo Agrícola,[1,2] Marwa Faheem,[1,2] Afrooz Habibi,[1] and Fernando Moreira da Silva[1,2]

[1] *Animal Reproduction, Department of Agrarian Sciences, University of the Azores, 9701-851 Angra do Heroísmo, Portugal*
[2] *CITA-A, University of the Azores, 9701-851 Angra do Heroísmo, Portugal*

Correspondence should be addressed to Fernando Moreira da Silva, jsilva@uac.pt

Received 24 September 2009; Accepted 2 November 2009

The present study was conducted to evaluate sire effect on the kinetics of in vitro cleavage and further embryonic development after in vitro fertilization (IVF) using bulls of an Azorean rare breed called "Ramo Grande" (RG), and examine whether a relationship exists between bull's sperm apoptosis and sire in vitro fertility. Results showed that cleavage and blastocyst rates were statistically different between bulls ($P < .05$), being possible to notice different ability to produce embryos with good development competence ($P < .05$), as assessed by the proportion of fertilized oocytes that reached blastocyst stage. The proportion of blastocysts that continued the development to hatched blastocysts stage ranged from 9.1 to 87.5% ($P < .05$). Differences in sperm apoptosis increased during the swim-up procedure, which was statistically different ($P < .05$) between bulls, being negatively correlated with the ability of those bulls to fertilize oocytes and resulting embryos to develop to hatched blastocysts ($R = -0.96$, $P \leq .01$). In conclusion, this study clearly demonstrated great differences between bulls, concerning apoptosis levels, which were negatively related to bull's ability to produce good quality embryos after IVF.

1. Introduction

It is well known that sperm from different bulls differ in their ability to fertilize oocytes in vitro, having an effect on preimplantation embryo development [1, 2]. Therefore, kinetics of early cleavage divisions can be used to discriminate between bulls of high and low field fertility [3]. Assays for apoptotic sperm detection can be used, together with in vitro fertilization (IVF), to reduce the risk of using poorfertility bulls in assisted reproduction programs. Chaveiro (2005) [4] demonstrated statistical differences in the sperm membrane permeability characteristics between semen of different bulls. This leads to different reductions in sperm viability among different bulls, since normally a general protocol is used to all bulls in freezing/thawing process. Thus, contributing to the general decline in fertility that is observed in semen samples after freezing/thawing process. Annexin V/PI assay serves as a sensitive probe that can be used for flow cytometric detection of cell death (apoptotic or necrotic) characterized by the loss of membrane asymmetry, allowing the detection of apoptotic cells. Said et al. [5] concluded that reduced sperm motility in the annexin V-positive fraction is an indicator that annexin V binding is more associated with cell damage/death rather than sperm capacitation.

Although exists an association between apoptotic activity in spermatozoa and blastocyst development and on pregnancy rates [6], this negative effect has not yet been fully investigated and understood. Therefore, the present study was conducted to evaluate (1) the effect of sire on the kinetics of in vitro cleavage rates and embryo development parameters after IVF, (2) to study the in vitro cleavage rates and embryo developmental competence for bulls of known sperm apoptosis levels to be able to investigate a possible association between individual apoptotic sperm levels and fertility potential of semen processed for artificial insemination.

2. Materials and Methods

Bovine semen (5 sires) from a local breed "Ramo Grande" (RG), numbered from 1 to 5, and a sixth bull of the Holstein

Frisian bread (used as control), numbered as 6, frozen in 0.25 mL of French straws were used in the present study. The 5 RG bulls had no field fertility records and were untested to the date.

Three straws per bull, per ejaculate were used for flow cytometric analysis and 4 replicates were carried out with each of the 6 bulls being represented in each replicate, for IVF experiments.

Bovine ovaries were collected at a slaughterhouse, regardless of the estrous cycle and hormonal stage, and transported in a bottle with phosphate buffered solution (PBS).

Unless otherwise all chemicals were purchased from Sigma Chemical Co. (Sigma, St, Louis, MO).

2.1. In Vitro Fertilization. Cumulus-oocyte complexes (COCs) were obtained by aspiration from 2 ± 8 mm follicles. For in vitro maturation (IVM), COCs ($n = 1227$) were cultured for 24 h in M199 medium supplemented with 25 mM $NaHCO_3$, 10% fetal calf serum (FCS), 10 mg ml^{-1} of FSH-LH (Stimufol, Belgium), and 1 mg ml^{-1} of estradiol—17b at 38.5°C and 5% CO2 in air.

After maturation, oocytes were divided in 6 groups, and frozen/thawed semen from each 6 bulls were used to fertilize oocytes of each group. Two different modified Tyrode's media (TALP) were used for capacitation and fertilization [7, 8]. Thawed sperm of each bull were selected by swim-up (SU) and prepared according to Parrish et al. [8], adjusted to a final concentration of 1×10^6 spermatozoa ml^{-1}. Groups of matured oocytes were inseminated in fertilization TALP medium containing 0.05 μg ml^{-1} of heparin (Marquant-LeGuienne, 1990), 10 μM hypotaurine, 1 μM epinephrine, and 20 μM penicillamine at 39°C and 5% CO_2 in air. For semen capacitation, low doses of heparin were employed (0.05 μg ml^{-1}) for discriminating individual bulls, found optimal by previous studies [9–11] and same numbers of spermatozoa were used per insemination for each bull (1×10^6 spermatozoa ml^{-1}) according to previous studies [3, 12, 13].

At 18 h postinsemination (hpi), cumulus cells were removed by vortex, and presumptive zygotes were then cocultured with monolayers of granulosa cells in 45 μl droplets of B2 medium (CCD Laboratory, Paris, France), supplemented with 10% (v/v) FCS under mineral oil, at 39°C and 5% CO2 in air. On Day 2 (Day 0 = day of in vitro fertilization), Day 7, Day 9, and 10 embryos were classified as developed (cleaved, blastocysts, and hatched blastocyst) or degenerated. The results of embryo production are expressed as cleavage rate, blastocyst rate (Blastocyst Yield/cleaved oocytes), hatched blastocyst rate (hatched blastocyst number/blastocyst number), and global development (blastocyst number/fertilized oocyte number).

2.2. Semen Apoptosis

2.2.1. Annexin-V/PI Binding Assay. An Annexin V-FITC Apoptosis Kit (catalog no. 6710KK; Pharmingen, San Diego, CA, USA), was used to detect the transition of phospholipids PS of the spermatozoa plasma membrane post-thaw as recommended by the manufacturer with slight modifications. Briefly, as previously described [14, 15], for both treatments (post-thaw and SU), aliquots of semen were diluted in Annexin-V Binding buffer (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) to a concentration of 2×10^6 spermatozoa ml^{-1}. Aliquots of extended semen (100 μL, corresponding to 2×10^5 cells ml^{-1}) were transferred to a 5 mL cultures tubes and supplemented with 5 μL Annexin-V –FITC and 5 μL of PI (50 μg ml^{-1}). Tubes were gently mixed and incubated for 15 minutes at room temperature in the dark. Additional 400 μL of Binding Buffer was added (final spermatozoa concentration of 1×10^6 cells ml^{-1}) to each tube prior to flow cytometric evaluation. Flow cytometric evaluation was conducted within 5 minutes.

2.2.2. Flow Cytometric Analysis. Samples were analyzed by a FacsCalibur flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, USA) equipped with standard optics. Briefly, as previously described by Chaveiro et al. [15], for each cell, forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1), and PI fluorescence (FL3) were evaluated using the Cellquest software. Acquisition gate applied in FSC/SSC two-dimensional histogram was used to restrict the analysis to spermatozoa, and to eliminate small debris and other particles for further analysis. The percentages of viable spermatozoa (Annexin-V$^-$, PI$^-$), apoptotic sperm (Annexin-V$^+$, PI$^-$), necrotic sperm (Annexin-V$^-$, PI$^+$), and early necrotic (Annexin-V$^+$, PI$^+$) were evaluated, based on regions determined from single-stained and unstained control samples.

Finally, the extend of damage provoked by SU procedure on spermatozoa from each bull was quantified, to evaluate the differences of Annexin-V$^+$/PI$^-$ levels before and after SU technique (diffSUapop) and relate it with IVF and EP results. For this related part of the study, only the apoptotic spermatozoa values were considered.

2.3. Statistical Analysis. Comparisons between bulls for cleavage rate, blastocyst rate, hatched blastocysts rate, and proportion of fertilized oocytes developing to blastocysts were analyzed using Chi-square analysis. Pearson correlation coefficients were calculated to test the relationships between bull fertility and Annexin-V$^+$/PI$^-$ levels in spermatozoa before and after SU. All data expressed as percentages were normalized with an arcsine transformation. Analyses were conducted using the statistical software SPSS (SPSS Inc., Chicago, IL, USA).

3. Results

Four different subpopulations of sperm cells were detected. Apoptotic spermatozoa were labeled with Annexin V-FITC, but not with PI, early necrotic spermatozoa labeled with Annexin-FITC and PI, and sperm only labeled with PI but not with Annexin-FITC representing necrotic sperm. The majority of the sperm cells before and after SU, on average, where nonfluorescent, meaning fully viable sperm

Table 1: Percentage of different sperm subpopulations (mean ± SEM) before and after swim-up procedure, assessed by flow cytometry.

		Viable	Apoptotic	Necrotic	Early necrotic	diffSUapop	% apop.	Variation
Bull 1	Before SU	57.2 ± 1.1^{b}	7.1 ± 0.4^{a}	8.7 ± 0.1^{a}	27.1 ± 1.0^{b}	17.2*		342.3
	After SU	55.0 ± 0.6^{B}	$24.3 \pm 0.3^{A*}$	$3.8 \pm 0.1^{A*}$	$14.2 \pm 0.1^{A*}$			
Bull 2	Before SU	39.9 ± 4.3^{a}	27.9 ± 8.1^{b}	8.2 ± 1.2^{a}	23.9 ± 2.6^{ab}	6.0		121.5
	After SU	41.7 ± 3.2^{A}	33.9 ± 4.4	$4.6 \pm 0.2^{A*}$	20.0 ± 1.3^{B}			
Bull 3	Before SU	32.9 ± 1.8^{a}	14.1 ± 3.1^{a}	11.4 ± 0.4^{b}	41.6 ± 1.3	1.2		108.5
	After SU	$40.3 \pm 0.7^{A*}$	15.3 ± 1.2^{B}	$6.3 \pm 0.4^{B*}$	38.1 ± 1.4			
Bull 4	Before SU	69.6 ± 0.5^{c}	7.0 ± 0.9^{a}	4.1 ± 0.3^{c}	19.6 ± 0.5^{a}	52.2*		845.7
	After SU	$22.5 \pm 0.1^{C*}$	$59.2 \pm 0.2^{C*}$	$2.5 \pm 0.2^{A*}$	$15.8 \pm 0.2^{A*}$			
Bull 5	Before SU	60.5 ± 1.2^{b}	6.4 ± 0.9^{a}	7.7 ± 0.7^{a}	26.0 ± 0.9^{b}	12.7*		298.4
	After SU	58.2 ± 1.5^{B}	$19.1 \pm 0.8^{AB*}$	$3.6 \pm 0.3^{A*}$	$19.0 \pm 0.5^{B*}$			
Bull 6	Before SU	71.3 ± 1.6^{c}	8.5 ± 1.2^{a}	4.3 ± 1.0^{c}	22.3 ± 2.9^{a}	1.6		118.8
	After SU	69.0 ± 2.4^{D}	10.1 ± 0.9^{B}	$1.6 \pm 0.1^{C*}$	21.5 ± 2.0^{B}			

Superscripts values in the same line, with different letter differ significantly ($P < .05$; uppercase letters indicate before SU and lowercase letters after SU).
Asterisks mark significant differences between before and after SU samples within the same bull ($P < .05$).
diffSUapop represents the difference of semen's apoptosis levels (Annexin-V^+, PI^-) before and after the SU procedure.

Table 2: Effect of bull on first cleavage following IVF and the subsequent blastocyst development.

	Oocytes fertilized	Cleavage rate		Blastocyst rate		Hatched blastocyst rate		Global development	
Bull	*n*	*n*	%	*n*	%	*n*	%	*n*	%
1	170	111	65.3^{a}	13	11.7^{ab}	7	53.9^{ab}	13	7.7^{ab}
2	172	144	83.7^{b}	24	16.7^{ab}	16	66.7^{ab}	24	13.9^{bc}
3	118	77	65.3^{a}	8	10.4^{ab}	7	87.5^{b}	8	6.8^{ab}
4	218	108	49.5^{c}	11	10.2^{a}	1	9.1^{c}	11	5.1^{a}
5	216	157	72.7^{a}	29	18.5^{ab}	14	48.3^{a}	29	13.4^{bc}
6	207	178	86.0^{b}	36	20.2^{b}	26	72.2^{b}	36	17.4^{c}

Values in the same column, with different superscripts (a–c) are significantly different ($P < .05$).

cells (Table 1). The most consistent effect observed was the increased fraction of apoptotic sperm (Annexin-V^+, PI^-) after swim-up treatment (SU) among the 6 bulls tested, being statistically ($P < .05$) in Bulls 1, 4, and 5. The highest increase of apoptotic spermatozoa after SU was observed in Bull 4, with a diffSUapop of 52.2. In addition, statistical differences ($P < .05$) among bulls were observed immediately after thawing, as well as after the SU procedure (Table 1). On opposite, the percentage of necrotic sperm population decreased ($P < .05$) after swim-up in all bulls (Table 1).

Swim-up treatment induced a statistical ($P < .05$) increase in the fraction of viable sperm population in Bull 3 and a decrease ($P < .05$) in Bull 4 (Table 1).

In the present study, there was a significant bull effect ($P < .05$) on the quantity and quality of produced embryos (Table 2). Cleavage rates were statistically different ($P < .05$) among bulls. The highest cleavage rate (86%) was observed in Holstein bull (Bull 6), used as control, which had a low diffSUapop (1.6). For the oocytes fertilized with semen of the RG bulls, cleavage rates varied from 49.5% (the lowest cleavage rate among all bulls) to 83.7% obtained, respectively for the oocytes fertilized with semen of Bull 4 and 2. Bull 4 had also the highest (52.2) diffSUapop (Table 1).

On the 7th day, at blastocyst stage, the highest results were also observed with control bull (20.2%) and the lowest results were observed in the RG bull number 4, in which only 10.2% of the fertilized embryos developed to blastocysts, existing a difference ($P < .05$) between them. The proportion of blastocysts that continued to expand and hatch was also statistically different ($P < .05$) among bulls. Bulls that produced higher and lower number of hatched blastocysts were, respectively, Bull 3 and Bull 4 (87.5% and 9.1%) ($P < .05$).

For Global Development (Blastocyst Yield/oocytes fertilized) the best results were achieved by bull used as control (17.4%). From RG bulls, the higher value was obtained for Bull 2 (13.9%) and the lowest form Bull 4 (5.1%) ($P < .05$).

Taken together, in all embryo development stages, a general negative correlation between semen apoptotic levels after SU and the IVF/EP results was observed (Table 3). For expanded and hatched blastocyst stages, correlation coefficients were significantly negatives ($R = -0.85$ and $R = -0.84$; $P < .05$, resp.). The same relationship was found between proportion of cleaved zygotes that hatched (HatchBl/Cleav) and semen apoptotic levels after SU ($R = -0.86$; $P < .05$). Analyzing the evolution of apoptotic levels

Table 3: Correlations between apoptotic values observed in sperm cells after semen thawing and sperm cell segregation and rates of in vitro embryo development after IVF.

	Correlation coefficients	
Parameters	ApoptoticSU	diffSUapop
Cleavage	−0.63	−0.80
Blastocyst Yield	−0.51	−0.50
Expanded Blastocyst	−0.85*	−0.96**
Hatched Blastocyst	−0.84*	−0.97**
HatchBl/Cleav	−0.86*	−0.95**

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
diffSUapop represents the difference of semen's apoptosis levels (Annexin-V^+, PI^-) before and after the SU procedure.

from each bull, before and after SU, and comparing results with those obtained for IVF, negative correlations between results obtained for these 2 variables were found. Significant negative correlations were found between diffSUapop and expanded and hatched blastocysts ($R = -0.96$ and $R = -0.97$; $P < .01$, resp.). For hatchedBL/cleav, the negative correlation was also observed ($R = -0.95$; $P < .01$).

4. Discussion

The main findings of the present study are that sire used in IVF can have a profound effect on the proportion of fertilized oocytes developing to the blastocyst stage ($P < .05$), and can give rise to variations in quality of the blastocysts produced as assessed by hatched blastocysts rates ($P < .05$), which were inversely related to the levels of apoptosis in bull spermatozoa. It was shown that sperm apoptotic levels from each bull may be an important contributor to this sire effect, and to our knowledge, this is the first report of an established relationship between apoptosis evolution potential (diffSUapop) in bull spermatozoa and IVF/EP assays. Evidence from this experience model, whereby the value of the difference from sperm apoptotic levels between before and after swim-up treatments is compared with embryo development, indicated that a negative correlation exists between blastocyst development ability and apoptotic sperm levels ($P < .01$).

In present experiments, we standardized, as much as possible, the parameters related to the fertilization process, to be able to highlight bull effect. Limited number of available straws restricted the possibilities of preliminary experiments in order to optimize the heparin and sperm concentrations for each individual bull. Furthermore, different heparin doses or sperm concentrations could represent sources of variation. As penetration rate has been found to depend on both the sperm and heparin, $0.05\,\mu g\,ml^{-1}$ of heparin and a concentration of 1×10^6 spermatozoa ml^{-1} were used based on previous studies [9–11]. Higher doses of heparin or sperm concentration could lead to a leveling process of fertilization which, in turn, could hide differences between bulls, as unknown fertility bulls (RG bulls) and a commercial bull were up to be compared in present research.

In fact, statistical differences ($P < .05$) between bulls on cleavage rates (48 hpi) were observed, in agreement with previous studies [16, 17]. According to our results, it can also be concluded that there is in fact a bull effect in the quality of resulting blastocysts ($P < .05$). Previous studies [2, 18] achieved similar conclusions, however assessed by other means.

Culture conditions could influence the quality of resulting blastocysts [19], however in present study it would be in the same proportion to all bulls, since culture conditions were identical to all bull's batches. The significant differences between bulls achieved in the quality of producing blastocysts are most likely a reflection of multiple spermatozoa characteristics and like previously found paternal genome effects on postembryonic genome activation [6, 20–22].

Flow citometry can easily and reliably detect PS expression on the outer leaflet of the spermatozoa membrane, which is considerer a hallmark of apoptosis [14, 23, 24]. A previous study associated annexin V staining with the acrosome reaction and not apoptosis [25], in human sperm. However, recently annexin V was used to separate apoptotic from nonapoptotic spermatozoa, being the last one considered with superior ability to capacitate and with maximum potential to perform acrosome reaction after stimulation [26]. Furthermore, when both populations were compared, no difference in spontaneous acrosome reaction was found, thus supporting our results that link annexin V staining with inferior IVF results.

It has been unequivocally demonstrated that inactive and active caspases are present in ejaculated sperm [23, 27–29] and can become activated, particularly after freezing and thawing [28]. Active caspases are more frequently found in sperm from infertile than fertile donors, and in the purified fractions of sperm with low motility compared with the ones with high motility [23, 28, 30]. Paasch et al. [27] showed that deterioration of sperm plasma membrane (characterized by PS externalization) is associated with activated caspases. Furthermore, correlations between increased sperm DNA damage, production of reactive oxygen species, higher levels of cytochrome *c* and caspases 8, 9 and 3, alterations of mitochondrial membrane potential integrity, and the PS externalization have been widely reported [23, 24, 31–33], suggesting a relationship between them.

Taken together, and with the general increasing degree of annexin V binding (an early apoptotic event) in post SU spermatozoa in relation to post-thawing spermatozoa observed in this study, it is tempting to speculate that this apoptotic marker may also appear after the ejaculation, in response to adverse conditions that would trigger apoptosis.

Furthermore, different individuals may differ in cellular characteristics, such as sperm cells. Cryopreservation have a dramatic effect on spermatozoa [14, 34] causing fragmentation of spermatozoa, overcondensation of spermatozoal DNA, and sperm apoptosis (translocation of PS from the inner to the outer leaflet of the sperm membrane) on bull spermatozoa [14, 35]. All these changes likely contribute to the general decline in fertility that is observed in semen samples after cryopreservation. As normally a general freezing protocol is used, that is not suitable in the same

manner to different bulls, it is likely that the tremendous chemical and physical stresses that spermatozoa undergo during cryopreservation result in different levels of semen damage, leading to different decreases in sperm viability post freezing/thawing process, that can explain the significant differences in fertility results among bulls.

The ultimate goal for the assessment of sperm quality is to define any relationship to fertility. Said et al. [5] showed a negative impact of apoptosis on sperm-oocyte penetration with significant negative correlations between annexin V-positive and sperm-oocyte penetration assays. Nevertheless, it must be kept in mind that their results were evaluated as the percentage of oocytes penetrated by sperm (SPA) and the average number of sperm penetrated per oocytes (sperm capacitation index, SCI).

Although in the present study we did find differences ($P < .05$) between bulls on cleavage rates, and apoptosis levels appear to have a negative impact on cleavage rates, the negative correlation between this 2 parameters was not significant. This may be explained by the fact that, at this stage, the paternal genome is not yet activated and the development is controlled by maternally inherited mRNA [21, 22].

The DNA repair machinery has been shown to be defective in spontaneously aborted embryos [36, 37]. It is therefore possible that more subtle DNA aberrations in sperm insufficient to induce gross responses in cell cycle arrest and apoptosis may be expressed in a later embryonic or even postnatal phase [22, 38].

Recently, Fatehi et al. [22] submitted sperm cells to irradiation and despite DNA damage, sperm cells were normally capable to fertilize the oocyte as the cleavage rates were not lower than in IVF experiments with non-irradiated sperm. Further embryonic development, however, was completely blocked; since at the time of onset of embryonic gene expression (4–8 cell stage), the paternal DNA damage becomes sensible for the apoptotic machinery of the early embryo and by blocking the mitosis, it arrests further embryonic development and only sporadic blastocyst formation was found [22]. Despite that this DNA damage cannot be considered a very usual event on sperm cells from healthy fertile males, it does highlight both 1 the possible sire influence on fertility, since DNA damage may vary from 0.1% in healthy fertile males [39] up to 50% in cases of testicular seminoma or asthenozoospermia [40] and 2 the importance of not useing only segmentation data or sperm binding to *zona pellucida* for fertility conclusions, since a paternal effect may be also pronounced at postembryonic genome activation.

Sperm DNA integrity is essential for the accurate transmission of genetic information. Upon entry into the oocyte, the sperm chromatin undergoes extensive remodeling, resulting in the reorganization of the paternal genome into the male pronucleus [3]. Thus, any form of sperm chromatin abnormality or DNA damage may result in male infertility [41]. In agreement with Seli et al. [6], our results indicate that higher sperm apoptosis after swim-up represents a negative effect on blastocysts development ability to hatch ($R = -0.84$; $P < .05$). Furthermore, bulls whoes semen samples were more affected by SU procedure, in what diffSUapop is concern, were also the ones with a profound decline in blastocyst development ability. We found negative correlations between diffSUapop and hatched blastocysts ($R = -0.97$; $P < .01$).

Recent studies demonstrated that a variable proportion of live, ejaculated spermatozoa under in vitro incubation conditions shows PS externalization [24, 42]. Since we already knew that sorted dual-stained spermatozoa die rapidly after incubation at 37–39°C [14], inducing detrimental effects during in vitro incubation of spermatozoa [24, 43, 44], these are interesting results, showing that distinct differences in apoptotic sperm levels evolution (diffSUapop) of each bull during SU are negatively linked with IVF results. Thus, indicating that differences in early embryo development may be paternally influenced and highlighting the important role that the quality of spermatozoa fertilizing the egg may play. The degree of apoptosis increase of each bull sperm cells population during the SU procedure seems to indicate the quality potential of that population, that is, the ability to fertilize oocytes and resulting embryos development to hatched blastocysts. One can speculate that this apoptotic levels increase may be equivalent or similar during the time interval in which the spermatozoa is coincubated with the oocytes during IVF procedure, leading to different embryo viability rates.

It is likely that multiple factors related to sire can influence embryo viability. The viability of high-apoptosis-levels-bull-derived embryos could have been negatively affected by multiple sperm factors as the very first cycle of DNA replication and mitotic division, as postulated by Eid et al. [45], entering the zygotic S-phase later compared with embryos derived from low-apoptosis-levels-bulls, and therefore explain the differences in blastocysts rates among bulls.

Even so, we believe that these data point toward a relationship between inferior quality of sperm plasma membrane and failures in reproductive parameters.

In conclusion, this study clearly demonstrated a negative correlation between blastocyst development ability and the level of apoptosis in the population of each bull spermatozoa prepared for IVF, indicating that sperm apoptosis, characterized by the translocation of PS on the outer leaflet of the plasma membrane, could be responsible in part for some forms of poor embryo quality and development. In addition, differences in the increasing degrees of apoptosis in sperm during SU were negatively correlated with the ability of those bulls semen to fertilize oocytes and resulting embryos to develop to hatched blastocysts. Furthermore, apoptotic markers found in ejaculated spermatozoa may represent an important tool for the study of male infertility, and combine with IVF, may be a valuable laboratory routine technique.

Acknowledgments

Thanks are due to Dr. Ana Luisa Pavão (Regional Agrarian Development Services, Azores) for providing the frozen semen. This study was partially supported by the Luso-American Foundation (FLAD), Grant no. 379/03.

References

[1] P. Comizzoli, B. Marquant-Le Guienne, Y. Heyman, and J. P. Renard, "Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes," *Biology of Reproduction*, vol. 62, no. 6, pp. 1677–1684, 2000.

[2] F. Ward, D. Rizos, M. P. Boland, and P. Lonergan, "Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress," *Theriogenology*, vol. 59, no. 7, pp. 1575–1584, 2003.

[3] F. Ward, D. Rizos, D. Corridan, K. Quinn, M. Boland, and P. Lonergan, "Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo," *Molecular Reproduction and Development*, vol. 60, no. 1, pp. 47–55, 2001.

[4] A. Chaveiro, *Bull sperm cryopreservation, fundamental and applied aspects*, Dissertation, Veterinary Faculty, Utrecht University, Utrecht, The Netherlands, 2005.

[5] T. Said, A. Agarwal, S. Grunewald, et al., "Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an in vitro model," *Biology of Reproduction*, vol. 74, no. 3, pp. 530–537, 2006.

[6] E. Seli, D. K. Gardner, W. B. Schoolcraft, O. Moffatt, and D. Sakkas, "Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization," *Fertility and Sterility*, vol. 82, no. 2, pp. 378–383, 2004.

[7] G. D. Ball, M. L. Leibfried, R. W. Lenz, R. L. Ax, B. D. Bavister, and N. L. First, "Factors affecting successful in vitro fertilization of bovine follicular oocytes," *Biology of Reproduction*, vol. 28, no. 3, pp. 717–725, 1983.

[8] J. J. Parrish, J. L. Susko-Parrish, M. L. Leibfried-Rutledge, E. S. Critser, W. H. Eyestone, and N. L. First, "Bovine in vitro fertilization with frozen-thawed semen," *Theriogenology*, vol. 25, no. 4, pp. 591–600, 1986.

[9] J. J. Parrish, J. Susko-Parrish, M. A. Winer, and N. L. First, "Capacitation of bovine sperm by heparin," *Biology of Reproduction*, vol. 38, no. 5, pp. 1171–1180, 1988.

[10] B. Marquant-Le Guienne, P. Humblot, M. Thibier, and C. Thibault, "Evaluation of bull semen fertility by homologous in vitro fertilization tests," *Reproduction Nutrition Development*, vol. 30, no. 2, pp. 259–266, 1990.

[11] T. G. Kroetsch and R. Stubbings, "Sire and insemination dose does effect in vitro fertilization of bovine oocytes," *Theriogenology*, vol. 37, p. 240, 1992.

[12] B. Marquant-Le Guienne, P. Humblot, N. Guillon, O. Gerard, and M. Thibier, "In vitro fertilization as a tool to evaluate fertility in the bovine," in *Proceedings of the 12th International Congress on Animal Reproduction*, pp. 662–664, The Hague, The Netherlands, August 1992.

[13] L. Vandaele, B. Mateusen, D. Maes, A. de Kruif, and A. Van Soom, "Is apoptosis in bovine in vitro produced embryos related to early developmental kinetics and in vivo bull fertility?" *Theriogenology*, vol. 65, no. 9, pp. 1691–1703, 2006.

[14] M. Anzar, L. He, M. M. Buhr, T. G. Kroetsch, and K. P. Pauls, "Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility," *Biology of Reproduction*, vol. 66, no. 2, pp. 354–360, 2002.

[15] A. Chaveiro, P. Santos, and F. M. da Silva, "Assessment of sperm apoptosis in cryopreserved bull semen after swim-up treatment: a flow cytometric study," *Reproduction in Domestic Animals*, vol. 42, no. 1, pp. 17–21, 2007.

[16] B. R. Zhang, B. Larsson, N. Lundeheim, and H. Rodriguez-Martinez, "Relationship between embryo development in vitro and 56-day nonreturn rates of cows inseminated with frozen-thawed semen from dairy bulls," *Theriogenology*, vol. 48, no. 2, pp. 221–231, 1997.

[17] F. Ward, P. Lonergan, D. Rizos, D. Corridan, and M. Boland, "Bull effects on kinetics of fertilization in vitro and the implications for subsequent embryo development," *Theriogenology*, vol. 55, p. 375, 2001.

[18] A. H. Walters, R. G. Saacke, R. E. Pearson, and F. C. Gwazdauskas, "The incidence of apoptosis after IVF with morphologically abnormal bovine spermatozoa," *Theriogenology*, vol. 64, no. 6, pp. 1404–1421, 2005.

[19] D. Rizos, F. Ward, P. Duffy, M. P. Boland, and P. Lonergan, "Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality," *Molecular Reproduction and Development*, vol. 61, no. 2, pp. 234–248, 2002.

[20] D. Sakkas, "The use of blastocyst culture to avoid inheritance of an abnormal paternal genome after ICSI," *Human Reproduction*, vol. 14, no. 1, pp. 4–5, 1999.

[21] T. A. L. Brevini Gandolfi and F. Gandolfi, "The maternal legacy to the embryo: cytoplasmic components and their effects on early development," *Theriogenology*, vol. 55, no. 6, pp. 1255–1276, 2001.

[22] A. N. Fatehi, M. M. Bevers, E. Schoevers, B. A. J. Roelen, B. Colenbrander, and B. M. Gadella, "DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages," *Journal of Andrology*, vol. 27, no. 2, pp. 176–188, 2006.

[23] S.-L. Weng, S. L. Taylor, M. Morshedi, et al., "Caspase activity and apoptotic markers in ejaculated human sperm," *Molecular Human Reproduction*, vol. 8, no. 11, pp. 984–991, 2002.

[24] G. Barroso, S. Taylor, M. Morshedi, F. Manzur, F. Gavino, and S. Oehninger, "Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations," *Fertility and Sterility*, vol. 85, no. 1, pp. 149–154, 2006.

[25] G. Martin, O. Sabido, P. Durand, and R. Levy, "Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction," *Human Reproduction*, vol. 20, no. 12, pp. 3459–3468, 2005.

[26] S. Grunewald, T. Baumann, U. Paasch, and H.-J. Glander, "Capacitation and acrosome reaction in nonapoptotic human spermatozoa," in *Annals of the New York Academy of Sciences*, vol. 1090, pp. 138–146, 2006.

[27] U. Paasch, S. Grunewald, G. Fitzl, and H. J. Glander, "Deterioration of plasma membrane is associated with activated caspases in human spermatozoa," *Journal of Andrology*, vol. 24, no. 2, pp. 246–252, 2003.

[28] U. Paasch, S. Grunewald, A. Agarwal, and H.-J. Glandera, "Activation pattern of caspases in human spermatozoa," *Fertility and Sterility*, vol. 81, supplement 1, pp. 802–809, 2004.

[29] G. Martin, N. Cagnon, O. Sabido, et al., "Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa," *Human Reproduction*, vol. 22, no. 2, pp. 380–388, 2007.

[30] S. Oehninger, M. Morshedi, S.-L. Weng, S. Taylor, H. Duran, and S. Beebe, "Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa," *Reproductive BioMedicine Online*, vol. 7, no. 4, pp. 469–476, 2003.

[31] X. Wang, R. K. Sharma, S. C. Sikka, A. J. Thomas Jr., T. Falcone, and A. Agarwal, "Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility," *Fertility and Sterility*, vol. 80, no. 3, pp. 531–535, 2003.

[32] U. Paasch, R. K. Sharma, A. K. Gupta, et al., "Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa," in *Biology of Reproduction*, vol. 71, no. 6, pp. 1828–1837, 2004.

[33] S. L. Taylor, S. L. Weng, P. Fox, et al., "Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality," *Molecular Human Reproduction*, vol. 10, no. 11, pp. 825–834, 2004.

[34] G. Martin, O. Sabido, P. Durand, and R. Levy, "Cryopreservation induces an apoptosis-like mechanism in bull sperm," *Biology of Reproduction*, vol. 71, no. 1, pp. 28–37, 2004.

[35] A. Januskauskas, A. Johannisson, and H. Rodriguez-Martinez, "Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility," *Theriogenology*, vol. 60, no. 4, pp. 743–758, 2003.

[36] D. A. Spandidos, E. Koumantakis, S. Sifakis, and G. Sourvinos, "Microsatellite mutations in spontaneously aborted embryos," *Fertility and Sterility*, vol. 70, no. 5, pp. 892–895, 1998.

[37] D. Nudell, M. Castillo, P. J. Turek, and R. R. Pera, "Increased frequency of mutations in DNA from infertile men with meiotic arrest," *Human Reproduction*, vol. 15, no. 6, pp. 1289–1294, 2000.

[38] D. Sakkas, G. Manicardi, D. Bizzaro, and P. G. Bianchi, "Possible consequences of performing intracytoplasmic sperm injection (ICSI) with sperm possessing nuclear DNA damage," *Human Fertility*, vol. 3, no. 1, pp. 26–30, 2000.

[39] B. Baccetti, G. Collodel, and P. Piomboni, "Apoptosis in human ejaculated sperm cells (notulae seminologicae 9)," *Journal of Submicroscopic Cytology and Pathology*, vol. 28, no. 4, pp. 587–596, 1996.

[40] S. Lopes, J. G. Sun, A. Jurisicova, J. Meriano, and R. F. Casper, "Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection," *Fertility and Sterility*, vol. 69, no. 3, pp. 528–532, 1998.

[41] A. Agarwal and T. M. Said, "Role of sperm chromatin abnormalities and DNA damage in male infertility," *Human Reproduction Update*, vol. 9, no. 4, pp. 331–345, 2003.

[42] N. K. Duru, M. Morshedi, A. Schuffner, and S. Oehninger, "Cryopreservation-thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine," *Fertility and Sterility*, vol. 75, no. 2, pp. 263–268, 2001.

[43] N. K. Duru, M. S. Morshedi, A. Schuffner, and S. Oehninger, "Cryopreservation-thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation," *Journal of Andrology*, vol. 22, no. 4, pp. 646–651, 2001.

[44] M. Muratori, M. Maggi, S. Spinelli, E. Filimberti, G. Forti, and E. Baldi, "Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation," *Journal of Andrology*, vol. 24, no. 2, pp. 253–262, 2003.

[45] L. N. Eid, S. F. Lorton, and J. J. Parrish, "Paternal influence on S-phase in the first cell cycle of the bovine embryo," *Biology of Reproduction*, vol. 51, no. 6, pp. 1232–1237, 1994.