Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 4 Issue 7 July 2022

Research Article

Integrity Analysis and Functional Sperm Assessments to Predict Boar Fertility Performance

Tainã F Cardoso[1], Antonio Sergio Varela Jr[2], Estela F Silva[1], Mariana B Jorge[2], Adalto Bianchini[2], Carine D Corcini[1,3]*

[1]Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, Brasil

[2]Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, Brasil

[3]ReproPel, Faculdade de Veterinária, Campus Capão do Leão, Universidade Federal de Pelotas (UFPel), Pelotas, RS, Brasil

***Corresponding Author:** Carine D Corcini, Programa de Pós-Graduação em Ciências Fisiológicas, Universidade Federal do Rio Grande (FURG), Rio Grande, RS, Brasil.

DOI: 10.31080/ASVS.2022.04.0364

Received: January 17, 2022

Published: June 24, 2022

Abstract

Methods for estimating the *in vitro* fertility quality of a semen sample for use in artificial insemination have been investigated for a long time; however, for animals with high reproductive performance, these methods still have many shortcomings. Furthermore, there have been few reports on the peculiarities of swine sperm cells, especially regarding factors that influence fertility. Thus, some characteristics of sperm integrity and functionality may serve as indicators of reproductive performance in swine. The present study evaluated the morphophysiology of spermatozoa from swine with high and low in vivo farrowing rates. Animals with high fertility had higher mitochondrial functionality ($p < 0.05$), lower plasma membrane fluidity ($p < 0.01$) and higher total antioxidant capacity ($p < 0.01$). The last two parameters were positively correlated with in vivo fertility ($r = 0.77$, $p = 0.0003$; and $r = 0.63$, $p = 0.0049$; respectively). These findings indicate that certain parameters affect fertility and are potentially useful factors for evaluating semen and indicating the farrowing rate in swine.

Keywords: Plasma Membrane Fluidity; Sperm Physiology; Farrowing Rate

Introduction

Intrauterine insemination and synchronizing ovulation in pigs aim to reduce the sperm concentration in each insemination dose to satisfy a greater number of females, without compromising the size of litters. Until now, animal fertility has been determined based on a traditional semen analysis that considers parameters such as sperm motility, concentration and morphology [33]. However, fertility is a complex and multifactorial phenomenon, especially in animals of proven fertility and prolificacy; and the physiological differences in these animals are poorly understood [11,12].

The characteristics required for spermatozoa to successfully traverse the female reproductive tract and bind to and fertilize oocytes include oxidative state, ionic concentration and cellular properties, such as cell membrane integrity and fluidity as well as acrosome reaction and DNA integrity [8,9,21, 43]. In addition, these factors are good indicators of the physiological state of spermatozoa and can provide valuable information regarding male fertilizing capacity.

Basic knowledge of swine spermatozoa physiology still has numerous gaps, which are a major obstacle for improving techniques aimed at increasing production, especially when considering genetically selected animals and their use as a biological resource [5]. As noted in several studies, results with swine have been far less significant compared to those with other species [16,47]. Thus conventional evaluation procedures of ejaculation allows identify only males with significant quality of commitment [21], no impairment of litter size depends on the use of compensatory doses with high sperm concentration.

Therefore, it is crucial to understand the structural and functional characteristics of swine spermatozoa and their relationships with the fertilization process. For that reason, the present study aimed to evaluate the structural and functional characteristics of boar sperm to predict boar fertility performance.

Methods

Experimental design

A retrospective study was performed based on the farrowing rate of 1520 females inseminated by 20 males (76 females per 1 male). These females were from a commercial artificial insemination center (51°57'59"W, 29°30'07"S), and they were housed under the same environmental and sanitary conditions. Estrus detection was performed by the same trained professional in the presence of the male. Artificially insemination was performed in the intracervical region with a dose of semen containing 3 x 10^9 cells/ml from the rich fraction that had been diluted in Beltsville Thawing Solution (BTS). Two semen samples, collected in distinctive days, from these animals were analyzed for spermatozoa characteristics, and the relationships between sperm characteristics and fertility were tested *in vivo* (based in farrowing rates) and *in vitro* (using penetration testing in homologous oocytes). All the chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sampling

The use of biological material in the present study was approved by the Ethics Committee for Animal Use (CEUA) of the Federal University Rio Grande.

Two distinct ejaculates from each of twenty Landrace males with proven fertility based on routine monitoring in a commercial artificial insemination center (51°57'59"W; 29°30'07" S) were evaluated. Animals were fed twice daily and handled under the same environmental and sanitary conditions. Semen samples from the rich fraction were collected using the gloved hand method. Immediately after collection, semen was diluted in isothermal conditions (1:1 v/v) using BTS as described by Pursel and Johnson [40].

Sample processing

Diluted samples were transported to the Laboratory of Animal Reproduction (School of Veterinary Medicine, Federal University of Pelotas, Capão do Leão, Rio Grande do Sul, Brazil) under controlled temperature conditions (18 ± 2 ºC). For further processing, samples were submitted at 37°C for 10 min. Semen were evaluated for total sperm motility, all samples presented motility greater than 70%, and it were evaluated in terms of plasma membrane integrity and fluidity, acrosome integrity, mitochondrial functionality, DNA integrity, and reactive oxygen species concentration. Additionally, the *in vitro* penetration test was performed.

An aliquot of each sample was stored (-80 °C) for further analysis of total antioxidant capacity, intracellular ionic concentration, lipid peroxidation and protein carboxylation levels.

Sperm motility

Total sperm motility was analyzed in a 20µl aliquot that was diluted 1:10 in Dulbecco's phosphate-buffered saline (D-PBS) [45] (pH 7.2, mOsm 305). Samples were submitted at 37°C for 10 min and warmed glass slide (37°C) covered with a cover slip. Visual evaluations were performed using optical microscopy at high magnification (400x). All the samples were analyzed by a single trained examiner.

DNA integrity

Spermatozoa DNA integrity was determined based on the properties of the metachromatic probe acridine orange using the sperm chromatin structure assay (SCSA) [18]. Analyses were performed by flow cytometry as described below.

Membrane integrity

Plasma membrane integrity was analyzed based on permeability using a combination of propidium iodide (PI; 7.3 µM) and carboxy fluorescein diacetate (CFDA; 20µM). The working solution used for semen staining contained D- PBS, Hoechst 33342 (16.2 µM), CFDA (50 µM) and PI (7.3 µM). Sperm cells were classified as normal/non-damaged (CFDA+/PI-) or nonviable/dead (CFDA+/PI+; CFDA-/IP+; CFDA-/IP-). Analyses were performed by flow cytometry as described below. Data are presented as the percentage of spermatozoa in each category [19,22].

Plasma membrane fluidity

Plasma membrane fluidity was analyzed using the hydrophobic fluorescent dye merocyanine 540 (M540), which binds preferentially to lipid membranes that are loosely bound due to increased plasma membrane permeability. Samples were prepared in PBS containing M540 (2.7 mM stock solution) and Hoechst 33342 (16.2 µM). Cells were sorted based on high versus low fluorescence. Analyses were performed by flow cytometry as described below. Data are presented as the percentage of spermatozoa in each category [19,38].

Acrosome reaction

Acrosome reaction was estimated using Arachis hypogaea agglutinin lectin (PNA) coupled to fluorescein isothiocyanate (FITC). Samples were prepared in PBS containing PNA-FITC (20 µM), PI (7.5 µM) and Hoechst 33342 (16.2 µM) [19]. Sperm cells were classified as having unreacted acrosomes (PNA-/PI-) or reacted acrosomes (PNA+/PI-; PNA-/PI+ and PNA+/PI+). Analyses were performed by flow cytometry as described below. Data are presented as the percentage of spermatozoa in each category.

Mitochondrial functionality

Spermatozoa mitochondrial functionality was evaluated using Rhodamine 123. This dye is easily sequestered in mitochondria and emits intense green fluorescence in active mitochondria (mitochondrial membranes with higher potential). Samples were prepared in PBS containing Rhodamine 123 (13.0µM) and Hoechst 33342 (16.2 µM) [22]. Analyses were performed by flow cytometry as described below.

Reactive oxygen species (ROS)concentration

The ROS concentration was measured using 2',7'-dichlorofluorescein diacetate (H_2DCFDA), which is oxidized to dichlorofluorescein (DCF) in the presence of ROS, thus emitting fluorescence (excitation: 488 nm; emission: 530 nm). Samples were prepared in PBS containing H_2DCFDA (1 µM) and Hoechst 33342 (16.2 µM) [17]. The median intensity of green fluorescence was used for the analysis. Analyses were performed by flow cytometry as described below.

Flow cytometry

All the samples were incubated for 30 min with the fluorochrome-containing solutions at 37 ºC; thereafter, the samples were diluted to a concentration of 10^7 with modified D-PBS (calcium-free) for the analysis. Analyses were performed using an Attune Acoustic Focusing Flow Cytometer (Life Technologies). The green fluorescence emitted by FITC, H_2DCFDA, Rhodamine 123, carboxy fluorescein and the metachromatic probe acridine orange was read using the BL1 photodetector (530/30 nm filter). The orange fluorescence from the merocyanine 549 metachromatic probe acridine orange was read using the BL2 photodetector (574/26 nm filter). The red fluorescence of PI was read using the BL3photodetector (640 LP filter). Fluorescence data are presented on a log scale. Data were analyzed using Attune Cytometer version 2.1.0 (Life Technologies). Ten thousand events were collected per sample at a flow rate of 200 cells/s using forward and side light scatter to exclude debris and aggregates. An analysis of one sample of cells stained with Hoechst 33342 was performed using the VL1 photodetector (450/40 nm filter). Non-sperm events (debris) were eliminated based on the scatter plots [38,39]. The matrix compensation voltages were as follows: direct laser light incidence (FSC), 2.7; laser light perpendicular incidence (SSC), 3.6; BL1, 2.6; BL2, 2.2; BL3, 2.6; and VL1, 1.2.

Oocyte penetration test

In vitro fertility was analyzed using oocytes from ovaries of prepubertal gilts collected in a local abattoir. Ovaries were placed in an isothermal bottle and transported to the laboratory, where they were punctured using a vacuum device (Aspire Max). Material was frozen at -20° C and 30 oocytes per dose were analyzed as described by Corcini., *et al.* (2011). After, it were visualized under an epifluorescence microscope (Olympus BX 103 51, América INC, São Paulo - Brasil) using violet light. The number of spermatozoa per oocyte and the number of penetrated oocytes (*in vitro* penetration rate) were assessed.

Total antioxidant capacity

Sperm cells were separated from the seminal plasma and extender by centrifugation (8000x g for 10 min). Total antioxidant capacity against peroxide radicals generated by thermal decomposition of 2,2'-azobis (2- methylpropionamidine) dihydrochloride (ABAP) at 37°C was determined as described by Amado [4]. Fluorescence (excitation: 488 nm; emission: 529 nm) was measured using a fluorometer (Victor, Perkin-Elmer, USA).

Thiobarbituric acid reactive substances

The extent of lipid peroxidation was measured by quantifying thiobarbituric acid reactive substances (TBARS) according to the protocol described by Oakes and Van Der Kraak [37]. TBARS content was expressed as equivalents of malondialdehyde (MDA) in $3x10^6$ sperm cells/ml. Fluorescence (excitation: 515 nm; emission: 553 nm) was measured using a fluorometer (Victor, Perkin-Elmer, USA).

Protein carbonyl concentration

Protein oxidative damage was evaluated based on the concentration of carbonyl compounds. Samples were diluted to a concentration of $1x10^7$sperm cells/ml, and measurements were performed as described by Levine., *et al.* [29]. Absorbance readings were obtained at 370 nm, and data are presented as nmol carbonyl/10^7 cells.

Intracellular ionic concentration

Sperm cells were separated from the seminal plasma and extender by centrifugation (8000xgfor 10 min). Calcium, magnesium, potassium and sodium concentrations were measured by Atomic Absorption Spectroscopy in flame mode (AAS 932 Plus, GBC, Hampshire, IL, USA) as previously described [30].

Sperm morphology

The sperm morphology analysis consisted of verifying all changes in the acrosome, head, midpiece and tail. The semen was diluted 1:10 in saline formaldehyde (fixative) at equal temperatures. Sperm morphology analysis was performed using phase contrast microscopy at 1000x magnification with a drop of immersion oil on the slide. We evaluated a total of 200 cells per slide [13]. For the statistical analysis, we only considered the percentage of cells classified as having normal morphology.

Statistical analysis

Data are presented as the mean ± standard error (SE). Data normality and homogeneity of variances were checked using the Shapiro-Wilk test. Mean values of dependent variables with a normal distribution were subjected to analysis of variance (ANOVA) followed by Tukey's test. Non-normally distributed variables were analyzed by the Wilcoxon test. Correlation analyses were performed using Pearson and Spearman indexes for parametric and nonparametric data, respectively. All the analyses were performed using Statistix 9.0.

Results

In vivo farrowing rate analysis revealed two very distinct groups of males (Figure 1): males with high fertility (≥ 68%) and those with low fertility (< 48%). Showing a significant difference in farrowing rate ($P < 0.01$). Therefore, the animals were divided into two groups: HIGH and LOW group. No significant difference was observed in the two groups in the sperm penetration test in homologous oocytes (Table 1). The sperm penetration test in homologous oocytes is not adequately sensitive for identifying males of high and low fertility because the data from the two fertility groups did not differ statistically (Table 1). Additionally, no significant differences were found in reproductive characteristics that are influenced by the female and/or farm, such as the number of total births or the number of live births.

Figure 1: Quantile plot to in vivo farrowing rate, demonstrate two very distinct groups of males. Animals with HIGH fertility (≥ 68%) and animals with LOW fertility (< 48%).

	HIGH	LOW
iFR	81.6 ± 1.9[a] (74.0/91.9)	30.6 ± 4.9[b] (4.1/48.8)
PR	63.5 ± 8.3 (28.5/88.8)	63.1 ± 9.3 (28.7/100.0)
SPO	1.9 ± 0.5	1.6 ± 0.4
NTB	13.1 ± 0.5	13.3 ± 0.7
NLB	12.5 ± 0.4	12.7 ± 0.7

Table 1: *In vivo* farrowing rate (iFR), *in vitro* penetration rate (PR), and number of spermatozoa per oocyte (SPO), number of total births (NTB) and number of live births (NLB) in Landrace pigs showing high and low farrowing rate. Data are expressed as mean ± SE (n = 10. Minimum and maximum values for iFR and PR are shown within parenthesis.

*Different letters in a same category indicate statistical differences between groups (Wilcoxon test; $P < 0.01$).

Pigs with a high *in vivo* fertility rate had sperm cells with lower plasma membrane fluidity ($P < 0.01$) and higher mitochondrial functionality ($P < 0.05$) than those with a low *in vivo* fertility rate (Table 2). No significant differences were observed in sperm motility, cell membrane integrity, acrosome reaction and sperm morphology between the groups of pigs (Table 2). In the same manner as no significant difference in ionic concentration was observed between the two groups of pigs (Table 3).

Regarding the oxidative stress parameters (ROS concentration and oxidative damage to lipids, proteins and DNA), no significant differences were observed between the high and low fertility rate groups. However, sperm from pigs with a high *in vivo* fertility rate exhibited a higher total antioxidant capacity against peroxyl radicals than that from pigs with a low *in vivo* farrowing rate ($P < 0.01$) (Table 4).

	High	Low
SM	72.2 ± 3.2	73.3 ± 2.3
PMI	66.8 ± 9.2	50.6 ± 10.9
NFPM	37.4 ± 6.5*	21.0 ± 2.6
ACR	20.5 ± 3.6	25.2 ± 3.7
MIF	46.7 ± 9.7*	18.3 ± 4.1
MORF	92.7±6.2	97.8 ± 2.2

Table 2: Sperm motility (SM), plasma membrane integrity (PMI), non-fluidity of plasma membrane (NFPM), acrosome reaction (ACR), mitochondrial function (MIF) and sperm morphology (percentage of normal cells) in sperm cells of Landrace pigs showing high and low farrowing rate (FR). Data are expressed as percentage of the total cells analyzed and are represented as mean values ± SE (n = 10). *Different letters in a same category indicate statistical differences between groups (Wilcoxon test; NFPM: $P < 0.01$; FMI: $P < 0.05$).

	High	Low
ROS	45.6 ± 5.4	41.9 ± 4.3
ACAP	$5.5 \times 10^6 \pm 2.8 \times 10^5$*	$4.6 \times 10^6 \pm 1.8 \times 10^5$
LPO	$1.1 \times 10^{-2} \pm 1.8 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 1.6 \times 10^{-3}$
PC	1.5 ± 0.4	1.2 ± 0.5

Table 3: Reactive oxygen species (ROS) concentration, total antioxidant capacity against peroxyl radicals (ACAP), lipid peroxidation (LPO), protein carbonylation (PC) and DNA integrity (DNAi) in sperm cells of Landrace pigs showing high and low *in vivo* fertility rate (FR). ROS and ACAP value is expressed as fluorescence units/10^7 cells. LPO and PC values are expressed as nmol of TMP equivalents/3 x 10^6 cells and nmol/10^7 cells, respectively. DNAi value is expressed as percentage of the total cells analyzed. Data are means ± SE (n = 10).

*Different letters in a same category indicate statistical differences between groups (ANOVA; $P < 0.01$)

	Na^+	Mg^{2+}	K^+	Ca^{2+}
High	150.6 ± 26.5	1.6 ± 0.3	2.2 ± 0.3	36.4 ± 7.9
Low	149.7 ± 28.5	1.5 ± 0.2	2.9 ± 0.5	39.8 ± 8.7

Table 4: Ion concentrationin sperm cells of Landrace pigs showing high and low farrowing rate (FR). Values are expressed as µg/ml. Data are expressed as mean values ± SE (n = 10). No significant difference was observed between pig groups.

Positive correlations (Figure 2) were found between total antioxidant capacity against peroxyl radicals and the percentage of cells with a plasma membrane that lacked fluidity ($r = 0.63$; $p = 0.0049$) and the *in vivo* fertility rate ($r = 0.77$; $p = 0.0003$).

Figure 2: Correlation between in vivo farrowing rate and non-fluidity of the plasma membrane (A) and total antioxidant capacity against peroxyl radicals (B) in sperm cells of Landrace pigs.

Discussion

Based on the *in vivo* farrowing rate of Landrace pigs belonging to a commercial stock, two distinct groups of animals were identified. The high fertility group of pigs had an *in vivo* fertility rate ranging from 74 to 91%. Thus, a fertility rate ≥ 70% can be an indicative parameter for the more precise selection of high-performance animals. In this context, the penetration test of sperm into homolo-

gous oocytes, which can be used to predict male fertility, was not sensitive in identifying males with high and low farrowing rates in the present study. *In vitro* penetration rates and the number of sperm per oocyte, using cooled semen (17 °C) for a maximum of 6 h, were not able to predict the *in vivo* fertility of swine in this study.

Analyses of characteristics such as sperm motility and sperm morphology are currently used to obtain information on the spermatogenesis status and fertilization potential of different animal species (CBRA, 1998). However, as shown in the present study (Table 2), these tests are often not sufficiently sensitive, and they do not enable an assessment of the actual spermatozoa fertilization ability [15,33].

Recently, structural and functional analyses have been employed to recognize specific structures of cells, and many correlations with fertility have been identified. However, many differences have been observed among species, and only a few studies on swine have been reported [34,36,42]. In the present study, sperm cells from pigs with a high *in vivo* fertility rate had lower plasma membrane fluidity than those from pigs with a low *in vivo* fertility rate (Table 2). However, sperm cells from less fertile pigs exhibited plasma membrane integrity despite higher fluidity. This is a characteristic related to the sperm capacitating process that occurs before entering the female reproductive tract [44]. The increase in membrane fluidity due to cholesterol efflux from the plasma membrane is a physiological phenomenon that must occur in a chronologically and spatially controlled sequence [25]. Dyssynchrony between this event and fertilization can interfere with selective permeability and other events in sperm cells. This can negatively affect the fertilizing capacity and metabolism. In fact, pigs with a low farrowing rate had sperm cells with higher membrane fluidity.

Sperm cells from pigs with a high *in vivo* fertility rate had greater mitochondrial functionality than those from less fertile pigs (Table 2). Some studies have described a correlation between low mitochondrial functionality and reduced fertility [23]. In fact, a reduction in mitochondrial functionality is related to a decrease in the cellular metabolic state [31]. In addition, this reduction usually parallels ROS production, which contributes to cell damage and death [27]. However, the sperm cells from less fertile pigs that were evaluated in the present study did not show increased ROS levels compared to those from more fertile pigs. Thus, the physiological difference responsible for the observed increased fertilization rate could be the increased mitochondrial functionality associated with augmented cellular metabolism that is necessary for the fertilization events.

In the analysis of oxidative stress parameters in the present study, no significant differences were observed between the two groups of pigs regarding ROS concentration and oxidative damage to lipids, proteins (carbonyl groups) and DNA (Table 3); these findings are discordant with the results obtained by Broekhuijse., *et al.* [10] and are not in agreement with certain studies that reported inferences on the relationship between infertility and oxidative stress [1,7,28]. However, these factors have not been reported for animals with associated genetic selection and of proven fertility, such as those used in the present study. Therefore, ROS and oxidative damage seem not to be associated with reduced fertility in these animals. However, it is important to note that sperm cells from pigs with higher fertility had a higher total antioxidant capacity against peroxyl radicals and increased mitochondrial activity (Table 3). These findings corroborate the idea that sperm cells from more fertile pigs have increased cellular metabolism [20,35]. Notably, such gametes are able to scavenge generated ROS and avoid the associated oxidative damage.

In general, spermatozoa have a lower antioxidant capacity than other cell types. In fact, these cells lose most of their cytoplasm during the terminal phase of spermatogenesis, thus losing many antioxidant compounds [24]. Therefore, cells with a higher fertilizing capacity are likely able to maintain a greater amount of cytoplasmic components during this process. This can significantly reduce the adverse effects of ROS produced by the rapid increase in metabolism.

Ion concentrations were similar in sperm cells from both groups of pigs (Table 4). The values reported in the present study are consistent with those reported in the literature for other animal species, especially the calcium and magnesium concentrations [46]. Sodium and potassium concentrations are primarily associated with Na^+/K^+-ATPase activity (Jimenez., *et al.* 2010), which varies among species [41]. However, our results are similar to those reported for spermatozoa of other mammalian species, including humans. In this case, there is a higher intracellular concentration of sodium than of potassium [2,41]. Intracellular Ionic balances

are possibly related to the degree of sperm motility and migration time. The required plasma membrane excitability is enabled by the influx of Na^+ into the cell and the efflux of K^+ from the cytosol. This increased frequency of action potential generation can be justified by the need for cells to traverse from the site of semen deposition to the site where fertilization occurs [3,6,32].

Finally, positive correlations were identified between the total antioxidant capacity against peroxyl radicals and the number of cells with lower plasma membrane fluidity as well as the *in vivo* farrowing rate. These correlations might be associated with a greater mobilization of compounds responsible for ROS scavenging in cells with higher metabolism, indicated by the observed higher mitochondrial functionality, to prevent oxidative stress. In addition, this situation might be associated with the lower membrane fluidity in these cells. In fact, these cells are potentially able to undergo cellular changes in a synchronized manner, i.e., increasing membrane fluidity only when confronting female gametes and not earlier, making them more suitable for fertilization. Thus far, a relationship between fertility and these characteristics has not been reported in swine. This is quite interesting when considering the possible use of these laboratory tests to predict *in vivo* fertility.

Conclusions

In conclusion, swine spermatozoa show morphological and physiological differences related to *in vivo* fertilizing capacity. Therefore, practices currently recommended for differentiating animal fertility, such as sperm motility and sperm morphology, are no longer sufficient for more precise selection. The present study is the first to relate parameters such as increased mitochondrial functionality, reduced membrane fluidity and higher total antioxidant capacity with the higher fertilizing capacity of swine spermatozoa. In fact, these parameters can be used as biochemical markers to evaluate the quality of semen for AI.

Competing Interests

The author(s) declare that they have no competing interest

Acknowledgments

The present study was financially supported by the Brazilian "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES, Brasília, DF, Brazil). A. Bianchini and C.D. Corcini are research fellows a tthe Brazilian "Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil). The authors also thank the members of the Comparative Animal Reproduction Research Team (Universidade Federal do Rio Grande, Rio Grande, RS, Brazil) for their assistance.

Bibliography

1. Agarwal A., *et al*. "Proteomics, oxidative stress and male infertility". *Reproductive BioMedicine Online* 29 (2014): 32-58.

2. Akpa GN., *et al*. "Relationships between Body and Scrotal Measurements, and Semen characteristics in Yankasa ram". *Journal of Veterinary Science* 4.1 (2012): 7-10.

3. Alavi SMH., *et al*. "Spermatozoa motility in the Persian sturgeon, Acipenserpersicus: effects of pH, dilution rate, ions and osmolality". *Reproduction* 128 (2004): 819-828.

4. Amado LL., *et al*. "A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity". *Science of the Total Environment* 407 (2009): 2115-2123.

5. Antunes RC. "Avanço tecnológico e aplicabilidade da técnica de congelamento de sêmen suíno". *Revista Brasileira de Reprodução Animal* 31 (2007): 60-63.

6. Behan JR., *et al*. "A field investigation of intra-cervical insemination with reduced sperm numbers in gilts". *Theriogenology* 66 (2006): 338-343.

7. Benedetti S., *et al*. "Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality". *Reproductive BioMedicine Online* 25 (2012): 300-306.

8. Birck A., *et al*. "*In vitro* induction of the acrosome reaction in bull sperm and the relationship to field fertility using low-dose inseminations". *Theriogenology* 73 (2010): 1180-1191.

9. Broekhuijse MLWJ., *et al*. "Field data analysis of boar semen quality". *Reproduction in Domestic Animals* 46 (2011): 59-63.

10. Broekhuijse MLWJ., *et al*. "Relationship of flow cytometric sperm integrity assessments with boar fertility performance under optimized field conditions". *Journal of Animal Science* 90 (2012): 4327-4336.

11. Broekhuijse MLWJ., *et al*. "The value of microscopic semen motility assessment at collection for a commercial artificial insemination centre, a retrospective study on factors explaining variation in pig fertility". *Theriogenology* 77 (2012a): 1466-1479.

12. Broekhuijse MLWJ., *et al*. "Application of computer-assisted semen analysis to explain variations in pig fertility". *Journal of Animal Science* 90 (2012b): 779-789.

13. CBRA. Colégio Brasileiro de Reprodução Animal. "Manual para exame andrológico e avaliação de sêmen animal". 2nd edition. Belo Horizonte (1998).

14. Corcini CD., *et al*. "*In vitro* assays for vesper mice (calomys-laucha) sperm using coincubation and oocyte's cryopreservation". *Journal of Experimental Zoology* 154 (2012): 96-102.

15. Correa JR., *et al*. "Relationships among frozen-thawed sperm characteristics assessed via the rotine semen analysis, sperm functional tests and fertility of bulls in artificial insemination program". *Theriogenology* 48 (1997): 721-731.

16. De Ambrogi M., *et al*. "Viability and DNA fragmentation in differently sorted boar spermatozoa". *Theriogenology* 66 (2006): 1994-2000.

17. Domínguez-Rebolledo AE., *et al*. "Response of thawed epididymal red deer spermatozoa to increasing concentrations of hydrogen peroxide, and importance of individual male variability". *Reproduction in Domestic Animals* 46 (2011): 393-403.

18. Evenson DP., *et al*. "Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility". *Theriogenology* 41 (1994): 637-651.

19. Fernández-Gago R., *et al*. "Seminal plasma applied post-thawing affects boar sperm physiology: A flow cytometry study". *Theriogenology* 80 (2013): 400-410.

20. Ferreira ALA and Matsubara LS. "Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estressee oxidativo". *Revista da Associação Médica Brasileira* 43 (1997): 1-16.

21. Gadella BM., *et al*. "Capacitation and the acrosome reaction in equine sperm". *Animal Reproduction Science* 68 (2001): 249-265.

22. Gillan L., *et al*. "Flow cytometric evaluation of sperm parameters in relation to fertility potential". *Theriogenology* 63 (2005): 445-457.

23. Graham JK. "Assessment of sperm quality: a flow cytometric approach". *Animal Reproduction Science* 68 (2001): 239-247.

24. Hu JH., *et al*. "Effect of GynostemmaPentaphyllum Polysaccharide on boar spermatozoa quality following freezing-thawing". *Cryobiology* 59 (2009): 244-249.

25. Hurtado LA., *et al*. "AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa". *Biochimica et Biophysica Acta* 1828 (2013): 2143-2151.

26. Jimenez T., *et al*. "Na,K-ATPase alpha4 isoform is essential for sperm fertility". *Proceedings of the National Academy of Sciences of the United States of America* 11 (2010): 644-649.

27. Kroemer G., *et al*. "Mitochondrial control of apoptosis". *Immunology Today* 18 (1997): 44-51.

28. Lanzafame FM., *et al*. "Oxidative stress and medical antioxidant treatment in male infertility". *Reproductive BioMedicine Online* 19 (2009): 638-659.

29. Levine RL., *et al*. "Determination of carbonyl content in oxidatively modified proteins". *Methods Enzymology* 186 (1990): 464-478.

30. Lopes TM., *et al*. "Effect of copper onion content in isolated mantle cells of the marine clam Mesodesmamactroides". *Environmental Toxicology and Chemistry* 30 (2011): 1582-1585.

31. Ly JD., *et al*. "The mitochondrial membrane potential (deltapsi (m)) in apoptosis; an update". *Apoptosis* 8 (2003): 115-128.

32. Martínez-López P., *et al*. "Mouse sperm K+ currents stimulated by pH and cAMP possibly coded by Slo3 channels". *Biochemical and Biophysical Research Communications* 381 (2009): 204-209.

33. Melo MIV., *et al*. "Teste hiposmótico para avaliação da viabilidade do sêmen equino resfriado com diferentes diluidores". *Arquivo brasileiro de medicina veterinária e zootecnia* 57 (2005): 757-763.

34. Ménézo Y., *et al*. "Stress oxydantetfertilité: faussesévidences et mauvaisesrecettes". *Gynécologie Obstétrique Fertilité* 4 (2012): 787-796.

35. Nordberg J and Arnér ESJ. "Reactive oxygen species, antioxidants and the mammalian thioredoxin system". *Free Radical Biology and Medicine* 31 (2001): 1287-1312.

36. Novak S., *et al*. "Biomarkers of *in vivo* fertility in sperm and seminal plasma of fertile stallions". *Theriogenology* 7 (2010): 956-967.

37. Oakes KD., *et al*. "Utility of the TBARS assay in detecting oxidative stress in white sucker (Catostomuscommersoni) populations exposed to pulp mill effluent". *Aquatic Toxicology* 63 (2003): 447-463.

38. Petrunkina AM., *et al*. "Functional significance of responsiveness to capacitating conditions in boar spermatozoa". *Theriogenology* 64 (2005): 1766-1782.

39. Piehler E., *et al*. "Dynamic quantification of the tyrosine phosphorylation of the sperm surface proteins during capacitation *in vitro"*. *Cytom* 69 (2005): 1062-1070.

40. Pursel VG and Johnson LA. "Freezing of boar spermatozoa: Freezing capacity with concentrated semen and a new thawing procedure". *Journal of Animal Science* 40 (1975): 99-102.

41. Quinn JP., *et al*. "Studies of the distribution of the major cations in semen and male accessory secretions". Journal of Reproduction and Infertility 10 (1965): 379-388.

42. Rajender S., *et al*. "Mitochondria, spermatogenesis and male infertility". *Mitochondrion* 10 (2010): 419-428.

43. Rodriguez-Martinez H and Larsson B. "Assessment of sperm fertilizing ability in farm animal". *Acta Agriculturae Scandinavica B* 29 (1998): 12-18.

44. Senger PL. "Pathways to pregnancy and parturition. 2nd edition". Pullman (WA): Current Conceptions (2003): 271.

45. Songsasen N and Leibo SP. "Cryopreservation of mouse spermatozoa: Effect of seeding on fertilizing ability of cryopreserved spermatozoa". *Cryobiology* 35 (1997): 240-254.

46. Valsa J., *et al*. "Split ejaculation study: semen parameters and calcium and magnesium in seminal plasma". *Central European Journal of Urology* 65 (2012): 216-218.

47. Watson PF. "The causes of reduced fertility with cryopreserved semen". *Animal Reproduction Science* 60.61 (2000): 481-492.