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A NEW MODEL OF BOAR SEMEN EVALUATION AND THE IMPACT OF CRYOGENIC FACTOR ON SPERMATIC CELLS

UN NOU MODEL DE EVALUARE A MATERIALULUI SEMINAL DE VIER SI IMPACTUL FACTORULUI CRIOGEN ASUPRA CELULELOR SPERMATICE

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Nowadays, sperm evaluation is mostly used to predict fertility and freezability. The aim of this study is to evaluate the possibility of investigating the effects of the cryogenic agent on boar spermatozoa, by identifying a set of laboratory tests for a rapid and efficient evaluation of semen quality. Usual sperm analysis such as sperm concentration, motility and spermatozoa morphology are not able to show subtle abnormalities, which are having a basic role in the fertilizing ability. Moreover, it seems that other sperm characteristics, involved in the fertilizing ability, can interfere with the freezing-thawing processes, being not evaluated or maybe not known. Morphological (microscopic analysis of stained spermatozoa), functional (motility analysis and hypo-osmotic swelling test) and chromatin integrity (Acridine Orange Test and Comet Assay) analysis were performed aiming to show the differences in spermatozoon integrity and functionality, caused by the cryogenic factor.
Keywords: sperm evaluation, freezability, DNA fragmentation.

Introduction

In swine, nevertheless, the use of frozen semen in artificial insemination and in vitro fertilization is quite limited. It is owed it, fundamentally because the capacity of the sperm is seriously affected by the cryopreservation process. There may be considerable differences between breeds and between males, in the 'freezability' and fertilization ability of the semen. As a consequence, frozen semen of some genetically interesting breeds or males may not be suitable as high quality resources, or can be used only with a poor efficiency. Sperm cryopreservation success depends on several factors, including the initial sperm quality, the cryopreservation protocol, and also the specific susceptibility of spermatic cells at thermal, mechanic and osmotic stress, during the freezing-thawing cycle (Fraser et. al., 2007); for such reasons a high sperm evaluation is required in cryopreservation processes. Nowadays, sperm evaluation is mostly used to predict fertility and cryopreservation pretability. Usual sperm analysis such as sperm concentration, motility and spermatozoa morphology are not able to show

subtle abnormalities, which are having a basic role in the fertilizing ability (Holt et. al., 2005), emphasizing the limits of the sperm evaluation tests (Baracaldo et. al., 2008). In order to increase the sperm evaluation efficiency, HOST (hypo-osmotic swelling test) analysis were performed for spermatic membrane functionality assay. Moreover, it seems that other sperm characteristics involved in the fertilizing ability can interfere in the freezing-thawing processes (Hermandez et. al., 2007), being not evaluated or maybe not known. Despite the maintaining of the membrane functionality and spermatozoa viability, genomic alterations can not be emphasized using such laboratory tests, DNA integrity analysis such as Comet Assay (Single Cell Gel Electrophoresis – SCGE) or AOT (Acridine Orange Test) being required. Genomic integrity is one of the main parameter recently studied, the maintaining of a high spermatic DNA integrity, during the storage period, present a major role for artificial inseminations and *in vitro* fecundation, as a high fragmentation level is interfering in fertilization and normal embryo development.

In the present study, for a high efficient sperm evaluation is taken into considerations the following analysis: usually spermogram, functional, morphological and genomic integrity evaluation.

Materials and Methods

BIOLOGICAL MATERIAL. Semen was collected using the manual method from Large White boars of the Didactic Farm- USAMV Cluj-Napoca. Diluted spermatic material 1:1 (negative control C-), frozen spermatic material with cryoprotector agent- glycerol 9% in LEY medium (cryopreserved C) and frozen spermatic material without cryoprotector agent (positive control C+) were used to study the cryogenic factor.

SPERM CONCENTRATION was determined using the photometric method (SDM 5 photometer).

SPERM MOTILITY was performed through classical microscopy assays (Karl-Zeiss, Peraval).

ALIVE SPERMATOZOA PERCENT DETERMINATION METHOD

Bishop-Smiles staining method was performed to establish the percent of alive sperm cells, based on acridine orange staining solution (0,1 g acridine orange / 100 ml 0,9% sodium chloride solution). The cells were analysed using 400X microscope objective, 200 spermatic cells being percentually reported to the alive/ dead cells (green fluorescent / yellow red color in fluorescent microscopy).

MORPHOLOGICAL INTEGRITY

Bloom assay (eosine- nigrosine)

Spermatic cells with abnormalities at head, midpiece or tail level and immature spermatozoa were analyzed at 400X and 1000X microscope objective (Microscope Karl-Zeiss Peraval), the abnormalities being percentually estimated.

Spermac assay is another method used to evaluate the acrosomal integrity. After centrifugation, the sperm was suspended in BTS. Three staining solutions (A

staining solution- red, B staining solution- light green, C staining solution- dark green) were used prior to smears microscopic analisys at 1000X.

FUNCTIONAL INTEGRITY

HOST test was used for functional integrity determination. After sperm centrifugation the cells were suspended in BTS and than re-suspended in the hypo-osmotic solution (0,375 g sodium citrate, 1.35 g fructose). the samples were incubated one hour at 37º C. The microscopic analysis were performed at 400X, the coiled spermatozoa were classified as hypo-osmotic positives (normal functional membrane integrity).

GENOMIC INTEGRITY

AOT assay

For chromatin integrity TAO method was performed. The spermatic material was centrifuged and the cells were washed in PBS solution. The smears were incubated over night at room temperature in the fixing solution (methanol – glacial acetic acid 3:1). Than the smears were immersed in the acridine orange staining solution (10 mg AO, 0,8405 g citric acid, 0,2686 g $NaHPO_4$). The spermatic cells were analyzed using the fluorescent microscopy at 400X. The spermatozoa presenting a normal DNA integrity were green colored and the cells with a decreased genomic integrity orange-red colored.

Comet assay

Sperm cells were washed with Ca^{2+} and Mg^{2+}- PBS free, to yield a concentration of 1×10^7 spermatozoa/cm^3. Aliquots of the same sperm samples, pre-treated with 2% beta-mercaptoethanol and washed in PBS, were mixed with 0.5% low-melting point agarose and placed onto frosted microscope slides pre-coated with 0.75% normal-melting point agarose. A final layer of 0.5% low-melting point agarose was applied, the slides were immersed in ice-cold lysis solution (3,656 g Na Cl, 0,930 g EDTA, 0,0394 g Tris-HCl, 500 µl Triton X and 2,5 ml DMSO) and incubated for 1 h at 4°C. Then the slides were treated with RN-ase buffer (NaCl 6,375 g, Tris- HCl 0,687 g, SDS 0,05 g, RN-ase 500 µl) and incubated for 4 h at 37°C. Thereafter, slides were transferred to proteinase K buffer (NaCl 6,375 g, Tris- HCl 0,687 g, SDS 0,05 g, proteinase K 100 mg) and left overnight at 37°C. Slides were equilibrated in an electrophoresis solution (acetat de Na 24,61g Tris HCl 15,75g) before being migrated in an horizontal electrophoresis system at 19 V , 100 mA for 1 h at room temperature. The slides were drained, flooded slowly with three changes of neutralization buffer (0.4 M Tris, pH 7.4), fixed in 70 % ethanol for 15 minutes and then air dried. The dried slides were stained with ethidium bromide (20 µg/ml). Spermatozoa analyzed for comets were visualized using the fluorescence microscopy 590 nm filter, 400X. Whole sperm heads, without a comet, were not damaged, whereas spermatozoa with fragmented DNA that migrated from the sperm head, causing a "comet" pattern, were considered damaged. A total of about 100 sperm cells per slide were assessed for comets.

STATISTICAL ANALYSIS

The obtained results were analyzed using ANOVA and multiple comparisons tests Tukey - Kramer (Tukey - Kramer Multiple Comparisons Test). All the analysis were performed using GraphPad InStat software.

Results and Discussion

Usual spermogram

The usual spermogram performed consisted in volume, agglutination, concentration and sperm motility determination.

Table 1.

Evaluated parameters in usual spermogram using fresh semen

Breed	Evaluated parameters($\overline{X} \pm s_{\overline{X}}$)			
	volume (ml)	concentration ($1x10^9$)	motility (%)	agglutination
Large White	198 ± 13.92	0.346 ± 0.11	72 ± 1.00	8.0 ± 1.22

The average values obtained are comprised into the specific limits of the specie, according to the required standard for AI practice.

Morphological integrity

The results obtained in morphological integrity analysis are showing the negative influence of the cryogenic factor on sperm cells, affecting the viability and the percent of spermatozoa abnormalities.

Table 2.

Percentual results obtained by morphological integrity evaluation

Experimental variants	AO	E-N					Spermac
	Alive ($\overline{X} \pm s_{\overline{X}}$)	Abnormalities ($\overline{X} \pm s_{\overline{X}}$)				Immature ($\overline{X} \pm s_{\overline{X}}$)	Intact acrosom ($\overline{X} \pm s_{\overline{X}}$)
		Head	Intermediate piece	Main piece	Terminal piece		
C-	97 ± 2.12	3.6 ± 1.14	1.4 ± 0.54	2.2 ± 0.44	1.8 ± 0.44	2.0 ± 1.58	95.8 ± 1.78
C	34.2 ± 5.45[a]	4.4 ± 2.51	1.8 ± 0.83	2.2 ± 0.83	2.8 ± 0.44	2.0 ± 1.22	67.2 ± 4.55[a]
C+	2 ± 2.12[b]	4.8 ± 0.83	1.6 ± 0.89	2.0 ± 0.70	3.0 ± 1.22	1.8 ± 1.30	45.2 ± 4.91[b]

*Differences between any variant followed by at least one common character are insignificant.

If in C- blank (fresh semen) a 97% of alive spermatozoa was established using Bishop Smiles staining method, in C+ (frozen semen without cryoprotector agent) the percent was only of 2%.

The acrosomal integrity is one of the main parameters for a high efficient morphological evaluation. Assays for acrosome and plasmatic membrane integrity represent good sperm quality indicators. The cryopreservation process, decrease the acrosomal integrity while at the other morphological pieces significant abnormalities were not signaled.

Functional integrity

Infertility is in general defined as a lower sperm concentration of 20 x 10^6 spermatozoa/ml, less of 50% presenting a straight line motility and less of 60% morphological integrity.

The most important fertility mechanisms, like capacity, acrosomal reaction, and sperm oocyte binding, are considered to depend on the spermatozoa membrane functional integrity (Tartagni et. al., 2002).

One of the best and efficient tests for the functional integrity is HOST (hipo-osmotic swelling test).

Table 3.

Percentual results obtained by functional integrity evaluation

Experimental variants	Motility ($\overline{X} \pm s_{\overline{X}}$)	HOST + ($\overline{X} \pm s_{\overline{X}}$)
C-	86 ± 5.47	36.4 ± 4.03
C	11 ± 2.23[a]	3.2 ± 1.30[a]
C+	0 ± 0.00[b]	0.2 ± 0.44[a]

*Differences between any variant followed by at least one common character are insignificant

HOST test was used in this study as routine tests like: motility, percent of intact spermatozoa regarding the acrosom or viability, are showing only the actual status of spermatozoa, but can not be used for correlation with the fertility results.

Considering the data shown in table 3, it is obviously that even having a 80% motility in fresh sperm, only 36,4% are presenting a high functional membrane integrity. An ejaculate classified as "good" related its motility, can be considered just medium or low from the membrane functionality point of view, motility, that at the beginning can offer wrong information regarding the evaluation and the fertilizing abilities of the boar. A low membrane functionality, represents one of the main parameter for decreased motility after freezing-thawing processes (from 80% to 11% in case of C and to 0% in the case of C+).

The data obtained for the functionality evaluation showed a negative effect of freezing-thawing processes on spermatozoa motility. Besides motility, the cryogenic factor decreased the number of spermatozoa with a good functional integrity of the membrane at the beginning, interfering in the fertilization success.

In 2003, B. Perez-Llano et. al., showed that HOST positive spermatic cells are characterized by a lower percent of acrosomal abnormalities comparing with those presenting a HOST negative reaction.

Chromatin integrity

One of the negative characteristics affected during this freezing-thawing processes of boar spermatozoa is genomic integrity (Hernandez et. al., 2007).

In 2006, Erenpreiss et. al., recommended chromatin integrity analysis as independent complementary parameter for a better sperm quality evaluation.

Boar spermatozoids present a highly condensed chromatin which contains protamine, that protect the haploid DNA (Cordova et. al, 2002). The optimum DNA packing demonstrated to be the very important for the expression of the male

fertilisation potential; spermatozoa resulted from dysfunctional spermatogenesis are presenting dispersions at nuclear chromatin level, related the single stranded DNA (Tarozzi et. al.,2007).

Table 4.

Spermatozoa number presenting dispersed chromatin

Experimental variants	Number of analized spermatozoa	TAO	Number of analized spermatozoa	Comet assay
C-	500	30	1000	2
C	500	135	1100	11
C+	500	182	1000	14

On the present paper, the results showed in Table 4, indicate the chromatin dispersion and spermatic DNA fragmentation, after spermatozoa exposure to the cryogenic environment. The tests performed on spermatic DNA integrity, proved as in other studies, that fresh semen (depending at individual level) could present genomic fragmentation. This could be the result of an altered spermatogenesis process.

As almost all the protocols found on literature regarding Neutral Comet Assay test contained Sodium Lauryl Sarcosine in lysis buffer, and for objective reasons we had no capacities to procure it, we tried to find an alternative lysis buffer with the same action; we have tested more than 15 solution, obtaining satisfying results using the solution containing: NaCl, EDTA, Tris-HCl, 10% DMSO, 2% Triton X. This could explain the differences between the two methods used for genomic integrity assays. The addition of egg yolk to the cryopreservation medium, decrease the chromatin dispersion during the freezing-thawing cycles.

Recent studies shown that the oxidative stress (ROS-reactive oxigen species) have a high impact on spermatic DNA fragmentation. The spermatozoa have only two mechanisms against the oxidative stress, which can modify the DNA integrity - DNA packing system and seminal plasma composition.

The acridine-orange test (AOT) demonstrated that the freezing-thawing cycles caused negative changes in the spermatozoa chromatin structure. The number of spermatozoa having an non-dispersed chromatin was 470 for C-, decreasing at 365 for C, and 318 for **C+**. So, these results obtained suggest that the cryoprotector medium is not the only one having an impact on normal chromatin structure maintaining, other aspects being involved. The DNA integrity of sperm represents an important tool and a necessary condition for obtaining good reproductive results, its stability being definitely required for spermatic cells viability.

Conclusions

In this paper a new model of swine semen evaluation was developed referring also to the new trends of the genetics applied in advanced reproduction laboratory techniques, by performing semen evaluation methods completed inclusively by DNA sperm analysis.

The present study showed a specific influence of the freezing-thawing cycle on the morphologic, acrosom, functionality and genomic integrity.

The results obtained by performing HOST analysis indicated that HOST positive spermatozoa are presenting a resistant and functional plasmatic membrane, both at acrosom and tail region.

Classical eosin negrosin assays should be completed by acrosom intergity analysis such as SPERMAC, for a high efficiency evaluation.

Further studies are required to elucidate the impact of the cryogenic factor on boar sperm chromatin integrity; the potential results could solve the issues regarding the chromatin integrity status, giving an answer regarding the cryoprotective effect of egg yolk lipoproteins , optimum cryopreservation protocol, influence of the oxidative stress, considering the problems of boar semen cryopreservation and evaluation methods.

The above semen evaluation strategy could serve as a model to be applied in another problematic species regarding its freezability and prediction of fertilization ability, too.

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