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Changes in subpopulations of boar sperm defined according to viability and plasma and acrosome membrane status observed during storage at 15 °C

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Abstract

Four boar ejaculates were preserved for 42 d at 15 °C to examine the changes produced in the quality of sperm membranes according to their response to a combined short hypoosmotic swelling test (sHOST) plus viability test designated the sHV test. Every 1 or 2 d, a sample from each ejaculate preserved in long-term extender was subjected to sperm motility determination and the sHV test. Through simultaneous examination by phase contrast and fluorescence microscopy, three subpopulations of sperm were identified according to their response to sHOST challenge and their viability status. In the subpopulations scoring positive in the sHOST, a further four sperm subpopulations were defined according to their viability and acrosome status. All the sperm subpopulations differed in terms of changes in their proportions produced during the course of preservation and individual differences among ejaculates were detected in terms of relationships shown among subpopulations. The combined sHOST/viability test was able to identify sperm subpopulations with the strongest plasma and acrosome membranes as well as a subpopulation of sperm that had undergone a true acrosome reaction.
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1. Introduction

The sperm has the highly specialized function of fertilizing the oocyte. To successfully achieve this, the sperm must be structurally intact and functionally active. Each cellular component, the head, intermediate piece, flagellum and DNA content, play a crucial role in sperm function. The plasma membrane protects and makes contact with all these structures and the acrosomal membrane protects the enzyme contents of the acrosome. Accordingly, the condition of both the plasma membrane and the acrosomal membrane is a good measure of the quality of the sperm in a given sample.

Several tests have been designed to assess the state of boar sperm plasma membranes. These tests range from the simplest hypoosmotic swelling test (HOST) [1] and the exclusion of impermeable dyes such as eosin by intact cells to more sophisticated techniques based on fluorescent stains [2,3].

When designing a sperm quality test, a major goal is to obtain data that will predict sperm fertility. However, because of the complexity of the sperm, most sperm tests developed to date cannot be correlated with

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fertility results. It therefore seems that several other factors not related directly to the sperm have an influence on the final fertility results.

These unknown factors are likely to act in every fertility trial and only through regression analysis of the combined results of several quality tests [4] or by using stress tests such as osmotic resistance test (ORT) [5], HOST [1] and short hypoosmotic swelling test (sHOST) [6], has it been possible to observe some measure of correlation with sperm fertility results [1,5,6]. This is because these stress tests challenge the resistance of sperm structures and are able to select the resistant sperm subpopulations, better than the evaluation of the sperm motility or the acrosomal status.

Given our ample experience with sHOST and viability tests on boar sperm, the aim of this study was to develop a new combined test capable of providing information on both sperm quality and functionality.

2. Materials and methods

The study was performed on four ejaculates obtained from four Pietrain boars aged 3 years. The boars were housed in individual stalls, fed once a day and provided water *ad libitum*. Ambient temperature was 18–22 °C during the experiment and the photoperiod was 12 h. Ejaculates were collected by the gloved-hand method and the sperm rich fraction diluted in ACROMAX® (Gestión Veterinaria Porcina, S.L., Madrid, Spain) long-term extender to a sperm concentration of 30×10^6 sperm/ml after discarding ejaculates with more than 20% abnormal sperm (mainly those with proximal and distal cytoplasmic droplets and coiled tails). Sperm preparations were placed in 90 ml semen plastic bottles for storage at 15 °C.

Our trial started on the second day of storage and ended when sperm motility was zero, between 37 and 42 d of storage.

The viability of the sperm samples was determined by examining sperm motility and plasma and acrosomal membrane quality were assessed using the combined sHOST-viability test designated the sHV test [7].

Every 1 or 2 d, sperm motility (M) was visually checked under a light microscope at a magnification of 200×, placing the samples on a warmed glass slide at 37 °C, and scoring the percentage of motile sperm in at least 4 microscopy fields per sample.

The sHV test is a new test that subjects the semen sample to sHOST [8] and at the same time evaluates sperm viability by means of the fluorescent stain SYBR-14-propidium iodide (PI) [3]. For each semen sample, an 1.5 ml Eppendorf tube was prepared containing 1 ml of hypoosmotic solution (75 mOsm/kg solution obtained by preparing serial dilutions of ACROMAX® extender), 5 μl of SYBR-14 and 5 μl propidium iodide (SYBR-14-propidium iodide, Invitrogen, Carlsbad, CA, USA, Live Dead Sperm Viability Kit L-7011). These Eppendorf tubes containing hypoosmotic solution plus fluorescence stain, and glass tubes containing 1 ml of semen sample, were introduced in a water bath at 20 °C. When the temperature of the water bath reached 37 °C, 500 μl of each semen sample was added to the corresponding sHOST-SYBR-14-PI Eppendorf tube. After 5 min, the tubes were taken out of the water bath and 10 μl of 2% glutaraldehyde solution added to fix the sperm structures, mainly the acrosomal structures [9].

Finally, 5 μl of each processed sample was placed on a glass slide, covered with a coverslip and observed at 1000× under epifluorescence (Nikon filter B-2A) and phase contrast microscope (Nikon Eclipse 50i, Nikon Corporation, Japan).

Using this technique, the sperm were sorted according to their sHOST response (positive: cells with an intact tail plasma membrane indicated by coiled tails; negative: cells with a damaged tail plasma membrane indicated by straight tails), their acrosomal status (resistant or damaged acrosomal ridge) and their viability (green: alive; red, orange or green-orange: dead).

In each sample, 200 sperm were evaluated. The results of the sHV test were then used to identify three subpopulations of sperm as follows: (1) sHOST positive/alive (sHPA); (2) sHOST positive/dead (sHPD); and (3) sHOST negative/dead (sHND).

The sperm scoring positive in the sHOST were also classified according to acrosome membrane state as: live cells with a resistant acrosomal membrane (sHPARA); live cells with a damaged acrosomal membrane (sHPADA) and dead sperm with a resistant (sHPDRA) or damaged (sHPDDA) acrosomal membrane.

All data are expressed as percentages. ANOVA on ranks (Holm-Sidak method) was performed. When normality test failed a Tukey test was performed. We determined the changes produced in the proportions of each subpopulation in each of the boar ejaculates during storage and also examined differences among the trends shown by these subpopulations in the four ejaculates.

To validate the sHV test, we performed a preliminary trial using 30 boar semen samples diluted and preserved for different periods of time (2–4, 5–8 and 15–16 d). In these samples, we assessed sperm motility, sHOST, and sperm viability using SYBR-14-PI, to identify the sHV

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