

Effects of Alpha-Lipoic Acids on Sperm Membrane Integrity during Liquid Storage of Boar Semen

Laura Parlapan Pîndaru*, Ioan Ștefan Groza

University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine, Cluj-Napoca, Calea Mănăștur 3-5, Romania

Abstract

Preliminary studies have shown that sperm membrane from swine shows high sensitivity to cryopreservation process, causing a dramatic reduction in sperm quality. This has been attributed to the production of reactive oxygen species that cause lipid peroxidation in sperm membranes. The aim of the present study was to minimize the oxidative attack by adding different concentration of alpha-lipoic acid into the sperm liquid storage at 17°C for 7 days. Freshly ejaculated boar semen was diluted with Beltsville Thawing Solution (BTS) and supplemented with 5 levels of alpha-lipoic acid (0.015, 0.02, 0.05, 0.1, 0.15 mmol/ml). The membrane integrity was evaluated at days 0, 1, 3, 5 and 7 of liquid preservation, using flow cytometer FACSCanto II (BD Biociencias) systems. The experiment indicate that supplementation of alpha-lipoic acid to the semen liquid storage extender improve sperm membrane

Keywords: alpha-lipoicacid, liquid storage semen, membrane integrity, flow cytometer.

1. Introduction

Artificial insemination (AI) is one of the first biotechnologies widely used in commercial software swine, playing an important role in improving productivity [1] Ideally, in terms of biosafety and international trade, AI should be performed with cryopreserved semen to the detriment of fresh or chilled [2, 3]. However, due to the extremely low efficiency and productivity, the semen subjected to freeze-thaw processes is rarely used [4, 5]. Freezing of boar sperm began in the 60's [6], but despite the efforts to achieve fertility and fecundity consecutive acceptable artificial insemination, cryopreservation of boar sperm is consistently low compared with other species, due to losses during processing, which is long, costly and a low of doses per ejaculate. Recently, Rodriguez-Martinez and Wallgren [7] presented the main advantages

of boar semen cryopreservation, with importance for research, but particularly to relieve pigs. The frozen sperm is commonly used for long-term preservation (if the individual has a high value) for international export (when the boar is in quarantine in a specific location) and for research, and fertilization in vitro (IVF), in which case small samples of the same ejaculate reduce the experimental variations [8]. The limiting factor for a successfully cryopreservation application in commercial software of artificial insemination in swine is the low percentage of sperm that survive thawing row [4, 9, 10]. Preliminary studies have shown that the sperm membrane from pigs shows a high susceptibility to freeze-thaw process. This is due to the high content of polyunsaturated fatty acids (PUFAs), as well as an impairment of protective enzymes (antioxidants) in the seminal plasma [11]. Furthermore, cryopreservation has a strong oxidative effect and is associated with the production of reactive oxygen species (ROS), which causes lipid peroxidation (LPO) in the sperm membrane and implicitly a variety of physical and chemical changes of sperm cells that

* Corresponding author: Pîndaru Laura,
parlapan_laura@yahoo.com

predispose DNA damage and apoptosis. Therefore the only alternative to counteract the harmful effects of ROS is the addition of antioxidants in freezing environments [12, 13].

Alpha-lipoic acid is a organo-sulfur compound, which was isolated in 1951, with a key role in the chemical reactions that allow the body to produce the necessary energy for cells, having the ability to convert oxidated glutathione to reduced glutathione. One of the important features is that the acid is soluble in both water and oil. This allows to provide antioxidant protection to a large number of physiological reactions of the body, which made the researchers to name it the "universal" antioxidant [14, 15].

2. Materials and methods

The biological material for the study was of 5 Landrace breed boars aged between 1 and 3 years. The boars taken in the study were commonly used for making various techniques of assisted reproduction, being clinically healthy and without a history that could have repercussions on genital system. They were housed in individual stalls with controlled temperature ($23\pm 2^{\circ}\text{C}$), natural light supplemented with artificial light to a regime for 16 hours / day. Feeding the experience animals was done with standardized and standardized feed, male specific use in artificial insemination program. Feeding the experience animals was done with standardized and typified forage, specific for the male used in artificial insemination program, the quantity varying according to the weight of each boar in part. The manoeuvres in batching, isolation and feed manage were made carefully, avoiding animal stressing and without creating a state of discomfort. The collection of the semen samples was carried out through manual method using a double-walled glass collector, heated at 37°C and provided with a sterile filter. From each ejaculated was collected only sperm-rich fractions, which was diluted with the environment BTS (Beltsville Thawing Solution) preheated to 37°C in a ratio of 1: 3 v/v. There were accepted for processing and sent to the andrology laboratory only semen samples without detectable pathological changes in macroscopic examination (color, smell) and microscopic examination [16]. At the laboratory the SERFs were diluted BTS in 2 ml Eppendorf tube at a final

concentration of 25×10^6 spermatozoa/mL and subsequently analyzed (day 0). After that, semen samples were treated without or with different concentration of alpha-lipoic acid (0.015, 0.02, 0.05, 0.1, 0.15 mmol/ml) and preserved at 17°C for 7 days. Sperm viability as well as acrosome membrane integrity was analyzed with flow cytometry at days 0, 1, 3, 5 and 7 of preservation. For this purpose we used FACSCanto II cytometer (BD Biosciences) equipped with three lasers "solid-state" and a digital signal processing system FACSDiva software. Samples analyzed by FACS were excited by an Argon-ion laser of 488 nm, and the fluorescence spectrums of PNA-FITC and PI were detected by a 670 a LA filter respectively BP 530/30 nm. Sperm populations analyzed were 10,000/sample, which is measured at a rate of 100 to 800 cells per second. The membrane integrity assessment and acrozomiale involved marking sperm cells with a combination of 3 fluorochromes Hoechst 33342 (H-42), Propidium iodide (PI) and fluorescein isothiocyanate-conjugated Peanut agglutinin of (PNA-FITC) , according to the method described by Martinez-Alborcia [17]. Thus a tube 75 μl semen (1.5×10^6 sperm) were added 4 μl H-42 (0.05 mg / ml in PBS), 2 μl of propidium iodide (PI, 0.5 mg / ml in PBS, Molecular Probes Europe BV, Leiden, The Netherlands) and 4 μl PNA-FITC (200 mg / ml in PBS), the tubes were then incubated at 37°C for 10 minutes. Because fluorochromes may lose their ability to emit fluorescence, is recommended to analyze the samples immediately after incubation time. To streamline the process of analysis, each sample was partly added 400 μl PBS. The results of flow cytometry for each sample separately were played as a two-dimensional graphic dot-plot in which the representation of each of the cells is achieved by a point on the graph. This way on a dot-plot on graph, sperm cells are grouped according to the fluorescent emission intensity. By plotting two perpendicular straights the graph is divided into four quadrants, denoted in order from left to right and from top to bottom by 1, 2, 3 and 4. In quadrant 1 will group sperm cells PI +/-PNA-FITC- in quadrant 2 the PI +/- PNA-FITC+, in quadrant 3 sperm cells which do not show any fluorescence emission, in PI/ PNA-FITC-, in 4 sperm cells PI/PNA-FITC+. The numerical values and percentage of events in each quadrant are calculated and generated automatically by

cytometer software. To bet on cytometer calibration for each fluorochrome and the placement of two perpendicular straight, to highlight the 4 quadrants is necessary to offset the fluorescent signals. Therefore we used four reaction tubes, a tube of unlabeled sperm and other three sperm tubes labeled with H-42, IP and PNA-FITC. Determination of detection limit for each part of the fluorochrome was carried out by pre-incubating the tubes at 37 ° C for 10 min.

3. Results and discussion

The results of the spermatozoa viability of the average values are shown in Table 1. The viability of sperm cells obtained from appreciation membrane and acrozomiale integrity, measured by flow cytometry and the combination of three fluorochromes H-42, IP and PNA-FITC, determined the distinguish of 4 cell subpopulations (Fig. 1): (1) live sperm with intact acrosome (PI / PNA-FITC-) - these are considered viable sperm, without any alteration of the membrane; (2) sperm with an intact plasma membrane and acrosome damage to the PI / PNA-FITC +); (3) sperm with damaged plasma membrane and acrosome intact (PI + / PNA-FITC-); (4) dead sperm with acrosome damage. It is known that the percentage of intact membranes sperm (viable) is an extremely safe factor in terms of predicting fertility semen concerned. Lipid peroxidation induced ROS, is considered the main cause of sperm dysfunction

especially in the loss of membrane fluidity, resulting viability decreasing. Alpha-lipoic acid is considered a powerful antioxidant with the ability to convert oxidated glutathione to reduced glutathione. Thus, as can be seen in the table above, storage at 17°C of semen from a boar in the presence of various alpha-lipoic acid concentrations (0.015 mmol/mL, 0.02 mmole/ml and 0.05 mmol/ml) resulted in no significant differences during the 7 days of storage compared with semen stored only with BTS. Regarding the percentage of live and membrane unaltered sperm stored with a concentration of 0.1 mmol/ml alpha-lipoic acid and that of 0.15 mmol/ml has shown a strong protective effect including until storage day 5, and in the day 7 to signal a slight decrease of this effect. Moreover Fatimah [15] has shown, in studies, an improvement of motility parameters in these doses.

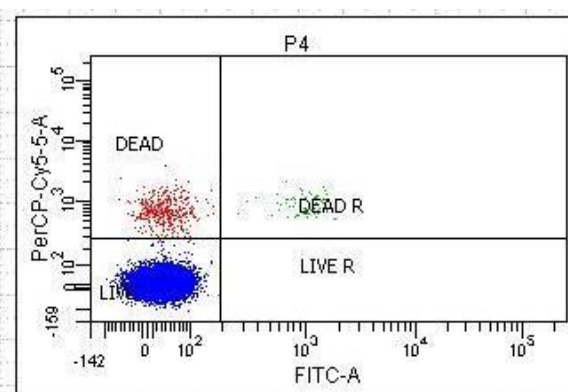


Figure 1. Flow cytometry histogram

Table 1. Spermatozoa viability after addition of different alpha-lipoic acid concentrations to boar semen doses preserved at 17°C for 7 days

	Cell viability (PI-/FITC-PNA-)*				
	Day 0	Day 1	Day 3	Day 5	Day 7
BTS	87.24±1.54	86.42±2.15	74.28±1.71	67.28±1.14	60.17±25
0.015 mmol/ml ALA		86.25±1.49	82.56±2.23	79.34±1.24	70.26±2.65
0.02 mmol/ml ALA		86.71±1.24	79.12±3.76	73.24±2.43	66.53±1.98
0.05 mmol/ml ALA		88.72±2.44	85.12±3.92	81.98±2.16	72.26±1.65
0.1 mmol/ml ALA		89.25±1.49	87.56±2.23	85.34±1.24	80.86±2.65
0.15 mmol/ml ALA		88.71±1.24	88.12±3.76	86.24±2.43	80.56±1.98

*Boar seminal doses were preserved at 17°C during 7 days in BTS in absence or presence of different concentrations of alpha-lipoic acid (ALA) (3 μM, 6 μM, 8 μM and 1 mM). Percentages of spermatozoa cell viability and spermatozoa acrosomal integrity were measured by flow cytometry as described in Materials and Methods. Results are expressed as mean ± standard error of the mean (SEM).

4. Conclusions

Flow cytometry, by the possibility of analyzing a large number of sperm cells in a short time is a very useful technology to assess their integricate and architectural character. Alpha-lipoic acid has proven to be a very good membrane integrity protective, protective effect depends on both the antioxidant concentration added to the environment, as well as during storage.

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