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Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm

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Abstract

The main aim of this work was to test the effects that freeze-thawing could have on the overall nuclear structure of boar sperm. This was done by analyzing both the DNA fragmentation and the protamine-1–DNA interaction of the boar-sperm nucleus. Our results indicate that freezing–thawing did not induce a significant degree of DNA fragmentation, as manifested through both the Sperm–Sus–Halomax® stain and a random primed analysis prior to partial DNA digestion with enzymes BamHI–HinDIII. On the other hand, freeze-thawing induced significant changes in the protamine-1–DNA interaction, as revealed through both Western blot analysis and immunocytochemistry for protamine-1. These alterations caused, in turn, significant changes in the overall nuclear structure of boar sperm after thawing. Protamine-1–DNA alterations started to be apparent during the cooling phase of the freeze-thawing protocol. These results imply that one of the alterations that may be responsible for the loss of fertilizing ability of boar sperm after freeze-thawing may be an alteration in the correct formation of the overall nuclear structure, which, in turn, would induce alterations in the correct formation of the first nuclear structure after oocyte penetration.

Keywords: Boar sperm; Freezing–thawing; Protamine-1; DNA fragmentation

1. Introduction

It is well established that freeze-thawing induces a clear decrease in the fertilizing ability of boar sperm. This decrease has been related to a wide range of both functional and structural alterations associated with the freeze-thawing process, such as changes in the cell membrane structure, increase of oxidative reactions, mitochondrial alterations and others [1–7]. Moreover, in the last few years, several authors have studied sperm DNA alterations as a source of decreased fertility of frozen–thawed boar sperm. Studies have been conducted being based on the previous work of several authors, which has shown a close relationship between DNA fragmentation and sperm fertilizing ability in several species (see [7] as a review). Following this, freezing-induced DNA fragmentation has been described in species such as horse [8]. However, results obtained in boar varied from authors that indicated that freeze-thawing induced a clear impairment of DNA integrity [9] to others that indicated that DNA integrity

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was not significantly affected by freezing [10,11]. A similar lack of effect has been shown in ram [12], opening doubts about a freezing-induced effect on sperm DNA integrity. This is an important question since, as indicated above, the relationship between DNA integrity and sperm fertilizing ability has been well established [7], and this could be a major point in explaining cases of lack of fertilizing ability of thawed samples despite reasonably good results in other parameters of semen quality, like viability and motility. In fact, it has been described that sperm with a high ratio of DNA fragmentation can maintain its motility characteristics and is even able to undergo oocyte penetration. However, embryos obtained after ''in vitro'' fertilization with DNA-damaged sperm died through an apoptotic process during the 4-to-8-cell stage, possible due to aberrant embryo DNA expression [7]. Thus the establishment of the mechanisms by which freeze-thawing can affect DNA integrity in species like the boar will be of the greatest importance to improve the fertilizing ability of these samples.

The mammalian sperm nucleus has a very peculiar structure, which differentiates it from all other eucaryotic cells. This differentiation is not only due to the fact that spermatozoa are haploid cells, but also to a very specific nucleoproteinic structure [13–15]. Thus, the most important nuclear proteins of mammalian sperm are from the protamine family, instead of histones commonly found in somatic, eucaryotic cells. Protamines are very small, acidic proteins that form very complex structures with sperm DNA, thus inducing a highly compacted DNA [13–15]. There are two different protamines associated with mammalian sperm DNA. They are named protamine-1, which is present in all of the studied species, and protamine 2, only present in species such as human, mouse and horse [15]. The maintenance of an appropriate protamine–DNA structure in the sperm nucleus is very important in order to maintain sperm fertilizing ability. In this sense, it has been described in humans that alterations in protamine–DNA and protamine-1–protamine-2 ratios are associated with a decrease of infertility [16,17]. This is probably due to the fact that decondensation of the sperm nucleus after oocyte penetration involves a strict, sequential process, which is initiated by the loss of nuclear protamines before sperm nuclear decondensation [18,19]. Notwithstanding, there is a noticeable lack of information regarding the effect of freeze-thawing on the specific sperm protamine–DNA structure. This is especially important in species such as boar, where a putative, freezing-induced destabilization of the protamine–DNA structure could cause a significant loss of sperm fertilizing ability without a great modification of the results of the routinely performed semen analysis. Taking this into account, the main aim of this work is to evaluate how a standard freeze-thawing protocol can affect the overall protamine–DNA structure of boar sperm. For this purpose, DNA integrity was analyzed through two separate techniques. First, via a specific stain for DNA fragmentation and, secondly, by a random priming analysis of sperm DNA after digestion with the combination of restriction enzymes BamHI and HinDIII. Finally, the protamine-1–DNA interaction was analyzed by Western blot and immunocytochemistry of samples utilizing a specific anti-protamine-1 antibody.

2. Materials and methods

2.1. Animals and samples collection

Twelve healthy boars of 2–3 years of age from a commercial farm were used in this study. The boars were from three separate lines (four Landrace, three Large White and five Pietrain). All boars had proven fertility after AI using extended, liquid semen. The sperm-rich fraction of each ejaculate utilized in this study was manually collected twice weekly using the gloved-hand method and analyzed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was suspended (1:2; v/v) in a commercial extender (MR-A; Kubus SA; Majadahonda, Spain). The extended semen samples were cooled and maintained at 17 °C for shipment to the laboratory of the Autonomous University of Barcelona within 24 h post-collection, for further processing and analyses.

2.2. Semen cryopreservation

Immediately after receiving the shipped semen samples, an aliquot was taken to perform the appropriate semen assessments, as well as those regarding protamine-1–DNA studies (fresh semen sample). Only those samples displaying a minimum of 70% progressive motile and 80% of morphologically normal spermatozoa were further processed by adapting a proven protocol [20]. The extended semen was centrifuged in a programmable refrigerated centrifuge (Medifriger BL-S; JP Selecta; Barcelona, Spain) set at 17 °C, at 800 × *g* for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose–egg yolk (LEY) extender (80 mL (80% (v/v) 310 mM) of β-lactose + 20 mL egg yolk), at a ratio that led to a final concentration of