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Fragmentation dynamics of frozen-thawed ram sperm DNA is modulated by sperm concentration

C. López-Fernández[a], S.D. Johnston[b,*], J.L. Fernández[c], R.J. Wilson[d], J. Gosálvez[a]

[a] *Departamento de Biología, Unidad de Genética, Universidad Autónoma de Madrid (UAM). 20849-Madrid. España.*
[b] *School of Animal Studies, The University of Queensland, Gatton, 4343, Australia*
[c] *INIBIC-Genética, Complejo Hospitalario Universitario Juan Canalejo, As Xubias, A Coruña, Spain*
[d] *School of Mathematics and Physics, The University of Queensland, St Lucia, 4072, Australia*

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Abstract

This study investigated the hypothesis that post-thaw incubation of ram sperm at high concentrations results in a faster rate of DNA fragmentation than when sperm are incubated at a lower concentration. Ejaculates from 10 rams were frozen-thawed, prepared in sperm concentrations of 100, 50, 25, 12, and 6 × 10^6 sperm/mL, and incubated for 6 h at 37 °C. Sperm DNA fragmentation was assessed using the sperm chromatin dispersion test (Sperm-Halomax®) at 1, 3, 4, and 6 h of incubation at 37 °C. On fitting a binary logistic regression with a cubic over time and treating ram and dilution levels as factors, there were significant effects with respect to the ram, dilution and time (all P-values were very much smaller than 0.001). Therefore, DNA fragmentation dynamics of incubated frozen-thawed ram sperm were not only dependent on the inherent sperm DNA fragmentation expressed immediately after thawing, but also on the concentration of sperm incubated in the sample. Although there was evidence of individual ram variation in SDF during the incubation period, the general finding of the current study was that lower sperm concentrations resulted in a slower rate of DNA fragmentation These findings have important implications for the post-thaw manipulation of ram sperm used for AI and advanced reproductive procedures that use sperm at low concentrations. Our data also emphasised the highly dynamic nature of sperm DNA fragmentation and the importance of conducting the procedure in a standardised manner.

Keywords: Sperm DNA fragmentation; Semen cryopreservation; Sheep; Dilution; *In vitro* incubation

1. Introduction

Three major hypotheses have been proposed to explain cellular mechanisms that result in the altered sperm DNA molecule. The first is related to torsional stress in unconstrained DNA supercoils and is a direct consequence of histone-protamine replacement during mid-spermiogenesis [1,2]. The second hypothesis regards DNA fragmentation as a consequence of oxidative stress in the male reproductive tract [3–6]. The third hypothesis concerns apoptotic-related DNA strand breakage, similar to that which occurs in abortive apoptosis in somatic cells; the presence of caspase 9 in the midpiece and the occurrence of activated caspases 8, 1 and 3 in the postacrosomal region appeared to support this view [4,6,7]. However, the etiologies of DNA damage are many and varied, ranging from bacterial infections [8], chemical toxicity [9,10], elevated temperature [11], diabetes [12,13], age

* Corresponding author. Tel.: 617 32026902; fax: 617 33655644.
E-mail address: stevejohnston@uqconnect.net (S. Johnston).

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[14,15], body mass [16], and genetic background [17]. Whereas many of the factors resulting in sperm DNA fragmentation are typically unavoidable, certain types of induced iatrogenic sperm DNA damage can become exacerbated when sperm are inappropriately manipulated in the laboratory. For example, we have previously shown how changes in temperature excursions can affect the rate of sperm DNA fragmentation during *in vitro* incubation [18].

Successful artificial insemination (AI) of frozen-thawed semen relies on the delivery of sufficient numbers of sperm into the female reproductive tract, which are morphologically normal, have a functional capacity in terms of motility, and are able to undergo an acrosome reaction and to successfully participate in syngamy. Although numerous studies have examined post-thaw survival characteristics (motility, membrane integrity, and acrosomal status) of incubated mammalian sperm, only recently has there been interest in the effect of post-thaw incubation on sperm DNA fragmentation and the potential importance of this phenomenon to the success of AI programs [19,20].

Cryopreservation induced capacitation-like changes in ram sperm, which resulted in membrane destablisation and cell death [21]; compounding this phenomenon, excessive dilution of sperm and the consequent removal of seminal plasma has also been shown to be associated with a loss of motility and viability [22]. In addition, highly diluted frozen-thawed ram sperm recovered from swim-up procedures had changes in their motility and chlortetracycline staining patterns which were indicative of an increase in the incidence of capacitation [23,24]. A study on canine sperm revealed that both motility and membrane integrity was improved with increasing dilution of the frozen-thawed sample, but that the proportion of reacted acrosomes was similar [25]. In the bull, dilution and incubation of thawed sperm at high and low concentrations at room temperature for 24 h resulted in little difference between these populations in terms of viability or susceptibility to osmotic stress, although sperm preparations with lower sperm concentration had a higher proportion of viable cells with reacted acrosomes [26]. Surprisingly, the effect of dilution on sperm DNA fragmentation of incubated frozen-thawed sperm has not been investigated. Therefore, the objective of the current study was to analyze the effect of sperm concentration and post-thaw incubation on the integrity of ram sperm DNA. This experiment was based on the hypothesis that the rate of DNA fragmentation of frozen-thawed ram sperm is dependent on the initial sperm concentration at which the thawed sample was extended. The results of this investigation will provide important insights and practical applications into the way in which thawed ram sperm should be processed and diluted prior to its use in laparoscopic AI or other advanced reproductive technologies.

2. Materials and methods

2.1. Animals, semen collection and sperm cryopreservation

Semen was collected by artificial vagina from 10 sexually mature (2–4 y) rams housed at the Ovigen Centre, Zamora, Spain; these animals were kept under controlled feeding and photoperiodic conditions for maximal reproductive performance and were clinically healthy throughout the semen collection period. In preparation for cryopreservation, the fresh ejaculate was diluted to a final concentration of 1000×10^6 total sperm in a commercial egg yolk-based extender, (Triladyl; Minitube Canada, Woodstock, ON, Canada) loaded into 0.25 mL PVC straws, and frozen in a programmable freezing chamber (Minitube Iberica, Tarragona, Spain).

2.2. Dilution and incubation of thawed sperm

Semen straws were thawed by plunging in a waterbath at 37 °C for approximately 30 to 60 s [27], after which time each sample was immediately diluted in a pre-warmed (37 °C) commercial extender (INRA 96, IMV Technologies, L'Aigle, France) to give a sperm concentration of $\sim 200 \times 10^6$ sperm/mL. At this time, the post-thaw survival of the thawed sperm was assessed; only samples with high post-thaw survival (>50% normal acrosomes, >35% positive to the HOS test, and >35% progressive motility) were used for subsequent incubation experiments. The semen sample was then allowed to cool to 15 °C over 30 min to reduce metabolism prior to being centrifuged ($300 \times g$ for 10 min); this step was included to remove the potential harmful influence of cryomedia which may have inadvertently affected DNA integrity during incubation. The supernatant was gently removed and resuspended with fresh diluent (INRA 96) to provide a standardised working sperm concentration of 100×10^6 sperm/mL (C100). Sperm DNA fragmentation (SDF) was assessed at this stage of post-thaw incubation and designated time T0. This diluted semen sample was subsequently serially diluted in INRA 96 to produce sperm concentrations of 50 (C50), 25 (C25), 12 (C12), and 6