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The addition of reduced glutathione to cryopreservation media induces changes in the structure of motile subpopulations of frozen-thawed boar sperm

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ABSTRACT

Adding cryopreservation media with reduced glutathione (GSH) has previously been shown to maintain the motility, membrane integrity and fertilizing ability of frozen-thawed boar sperm, although the effects of GSH on good (GFE) and poor freezability (PFE) ejaculates rely upon the intrinsic ejaculate freezability. The resilience to withstand freeze-thawing procedures has previously been related to the existence of a specific distribution of motile sperm subpopulations, which differs between GFE and PFE. Thus, the main aim of this study was to determine whether the addition of GSH to freezing media has any impact on the distribution of motile sperm subpopulations in GFE and PFE. With this purpose, 18 GFE and 13 PFE were cryopreserved with or without 2 mM GSH. Sperm quality and motile subpopulations were evaluated at 30 min and 4 h post-thawing. Three subpopulations were identified and the percentages of spermatozoa belonging to the fastest and most linear subpopulation, which was referred as 'SP1', decreased over post-thawing time. Good freezability ejaculates that were cryopreserved in the presence of 2 mM exhibited a significantly higher percentage of spermatozoa belonging to SP1 than the other combinations of treatment and freezability both at 30 min (mean ± SEM: GFE-C: 16.6 ± 0.4; GFE-GSH 27.7 ± 0.6) and 4 h post-thawing (GFE-C: 7.8 ± 0.2 *vs.* GFE-GSH: 16.7 ± 0.4). In conclusion, the positive effect of GSH on the motility of frozen-thawed sperm is related to a specific sperm subpopulation (SP1), which could coincide with the fertile sperm one.

1. Introduction

Cryopreservation is known to impair boar spermatozoa (see Ref. [43] for review), but the extent of that cryodamage differs between boar ejaculates [37]. For this reason, ejaculates are classified as of good (GFE) or poor freezability (PFE) based upon their sperm quality at post-thawing [37,38].

Previous works attempted to increase the resilience of boar sperm to cryopreservation by adding antioxidants to freezing and thawing media (see Ref. [42] for review). Supplementing cryopreservation media with reduced L-glutathione (GSH) maintains better the integrity of nucleus and plasma membrane, and the fertilizing ability of frozen-thawed boar spermatozoa [6,7,13,15,45]. Interestingly, the GSH-positive effect differs between ejaculates, so that the improvement mediated by this antioxidant is more apparent in GFE than in PFE [47]. However, while previous works on the effects of GSH upon cryopreserved boar sperm assessed different sperm parameters, no study has investigated whether this antioxidant affects the structure of motile sperm subpopulations.

Abaigar et al. [2] were the first to use advanced statistical methods for the analysis of Computer Assisted Sperm Analysis system (CASA) data. Following this landmark paper, motile sperm subpopulations have been identified in the ejaculates of separate mammalian species and have been suggested to have a functional role [33]. It is worth noting that sperm motile subpopulations rely on the kinetic characteristics of single sperm cells, which depend on their surrounding environment.

The number of sperm subpopulations varies across species and studies. For example, Santolaria et al. [34] identified three motile subpopulations in the sheep, whilst Luna et al. [19] identified four. Three motile sperm subpopulations were identified in the goat

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[39], and four in rabbits, donkeys and dogs [5,11,21–23,27,32]. In bulls, four sperm subpopulations were distinguished [25], the fastest one showing positive correlation with sperm binding to the zona pellucida, percentages of penetration and rates of pronucleus formation [9]. While Quintero-Moreno et al. [30] described four subpopulations in refrigerated stallion sperm, Ortega-Ferrusola et al. [26] found six and four subpopulations in fresh and frozen-thawed stallion sperm, respectively. In boars, whilst some reports identified three motile sperm subpopulations [2,4,8], others found four [11,12]. However, it is worth mentioning that the number and characteristics of those sperm subpopulations heavily relies upon the statistical method.

While the relevance of studying the structure of sperm subpopulations during freeze-thawing procedures of boar spermatozoa has been previously reported [4,12,36], the effects of GSH on that subpopulation structure warrant further research. In addition, as GSH-effects depend on the intrinsic ejaculate freezability [47], it would be interesting to address whether the extent of changes in the motile subpopulations of frozen-thawed boar sperm induced by GSH differs between GFE and PFE. Therefore, the present study aimed at investigating the structure of motile subpopulations in frozen-thawed boar sperm of GFE and PFE, following supplementation of cryopreservation media with or without 2 mM GSH.

2. Materials and methods

2.1. Semen samples

The current study involved a total of 36 ejaculates, which came from 25 separate boars. The ejaculates were obtained from a local farm (Selección Batallé, S.A.; Riudarenes, Spain), where animals were kept under adjusted conditions of temperature and humidity and were fed a standard diet with water provided *ad libitum*. These boars were routinely used for producing and selling semen doses for artificial insemination (AI). No fertility problems for these boars were recorded by the AI station. Ejaculates were collected twice a week through the gloved-hand technique [16]. Briefly, the sperm-rich fraction was collected and filtered through gauze and an insulated container containing 50 mL pre-warmed commercial extender (Duragen, Magapor, S.L.; Zaragoza, Spain) was used. After collection, the diluted sperm-rich fraction was re-diluted 1:1 (v/v) with the same extender (Duragen, Magapor, S.L.) and cooled down to 17 °C. Ejaculates were transported to the laboratory within 4 h post-collection.

Upon arrival, all semen samples were evaluated and sperm quality parameters were confirmed to be above the following standard thresholds in 31 out of 36 ejaculates: viable spermatozoa ≥85%; morphologically normal spermatozoa ≥85%; total motile spermatozoa ≥80%. The five ejaculates that did not satisfy the quality standards were excluded from the study and the other 31 were cryopreserved as described below.

The current study was designed following the Animal Welfare Directive issued by the Regional Government of Catalonia, Spain (D 214/1997, DOGC 1997; 2450: 9169–9174) and the Spanish welfare and protection standards in swine (RD 1392/2012, BOE 2012; 241: 71380–71382).

2.2. Sperm cryopreservation

Prior to cryopreservation, all ejaculates were stored for a 24 h-period at 17 °C as this holding time has been reported to increase sperm survival at post-thawing [48]. Next, ejaculates were split into separate falcon tubes of 50-mL each and then centrifuged at 17 °C and 600×*g* for 5 min. The resulting pellets were re-suspended in freezing medium (LEY) made up of 80% (v/v) 310 mM β-lactose (Sigma-Aldrich®; St Louis, MO, USA), 20% (v/v) egg yolk, and 100 μg mL^{-1} kanamycin sulphate (Gibco®; Life Technologies, Carlsbad, CA, USA). Final concentration, which was 1.5×10^9 spermatozoa·mL^{-1} at this step, was adjusted with LEY medium after determining sperm concentration with a Makler counting chamber (Sefi-Medical Instruments; Haifa, Israel). The resulting volume was split into two fractions of equal volume. One of these fractions (referred as FT-GSH) was supplemented with GSH ($C_{10}H_{17}N_3O_6S$; Sigma-Aldrich®; final concentration: 2 mM), whereas the other (called FT-Control) did not contain GSH (FT-Control). The two aliquots were frozen in parallel. Spermatozoa were cooled from 17 °C to 5 °C at a rate of −0.1 °C·min^{-1} through a controlled-rate freezer (Icecube14S-B; Minitüb GmbH, Tiefenbach, Germany). Spermatozoa were re-diluted to a final concentration of 1×10^9 spermatozoa·mL^{-1} with LEYGO medium, which consisted of LEY medium containing a cryoprotectant (glycerol; Sigma-Aldrich®) and a surfactant (Orvus ES Paste; OEP, Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA). After dilution, final concentrations of glycerol and OEP within straws were 2% and 0.5%, respectively. The LEYGO medium added to the GSH-supplemented aliquot also contained 2 mM GSH. Following this, the volume of each aliquot was packed in separate 0.5-mL plastic straws (Minitüb GmbH). Each straw was labeled using an automatic printer for plastic straws (Easycoder; Minitüb GmbH) with the following information: boar, ejaculate code, freezing date and treatment (FT-C or FT-GSH). In order to avoid any disturbance in the temperature of sperm samples, straws were cooled to 5 °C prior to sperm packaging. Upon sperm packaging, straws were transferred to the same controlled-rate freezer (Icecube14S-B; Minitüb GmbH) used for cooling the samples. A freezing curve specifically designed for boar sperm was run and cooling rates and times were as follows: −6 °C·min^{-1} for 100 s (from 5 °C to −5 °C); −39.82 °C·min^{-1} for 113 s (from −5 °C to −80 °C), holding step at −80 °C for 30 s, and −60 °C·min^{-1} rate for 70 s (from −80 °C to −150 °C). Straws were then plunged into liquid N_2 and evaluated within the next two months.

For thawing, and as recommended by Casas et al. [3], four straws per treatment (FT-C or FT-GSH) and ejaculate were taken and shaken vigorously in a water bath at 37 °C for 20 s [10]. The volume contained in the four straws was diluted with three volumes of Beltsville Thawing Solution (BTS; [29]) previously warmed at 37 °C. Sperm motility and membrane integrity were evaluated at 30 min and 4 h post-thawing, as 4 h is the insemination-to-ovulation interval recommended for cryopreserved boar sperm [40].

2.3. Evaluation of sperm concentration and morphology

Sperm concentration was only assessed when ejaculates were received in our laboratory and during cryopreservation steps (i.e. dilution in LEY, dilution in LEYGO, and at post-thawing). Briefly, three replicates were evaluated using a Makler counting chamber (Sefi-Medical Instruments) after dilution with 4% formalin buffered solution. Sperm count was adjusted to the dilution factor.

Sperm morphology was only assessed upon arrival of ejaculates in our laboratory in order to ensure that percentages of morphologically normal spermatozoa were above the threshold (85%) set as a quality standard. Sperm cells were previously fixed with 4% paraformaldehyde (Sigma-Aldrich®) and a drop of 5 μL was placed onto a slide and then mounted with a cover slip. Samples were evaluated under a phase-contrast microscope (Olympus BX41) at 200 × magnification (Olympus 20 × 0.40 PLAN objective lens, positive phase-contrast field) and three counts of 100 sperm each were made prior to calculating the mean ± standard error of the mean (SEM). Each spermatozoon was classified into one of the following categories: morphologically normal, with proximal