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The improving effect of reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability ☆

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ABSTRACT

Reduced glutathione (GSH) improves boar sperm cryosurvival and fertilising ability when added to freezing extenders. Poor freezability ejaculates (PFE) are known to present lower resistance than good freezability ejaculates (GFE) to cryopreservation procedures. So far, no study has evaluated whether the ability of GSH to counteract the cryopreservation-induced injuries depends on ejaculate freezability (i.e. GFE *vs.* PFE). For this reason, thirty boar ejaculates were divided into three equal volume fractions and cryopreserved with or without GSH at a final concentration of either 2 or 5 mM in freezing media. Before and after freeze–thawing, sperm quality was evaluated through analysis of viability, motility, integrity of outer acrosome membrane, ROS levels, integrity of nucleoprotein structure, and DNA fragmentation. Ejaculates were classified into two groups (GFE or PFE) according to their post-thaw sperm motility and viability assessments in negative control (GSH 0 mM), after running cluster analyses. Values of each sperm parameter were then compared between treatments (GSH 0 mM, GSH 2 mM, GSH 5 mM) and freezability groups (GFE, PFE). In the case of GFE, GSH significantly improved boar sperm cryotolerance, without differences between 2 and 5 mM. In contrast, PFE freezability was significantly increased when supplemented with 5 mM GSH, but not when supplemented with 2 mM GSH. In conclusion, PFE need a higher concentration of GSH than GFE to improve their cryotolerance.

Introduction

Sperm cryopreservation is currently the most efficient method for long-term storage of boar spermatozoa [11]. However, freeze–thawing protocols are known to affect boar sperm function and survival and reduce sperm fertilising ability [38]. Indeed, sperm cryopreservation damages plasma membrane and acrosome, and reduces sperm motility [15,19,21]. In addition, freeze–thawing of boar spermatozoa also alters the sperm nucleus through destabilization of its nucleoprotein structure by disrupting disulfide bonds rather than increasing DNA fragmentation [41].

As other mammalian species, the sperm ability to sustain cryopreservation procedures, namely freezability, differs between individuals and ejaculates coming from the same boar [22,30,31,37]. In this way, ejaculates can be classified into 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE; see [9]). These two groups have been reported to differ in their resistance to cryopreservation as evaluated through several sperm parameters, like viability, acrosome integrity, motility, or nucleoprotein structure [42].

The addition of antioxidants has been reported to partially counteract the deleterious effects that cryopreservation protocols may inflict upon boar spermatozoa [43]. In this context, reduced glutathione (GSH) has been shown to improve boar sperm cryotolerance, as protects nucleoprotein structure, maintains better sperm motility and viability [17,41], and increases the fertilising ability of frozen–thawed (FT) spermatozoa [14]. It is worth mentioning that this antioxidant is one of the most abundant thiol in live cells and plays a prominent role in maintaining intracellular redox balance [23].

To date, no study has been conducted to evaluate whether the intrinsic freezability of a given boar ejaculate affects the ability

Abbreviations: GFE, good freezability ejaculates; PFE, poor freezability ejaculates; ROS, reactive oxygen species; GSH, reduced glutathione; FT, Frozen–thawed spermatozoa.

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of GSH to improve sperm cryosurvival. Against this background, the main aim of the present study was to evaluate whether the improving effects of supplementing freezing extenders with GSH are of different intensity when comparing GFE with PFE. With this purpose, a total of 30 ejaculates were frozen–thawed in the absence (negative control, 0 mM) or presence of either 2 mM GSH or 5 mM GSH. Ejaculates were classified into GFE and PFE groups according to post-thaw sperm viability and motility of their respective 0 mM GSH aliquots. In addition, other sperm parameters were analysed. These parameters were sperm chromatin packaging, assessed as levels of free cysteine radicals in sperm nucleoproteins and DNA fragmentation, integrity of the outer acrosome membrane, and ROS levels. Sperm analysis of the FT samples was performed at four cryopreservation steps: before starting cryopreservation, after cooling step of the cryopreservation procedure (5 °C), and at 30 and 240 min post-thawing. Results indicate that PFE require higher concentrations of GSH than GFE to increase their resistance against cryoinjuries. This suggests that the beneficial effects of additives used to supplement cryopreservation extenders may depend on the intrinsic freezing ability of a given boar ejaculate.

Materials and methods

Animals and semen collection

A total of 33 ejaculates, each ejaculate coming from a different boar (Pietrain breed), were used in this study (age: 21.6 ± 0.9 months; mean ± standard error of the mean, SEM). Animals were housed in climate-controlled buildings at a local farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Barcelona, Spain), fed with an adjusted diet (2.3 kg day^{-1}) and provided with water *ad libitum*. Boars were collected by the gloved-hand technique twice per week with an interval of at least three days between collections. The total volume of the sperm-rich fraction was diluted 1:1 (v:v) in a long-term extender (Duragen®; Magapor S.L.; Zaragoza, Spain) after removing the gelatinous fraction by filtering through gauze. Within 2 h after collection, ejaculates were transported in an insulated container to the laboratory of the Autonomous University of Barcelona (UAB) at 17 °C.

Experimental design

Upon arrival to the UAB laboratory, sperm quality of all ejaculates was evaluated to check that they satisfied minimal quality standards, i.e. total sperm motility >80%, progressive sperm motility >60%; morphologically normal spermatozoa >85%; sperm viability >85% (see [42]). From the total of thirty-three ejaculates, three were below these thresholds and were thus discarded. The other thirty ejaculates were stored in our laboratory at 17 °C up to 24 h post-collection, when they were cryopreserved.

Ejaculates were divided into three aliquots of equal volume that were simultaneously subjected to cryopreservation using the Westendorf method [39], as modified in Casas et al. [9]. The main difference among these three aliquots was the presence or absence of GSH ($C_{10}H_{17}N_3O_6S$; Sigma®, St Louis, Missouri, USA) in the cryopreservation media (i.e. LEY and LEYGO). Thus, the first aliquot was processed in the absence of GSH in both media (GSH 0 mM samples). In the second aliquot, GSH was added to cryopreservation media to a final concentration of 2 mM (GSH 2 mM). Finally, GSH was added to cryopreservation media to a final concentration of 5 mM (GSH 5 mM) in the third aliquot. Several sperm parameters (sperm viability and integrity of outer acrosome membrane, levels of peroxides and superoxides, sperm motility, levels of free cysteine radicals in sperm nucleoproteins and sperm DNA fragmentation) were evaluated for each aliquot. The analyses of these parameters were carried out after the four following cryopreservation steps: (1) before starting the cryopreservation procedure (i.e. at 17 °C); (2) at the end of the cooling step (i.e. after sperm being cooled at 5 °C in LEY extender for 120 min); (3) after 30 min post-thawing (FT 30 min); and (4) after 240 min post-thawing (FT 240 min). With this purpose, an aliquot of 20 mL per ejaculate and treatment was taken to assess all the mentioned sperm parameters in the first step (before starting the cryopreservation protocol, i.e. at 17 °C). The remaining volume was cooled to 5 °C for 120 min and an aliquot of 20 mL was then taken to assess the mentioned sperm parameters (second step). Finally, the remaining sperm volume was cryopreserved and stored in liquid nitrogen at −196 °C for at least two months. Samples were thawed and sperm parameters evaluated after 30 and 240 min post-thawing (steps 3 and 4).

Freeze–thawing procedure started with the centrifugation of semen aliquots at 17 °C and 600×*g* for 5 min. Pellets were then recovered with 3–4 mL of the remaining supernatant and diluted to a concentration of 1.5×10^9 spermatozoa mL^{-1} (using a Makler counting chamber; Sefi-Medical Instruments; Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Next, spermatozoa were cooled down to 5 °C for 120 min (cooling ramp: 0.1 °C min^{-1}) using a programmable freezer (Icecube14S-B; Minitub Ibérica, SL; Tarragona, Spain) and subsequently diluted at 1×10^9 spermatozoa mL^{-1} in LEYGO extender that contained 6% glycerol (Sigma®) and 1.5% Orvus ES Paste (OEP, Equex STM; Nova Chemical Sales Inc.; Scituate; MA, USA). Final concentrations of glycerol and OEP in cryopreserved samples were 2% and 0.5%, respectively. Spermatozoa were finally packed in 0.5-mL plastic straws (Minitub Ibérica, SL) and transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica, SL). The freezing programme (SY-LAB software; Minitub Ibérica, SL) consisted of 313 s of cooling at the following rates: −6 °C min^{-1} from 5 to −5 °C (100 s), −39.82 °C min^{-1} from −5 to −80 °C (113 s), maintained for 30 s at −80 °C, and finally cooled at −60 °C min^{-1} from −80 to −150 °C (70 s). The straws were then plunged into liquid N_2 (−196 °C) for further storage.

After being stored in liquid N_2 for at least 2 months only for schedule reasons, four straws per ejaculate and treatments were thawed by heating at 37 °C for 20 s, pooled, and diluted with three volumes of warmed BTS [33] at 37 °C (final dilution: 1:4, v/v). After thawing, aliquots were incubated for 30 or 240 min at 37 °C, and the same parameters determined in the previous steps were evaluated. These two incubation times (30 and 240 min) were chosen to assess the survival of FT spermatozoa within the insemination-to-ovulation interval recommended for cryopreserved doses, and as a test to determine the sperm resistance after freeze–thawing [9,11,42].

Data obtained from all sperm parameters in all ejaculates, treatments and cryopreservation steps were statistically analysed as follows. First, ejaculates were classified as good (GFE) or poor freezability ejaculates (PFE) based upon assessments of sperm viability and motility after 30 and 240 min post-thawing in negative control (unsupplemented treatment, GSH 0 mM), as described in Casas et al. [9]. Subsequently, the effects of supplementing freezing media with GSH at final concentrations of 2 or 5 mM on the mentioned sperm parameters of GFE and PFE, and over the four cryopreservation steps (extended semen (17 °C), cooled semen (5 °C), FT 30 and FT 240) were tested. Statistical analyses are described with a much greater detail in Supplementary section 2.6.

Flow cytometric analyses

General information about the flow cytometric analyses performed in this work

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement