Evaluation of L-glutamine for cryopreservation of boar spermatozoa

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\textbf{Abstract}
The aim of the present study was to evaluate the protective effect of L-glutamine (L-Gln) against cryopreservation injuries on boar sperm. In Experiment 1, L-Gln from 20 to 80 mM was evaluated as a supplement for a standard freezing extender (egg yolk – EY – 20%, and glycerol 3%). No significant improvement ($P > 0.05$) was obtained for any post-thaw sperm parameter assessed (objective sperm motility – CASA system – and flow cytometric analysis of plasma and acrosomal membrane integrity $-\text{SYBR14}/\text{PI}/\text{PE-PNA}$ and plasma membrane stability $-\text{M540}/\text{YoPro1}$). In Experiment 2, L-Gln was evaluated as a partial glycerol substitute in the freezing extender. Significant ($P < 0.05$) enhancement of post-thaw sperm motion parameters was achieved in sperm frozen in the presence of 2% glycerol and 80 mM L-Gln compared to control (3% glycerol). In Experiment 3, L-Gln was evaluated as an EY substitute in the freezing extender, and no functional sperm were recovered after thawing sperm frozen in the presence of L-Gln and the absence of EY. In conclusion, L-Gln has the ability to cryoprotect boar sperm when it is used as a partial glycerol substitute in the freezing extender.

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1. Introduction

In recent years, a wealth of new research has been conducted on different aspects of the cryopreservation process of boar semen (see review by Roca et al., 2006), helping to improve the fertility results...
of frozen–thawed (FT) semen in artificial insemination (AI) programs (Eriksson et al., 2002; Roca et al., 2003; Bolarin et al., 2006). However, there are still many weaknesses in the sperm cryopreservation process that should be improved before FT boar semen can be applied with the same efficiency of liquid semen in routine AI programmes in commercial pig farms. One important improvement should be adjustments in the composition of the freezing extenders (FEs).

The goal of developing more effective freezing extenders to both stabilize the sperm membranes and extend the functional life span of thawed sperm has still not been achieved (Roca et al., 2006). The basic ingredients of current sperm freezing extenders are the same as those used 35 years ago, including the cryoprotectant agents egg yolk (EY) and glycerol (Crabo and Einarsson, 1971; Pursel and Johnson, 1971). Cryoprotectant agents are the most important constituents of the freezing extender, and have a large influence on post-thaw sperm survival. Glycerol has chemical and osmotic toxic effects on sperm (Fahy, 1986; Woods et al., 2004) that can cause loss in post-thaw sperm motility and viability with subsequent decline in fertility when FT semen is used in AI programs. EY is the most effective agent to protect sperm against cold shock (Bergeron and Manjunath, 2006). However, at present, there are important arguments against the use of EY as a sperm freezing extender constituent, including the wide variability in egg composition (Kuksis, 1992) and the potential risk of contamination of semen if microbial or other contaminants are present in the raw product (Bousseau et al., 1998). Consequently, there is a need to evaluate alternative cryoprotectant agents to find agents that are less toxic than glycerol and that do not have the potential contamination risk of EY.

Some amino acids (AAs), particularly l-glutamine (l-Gln), have been employed in sperm cryopreservation for mammalian species. They have been successfully used for freezing goat (Kundu et al., 2001; Ali Al Ahmad et al., 2008), stallion (Trimeche et al., 1999), ram (Sanchez-Partida et al., 1998), monkey (Li et al., 2003) and human (Renard et al., 1996) sperm. However, no studies have been published on boar sperm cryopreservation using AA. The ability of AA to improve sperm cryosurvival has been related to their metabolic, cryoprotective, oxidative or osmoregulative properties (Martins-Bessa et al., 2007). Therefore, the objectives of the following experiments were to evaluate l-Gln as a freezing extender ingredient for cryopreserving boar sperm. We hypothesized that l-Gln would improve the cryosurvival of boar sperm when used in the freezing extender either as an additive to or a replacement for glycerol or EY.

2. Materials and methods

2.1. Reagents and media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma–Aldrich Quimica, S.A. (Madrid, Spain) and were prepared under sterile conditions (HH48, Holten LaminAir, Allerod, Denmark) with purified water (18.2 MΩ-cm, Milli-Q water purification system; Millipore Co., Billerica, MA). The basic medium used for sperm extension was Beltsville thawing solution (BTS, composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM NaHCO3, and 3.35 mM EDTA, pH 7.2 and 290 ± 5 mOsmol/kg, Johnson et al., 1988), containing kanamycin sulfate (50 μg/mL). The basic medium used for sperm freezing was Tris–citric acid–glucose (TCG) composed of 111 mM Tris (Trizma® Base), 31.4 mM monohydrate citric acid, 185 mM glucose and 100 mg/mL kanamycin sulfate (pH 7.2 and 312 ± 6 mOsmol/kg). The freezing extenders were made by adding EY (FE-1) and EY and glycerol (FE-2) to the TCG extender. The concentrations of EY and glycerol in the FE vary according to the experiment (see Section 2.4). The final concentrations of EY and glycerol in the standard FE were 20 and 3%, v/v, respectively. In addition, all the FE used incorporated 0.5% of Orvus ES Paste (Equex STM®, Nova Chemical Sales Inc., Scituate, MA, USA). The osmolarity of the FE-1 was 308.5 ± 3.8 mOsmol/kg and the FE-2 ranged from 807.4 ± 4.5 to 1772.7 ± 9.1 mOsmol/kg according to the final glycerol concentration used (from 1 to 3%, v/v). The addition of l-Gln increases the osmolarity of FE-1 and FE-2 between 7.2 ± 0.2 and 62.1 ± 0.9 mOsmol/kg according to the final concentration used (from 10 to 80 mM). The pH of the FE was adjusted to 7.1.
2.2. Source, handling, evaluation and processing of ejaculates

Five healthy mature (2–4 years of age) and fertile crossbreed boars housed at a commercial insemination station (AIM Ibérica S.A, Burgos, Spain) were used as ejaculate donors. The boars were housed in individual pens in an environmentally controlled (15–25 °C) building with windows exposed to a natural day length and supplementary light for a 16 h daily total of light. They were given ad libitum access to water and were fed a commercial diet according to the nutritional requirements for adult boars.

Sperm-rich ejaculate fractions were collected using the gloved-hand method, extended (1:1, v/v) in BTS and evaluated for conventional semen characteristics. Only ejaculates with $\geq 200 \times 10^6$ sperm/mL, $\geq 85\%$ sperm with normal morphology, and with $\geq 75$ and $\geq 80\%$ of motile and viable sperm, respectively, were selected for cryopreservation. The extended semen was transferred to 50 mL plastic tubes, cooled to 17 °C, packaged in insulated containers and sent (in under 2 h) to the laboratory (Pig Research Centre, Instituto Tecnológico Agrario de Castilla y León, Spain) for cryopreservation.

At the laboratory, extended sperm-rich fractions from were pooled and centrifuged at 2400 × $g$ for 3 min at 17 °C (Carvajal et al., 2004), and the pellet was suspended in FE-1 to yield a concentration of $1.5 \times 10^9$ cells/mL. After further cooling to 5 °C within 90 min, the extended sperm were re-suspended in FE-2 to yield a final concentration of $1 \times 10^9$ cells/mL. The cooled sperm were packed into 0.5 mL PVC-French straws (Minitüb, Tiefenbach, Germany), which were frozen using a controlled-rate freezer (IceCube 14S, Minitüb) as follows: from 5 to $-5$ °C at a rate of 6 °C/min, from $-5$ to $-80$ °C at 40 °C/min, held for 30 s at $-80$ °C, then cooled at 70 °C/min to $-150$ °C and plunged into liquid nitrogen (LN2). The straws remained in the LN2 tank for at least 2 weeks before thawing. Thawing of straws was done in a circulating water bath at 37 °C for 20 s. Thawed sperm samples from three straws were extended in BTS (1:2, v/v, 37 °C) and incubated in a water bath at 37 °C for 150 min.

2.3. Post-thaw sperm quality assessments

In each experiment, sperm quality was determined by assessing motility, sperm viability (plasma membrane integrity together with acrosomal status) and plasma membrane stability at 30 and 150 min after thawing.

2.3.1. Assessment of sperm motility

Sperm motility was objectively evaluated using a computer-aided sperm analysis system (ISAS®, Proiser R+D, Paterna, Spain) following the procedure described by Cremades et al. (2005). Briefly, semen samples were suspended in BTS to a concentration of 20–30 $\times 10^6$ sperm/mL. For each evaluation, a 4 μL aliquot of sperm sample was placed in a pre-warmed (39 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and three fields were analyzed at 39 °C, assessing a minimum of 100 sperm/sample. The proportions of total motile sperm (%TMS) and rapid progressively motile sperm (%RPM, VAP > 50 μm/s and STR > 75%) were recorded.

2.3.2. Assessment of plasma membrane and acrosome integrity

Plasma membrane and acrosome integrity were evaluated simultaneously following the procedure described by Nagy et al. (2003). Briefly, 100 μL of semen sample (30 × $10^6$ sperm/mL in Heps buffer: 10 mM HEPES/NaOH pH 7.5, containing 0.14 M NaCl and 2.5 mM CaCl2) were transferred into culture tubes, and dyes were added at a final concentration of 100 nM SYBR-14 (100 μM stock solution in dimethyl sulfoxide [DMSO], Component A of LIVE/DEAD Sperm Viability Kit, L-7011, Molecular Probes Europe BV, Leiden, The Netherlands), 1 μg/mL of peanut agglutinin conjugated with phycoerythrin solution (1 mg/mL stock solution Phycoprobe-PE-PNA, P44, Biomeda Co., Foster City, CA), and 12 μM propidium iodide (PI) (1.5 mM in phosphate buffer [PBS], Component B of Sperm Viability Kit). Samples were mixed and incubated at 37 °C in the dark for 10 min. Just before analysis, 400 μL PBS was added to each sample and remixed before they were run through a flow cytometer. Analyzed sperm were allocated into the following four categories: live with intact acrosome (SYBR-14+/PI−/PNA−), live with damaged acrosome (SYBR-14+/PI−/PNA+), dead with intact acrosome (SYBR-14−/PI+/PNA−) and dead
with damaged acrosome (SYBR-14−/PI+/PNA+). Only live sperm with an intact acrosome (LIA) were used for further analysis.

2.3.3. Assessment of plasma membrane stability

Plasma membrane stability was assessed after staining the sperm with Merocyanine 540 (M-540, Molecular Probes Europe BV) and YoPro-1 (Molecular Probes Europe BV) (Harrison et al., 1996). Aliquots of 100 µL of semen sample (30 × 10⁶ cells/mL) were diluted in 400 µL of phosphate buffer saline containing 0.5 µL of YoPro-1 (25 µM in DMSO), then mixed and incubated at 37 °C in the dark for 10 min. Just before analysis, 1.3 µL of M-540 (1 mM in DMSO) were added to each sample, incubated for 2 min and remixed before flow cytometer analysis. Analyzed sperm were allocated into the following three categories: viable sperm with a stable plasma membrane (YoPro-1−/M-540−), viable sperm with an instable plasma membrane (YoPro-1−/M-540+), and dead cells (YoPro-1+). Only viable sperm with a stable plasma membrane (VSM) was considered in results.

2.3.4. Flow cytometry analyses

All flow cytometry analyses were performed using an EPICS XL (Coulter Corporation Inc., Miami, FL, USA) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA) with 15 mW laser power at 488 nm and EXPO 2000 software (Coulter Corporation). Forward and side scatter were recorded in linear mode for a total of 10,000–25,000 events per sample, and only sperm-specific events, which appeared in a typically L-shaped scatter profile, were positively gated for the analysis. The green fluorescence of SYBR-14 and YoPro-1 was measured using a 525 nm band pass (BP) filter, the red fluorescence (PI) by a 620 nm BP filter, and PE-PNA and M-540 fluorescence was detected through a 575 nm BP filter. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified.

2.4. Experimental design

Three separate experiments, each using a completely randomized design, were performed to evaluate the cryoprotective effect of L-Gln on boar sperm. The semen source was always a pool of sperm-rich ejaculate fractions from five boars. The range of L-Gln concentrations tested (from 10 to 80 mM) include those that had effective results for freezing goat (Ali Al Ahmad et al., 2008), monkey (Li et al., 2003), stallion (Trimeche et al., 1999) and ram (Sanchez-Partida et al., 1998) sperm.

2.4.1. Experiment 1: L-glutamine as a supplement in the freezing extenders

Once the pooled sperm-rich fractions were centrifuged, the sperm pellet was split into five aliquots. Aliquots were diluted with the standard freezing extender (with final EY and glycerol concentrations of 20 and 3%, v/v, respectively) supplemented with L-Gln for a final concentration of 0 (as control), 10, 20, 40 or 80 mM. The extended aliquots were then cryopreserved according to the protocol described previously.

2.4.2. Experiment 2: L-glutamine as a partial substitute of glycerol in the freezing extender

The sperm pellet was split into seven aliquots. One aliquot, as the control, was diluted with the standard freezing extender (to get final EY concentration of 20% and different glycerol concentrations) supplemented with L-Gln for a final concentration of 0 (as control), 10, 20, 40 or 80 mM. The extended aliquots were then cryopreserved according to the protocol described previously.

2.4.3. Experiment 3: L-glutamine as a substitute of the egg yolk in the freezing extender

To carry out this experiment, the sperm pellet was split into six aliquots. Five aliquots were diluted with the freezing extender without EY and a final glycerol concentration of 3%, v/v, supplemented with L-Gln to get a final concentration of 0 (as control), 10, 20, 40 or 80 mM. The sixth aliquot, as the control,
Table 1
Post-thaw assessments of boar sperm samples frozen using a standard freezing extender supplemented with different concentrations of L-glutamine.a

<table>
<thead>
<tr>
<th>Post-thaw sperm assessmentb</th>
<th>Concentration of L-glutamine (mM)</th>
<th>S.E.M.</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>TMS (%)</td>
<td>47.8</td>
<td>46.0</td>
<td>47.3</td>
</tr>
<tr>
<td>RPMS (%)</td>
<td>33.0</td>
<td>29.8</td>
<td>29.6</td>
</tr>
<tr>
<td>LIA (%)</td>
<td>51.8</td>
<td>52.0</td>
<td>53.4</td>
</tr>
<tr>
<td>VSM (%)</td>
<td>49.4</td>
<td>49.8</td>
<td>49.5</td>
</tr>
</tbody>
</table>

a Data are from five replicates of pooled sperm from five boars. Values are mean ± S.E.M. of two separate measurements at 30 and 150 min after thawing sperm samples in a water bath at 37 °C.

b TMS indicates total motile sperm; RPMS, rapid progressively motile sperm (0.50 μm/s); LIA, live sperm with intact acrosome; VSM, viable sperm with stable plasma membrane.

§ No statistical differences to P > 0.05.

was diluted with the standard freezing extender (to final EY and glycerol concentrations of 20 and 3%, v/v, respectively). Samples were then cryopreserved according to the protocol described previously.

2.5. Statistical analysis

Statistical analyses were performed by SPSS Version 14 (SPSS Inc., Chicago, IL). Data from five replicates of each experiment were analyzed as a split plot design using a mixed model analysis of variance (ANOVA). To fulfill the assumption of a normal distribution, percentage data of post-thaw sperm quality assessments were log-transformed before statistical analysis. Data were examined according to a statistical model that includes the fixed effects of L-Gln concentration and post-thaw evaluation times (30 and 150 min), and the random effect of replication. There were no interactions (P > 0.05) between L-Gln concentration and post-thaw evaluation times for any of the sperm assessments evaluated. Therefore, means were averaged throughout post-thaw evaluation times to evaluate the main effect of L-Gln concentration. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when P was less than 0.05. Results are presented as least-squares means ± S.E.M.

3. Results

3.1. Experiment 1: L-glutamine as a supplement in the freezing extenders

The effects of the supplementation of standard FE with L-Gln at 0, 10, 20, 40 or 80 mM on post-thaw sperm quality are shown in Table 1. None of the L-Gln concentrations added to the standard FE significantly affected (P > 0.05) any of the post-thaw sperm parameters evaluated. These results show that L-Gln, at the concentrations tested, is not able to improve the freezability of boar sperm frozen in a standard FE containing 20 or 3% EY or glycerol, respectively.

3.2. Experiment 2: L-glutamine as a partial substitute of glycerol in the freezing extender

The effectiveness of L-Gln in reducing the glycerol concentration of the FE is summarized in Table 2. When L-Gln was not added to the FE, the reduction of glycerol concentration from 3 to 2 or 1% negatively affected (P < 0.05) post-thaw sperm survival. The addition of L-Gln (20 or 80 mM) to the FE improved post-thaw sperm quality independently of the glycerol concentration used (1 or 2%). However, the magnitude of the improvement was influenced by the glycerol and L-Gln concentrations. The best post-thaw sperm quality was achieved in the sperm samples frozen using FE with 2% glycerol and 80 mM L-Gln, but the improvement was only significantly different (P < 0.05) from the control (with a 3% of final glycerol concentration and without L-Gln) for the motion parameters (TSM and RPMS).
Table 2
Post-thaw assessments of boar sperm frozen using extenders with reduced glycerol concentration and supplemented with L-glutamine.a.

<table>
<thead>
<tr>
<th>Freezing extender composition</th>
<th>Post-thaw sperm assessments (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (%): l-Glutamine (mM)</td>
<td>TMS</td>
</tr>
<tr>
<td>3 0</td>
<td>51.0b</td>
</tr>
<tr>
<td>2 0</td>
<td>39.8c</td>
</tr>
<tr>
<td>20</td>
<td>51.1a,b</td>
</tr>
<tr>
<td>80</td>
<td>57.4a</td>
</tr>
<tr>
<td>1 0</td>
<td>37.8c</td>
</tr>
<tr>
<td>20</td>
<td>36.9c</td>
</tr>
<tr>
<td>80</td>
<td>47.4b</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>1.7</td>
</tr>
<tr>
<td>Probability</td>
<td>0.001</td>
</tr>
</tbody>
</table>

†TMS indicates total motile sperm; RPMS, rapid progressively motile sperm (0.50 μm/s); LIA, live sperm with intact acrosome; VSM, viable sperm with stable plasma membrane.

a Data are from five replicates of pooled sperm from five boars. Values are mean ± S.E.M. of two separate measurements at 30 and 150 min after thawing sperm samples in a water bath at 37 °C. a–c Statistical differences to P < 0.05.

3.3. Experiment 3: L-glutamine as a substitute of the egg yolk in the freezing extenders

Neither motile, live nor even stable sperm could be recovered in thawed sperm samples frozen using FE without EY and supplemented with L-Gln, independent of the L-Gln concentration used. In contrast, the percentages of TMS, RPMS, LIA, and VIA sperm were 49.5, 36.5, 51.8 and 53.5%, respectively, in the sperm samples frozen using the standard FE. These results indicate that L-Gln, at the concentration tested, cannot replace the cryoprotective effect of EY on boar sperm.

4. Discussion

The cryoprotective properties of L-Gln for mammalian cells were first noticed 20 years ago by Kruuv et al. (1988) and were later confirmed for sperm by Kruuv and Glofcheski (1992). Nowadays, the cryoprotective ability of L-Gln has been confirmed for human (Renard et al., 1996), equine (Trimeche et al., 1999) and goat (Ali Al Ahmad et al., 2008) sperm. Surprisingly, L-Gln has never been tested in swine semen, in spite of the increasing interest in improving the sperm freezability in this species (Roca et al., 2006).

Looking at the above reports and others (Kundu et al., 2001; Khelifaoui et al., 2005), the following three features can be deduced with respect to the cryoprotective properties of L-Gln to mammalian sperm: (1) the effective concentration varies according to the species, indicating species differences in the sensitivity of sperm to L-Gln, (2) concentrations above 100 mM are either ineffective or toxic, indicating that the effects are biphasic and that L-Gln at higher concentrations may have a cytotoxic effect, and (3) the cryoprotective effect is mainly seen in an enhancement of the percentage of motile sperm after thawing. Keeping in mind these first two features, the concentration range evaluated in our experiments (from 20 to 80 mM) included those concentrations effective for the sperm of other mammalian species and avoided concentrations that could be ineffective or toxic.

In the first experiment, there was no significant improvement of post-thaw sperm parameters evaluated when L-Gln was added to a standard freezing extender containing 3% glycerol, indicating that L-Gln did not provide additional cryoprotection to boar sperm when they were frozen in extenders with optimal glycerol concentration. Three percent was demonstrated to be the optimal glycerol concentration to freeze boar sperm packaged in 0.5 mL straws (Hernandez et al., 2007). These results are in contrast with those achieved in goat (Ali Al Ahmad et al., 2008), equine (Trimeche et al., 1999) and human (Renard et al., 1996) sperm, in which L-Gln improved the motility rates of thawed sperm frozen in extenders with optimal glycerol concentration. Consequently, there are species differences in the response of sperm to L-Gln when it is used as an additive in a standard freezing extender. For
boar sperm, it seems that the potential cryoprotective effects of L-Gln are masked by the effects of the optimal glycerol concentration.

Kruuv and Glofcheski (1992) suggested that L-Gln could partially substitute for standard cryoprotectant agents. With this in mind, we carried out a second experiment using freezing extenders combining L-Gln together with suboptimal glycerol concentrations. Reducing the glycerol concentration in freezing extenders should always be an objective because boar sperm are particularly affected by the toxic effects of glycerol due to their membrane sensitivity to hyperosmotic stress. Despite that, glycerol is still essential for boar sperm cryopreservation, since less toxic cryoprotectant agents, that are equally as effective, have not yet been found (Bwanga, 1991). Therefore, one interesting alternative could be to partially substitute glycerol with other substances with potential cryoprotective effectiveness, such as L-Gln. In our experiment, L-Gln, particularly at a concentration of 80 mM, enhanced the post-thaw proportion of motile, progressive, viable and membrane stable sperm in the samples frozen with decreased glycerol concentration, reaching similar results to control samples frozen in the presence of 3% glycerol. This data indicates that L-Gln is able to partially replace glycerol as a cryoprotectant for boar sperm. However, our results differ with those of Renard et al. (1996), who did not find any beneficial effect of L-Gln for freezing human sperm when suboptimal glycerol concentration was used. Nevertheless, our findings are in concordance with those achieved by Khlifaoui et al. (2005) in stallion sperm. It is interesting to emphasize that these authors proposed a singular cryoprotectant method in which L-Gln would be able to substitute partially for glycerol for freezing ejaculates with intermediate and good sperm freezability, but not for those ejaculates with bad sperm freezability. This may be a reasonable explanation for our results, because the ejaculates cryopreserved in the present experiments showed an acceptable sperm freezability. In this context, it seems that the cryoprotective effects of L-Gln would be weaker than those of glycerol, so L-Gln would be a poor partial substitute for glycerol for cryoprotecting sperm with bad freezability.

Khlifaoui et al. (2005), by means of the radioactive isotope 3H-Gln, demonstrated that L-Gln did not penetrate the sperm membranes, and suggested that their cryoprotective action is at the extracellular level. Consequently, it appears that L-Gln probably carried out cryoprotective actions on the sperm membranes in either a direct or indirect way. However, the mode of action still remains unclear. One possibility is that L-Gln could prevent lipid peroxidation by protecting the sperm antioxidant enzymatic defenses, such as superoxide dismutase or glutathione peroxidase (Renard et al., 1996). This does not seem to be the explanation for our results for two reasons. First, because the antioxidant properties of L-Gln should be independent of the glycerol concentration of the freezing extender, and this was not the case in our study, where the sperm cryoprotective effectiveness of L-Gln was only evident when the glycerol concentration of freezing extender was lowered. Secondly, the addition of the mentioned enzymes to the freezing extender significantly improves post-thaw sperm quality (Roca et al., 2005), indicating that the levels of these enzymes in the boar ejaculate are not sufficient for protecting sperm from lipid peroxidation during the cryopreservation process. Another hypothesis is that L-Gln could play a direct role in the cryoprotective action on the sperm membranes, for example, as a membrane stabilizer (Anchordoguy et al., 1988; Kundu et al., 2001). Our results could agree with this possibility because the percentage of sperm with stable membranes in the second experiment was enhanced in the sperm samples frozen in presence of L-Gln, with respect to those control sperm samples frozen in extenders with low glycerol concentration alone. In order to verify this possibility, a third experiment was carried out to determine whether L-Gln alone is able to substitute for EY, demonstrating the above suggested membrane stabilizer properties. In this context, note that EY is still an indispensable constituent of semen extenders due its ability to protect sperm membranes against cold shock and freezing damage (Watson and Martin, 1975). However, EY should ideally be avoided due to serious concerns regarding bacterial and viral contamination, and alternatives to EY are subjects of active investigation. Considering the results of the third experiment, it seems clear that L-Gln alone is unable to substitute for EY in freezing extenders for protecting boar sperm membranes. Moreover, this experiment suggests that the protective properties of L-Gln for sperm membranes during the freezing and thawing processes do not work with L-Gln alone, because no sperm with either intact or stable membranes were recovered after thawing in the sperm samples frozen using freezing extenders with L-Gln but not containing EY. In this sense, it is interesting to
remember that other membrane stabilizers such as butylated hydroxytoluene have the ability to protect boar sperm membranes during the cryopreservation process in the absence of EY (Rodriguez-Vilar et al., 2007).

As previously indicated, reports evaluating the cryoprotective effectiveness of L-Gln for mammalian sperm demonstrated that the presence of L-Gln in the freezing extender enhanced post-thaw sperm motility. Our results are partially in agreement with this observation. Higher post-thaw motility assessments were achieved in sperm frozen with extenders with a suboptimal concentration of glycerol and supplemented with 80 mM L-Gln. This indicates that L-Gln is not only able to partially substitute for glycerol as a sperm cryoprotectant, but also provides additional sperm cryoprotection, which is visualized by an enhancement of post-thaw sperm motility assessments. The mechanisms by which L-Gln protects the motility properties of cryopreserved sperm remain unclear. While it seems clear that the cryoprotective mechanisms are different from those of glycerol, it is unclear whether L-Gln and glycerol are dependent on one another. For human and equine sperm, it has been suggested that the cryoprotective mechanisms would be independent (Trimeche et al., 1996; Renard et al., 1996). We disagree with this suggestion, because the cryoprotective effect of L-Gln on boar sperm was only visualized when the glycerol concentration of freezing extender was lowered. Consequently, it seems that the mechanisms are dependent, or at least complementary. Keeping this in mind, we suggest the following mechanism of action: L-Gln would partially substitute the cryoprotective capacity of glycerol in its role as a non-penetrating cryoprotectant agent, protecting sperm membranes during freezing and thawing processes. Then, the adverse chemical and/or osmotic toxicity of glycerol to sperm would be minimized because its concentration in the freezing extender is decreased, which would be evidenced by an enhancement of sperm motion parameters after thawing. As previously described, lowering glycerol concentration in the freezing extender improves post-thaw sperm motility (Critser et al., 1988).

5. Conclusion

In conclusion, these results suggest that L-Gln has the ability to cryoprotect boar sperm, which was particularly shown by an enhancement of post-thaw sperm motility. However, the cryoprotective effect is dependent on the glycerol concentration of the freezing extender, and is only evident when the glycerol concentration is lowered. Additional studies will be required to elucidate the specific cryoprotective mechanisms of L-Gln for boar sperm, which could be different from those suggested for the sperm of other mammalian species.

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