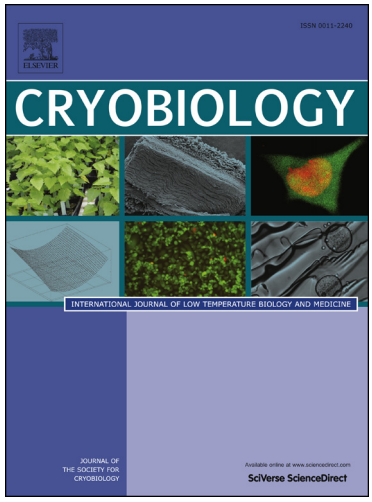

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Improved cryopreservation protocol for Blanca-Celtibérica buck semen collected by electroejaculation

Pilar Jiménez-Rabadán[a], Manuel Ramón[a], Olga García-Álvarez[b], Alejandro Maroto-Morales[b], Pablo J. Álvaro-García[a], Enrique Del Olmo[b], Mª Dolores Pérez-Guzmán[a], Mª Rocio Fernández-Santos[b] , J. Julián Garde[b], Ana J. Soler[b]

[a]Regional Center of Animal Selection and Reproduction (CERSYRA) JCCM, 13300 Valdepeñas, Spain

[b]SaBio (IREC, CSIC-UCLM-JCCM), 02071 Albacete, Spain

*Corresponding author:

Ana J. Soler Valls

ETSIA. Campus Universitario s/n, 02071-Albacete (España)

Phone: +34 967 599 200+2552

Fax: +34 967 599 238

e-mail: anajosefa.soler@uclm.es

Abstract

The collection of sperm samples by electroejaculation (EE) leads to an increase of the production of seminal plasma which could modify the tolerance of spermatozoa to the cryopreservation procedure. This study aims to compare a standard sperm cryopreservation protocol for samples collected by artificial vagina (AV) with the same protocol and modifications to this for samples obtained by EE. Semen from six males of Blanca-Celtibérica goat breed was collected by AV (control) and EE, and three experiments were conducted. In Experiment 1, it was examined the effects of egg yolk concentration contained in freezing extender (0, 1.5, 10 and 20% of egg yolk); in Experiment 2, it was evaluated the cooling rate from 30 ºC to 5 º C (*fast*: 10 min and *slow*: 90 min) and the temperature of glycerol addition (30 ºC and 5 ºC); and in Experiment 3, it was examined the time of equilibration at 5 ºC (0, 1, 2 or 3 hours). A heterologous *in vitro* fertilization test was carried out in order to compare the fertility of control samples with that resulting from the EE protocol which showed the highest sperm quality. Results showed greater sperm motility parameters after thawing for control samples cryopreserved in standard conditions in the three experiments. For samples collected by EE, extender with 20% egg yolk, a slow cooling rate and a longer equilibration time (3 h) provided higher sperm quality, and no differences were observed between temperatures of glycerol addition. Samples collected by EE and cryopreserved with the protocol which yielded the best sperm quality after thawing showed higher fertility compared to AV.

Keywords: electroejaculation, egg yolk, cooling rate, glycerol, equilibration, Blanca-Celtibérica buck

Introduction

The Blanca-Celtibérica goat is an autochthonous breed from Spain considered to be endangered (RD 2129/2008) and its conservation by genetic resource banks through semen cryopreservation is a key tool to ensure the long-term availability of genetic resources. Hence, the importance of defining optimal cryopreservation protocols in order to obtain the best post-thaw sperm characteristics.

It is important to note that the first step in the sperm cryopreservation is the collection of ejaculates. The electroejaculation (EE) is an alternative collection method to obtain semen in species or breeds which are bred and reared in extensive systems being necessary using different strategies instead of routine reproductive techniques. To date, the majority of studies carried out for cryopreserving buck semen have used samples collected by AV and few of them have used other semen collection methods. Differences on characteristics of ejaculates obtained by different collection methods have been observed in various species [16,17,18,21] and it has been suggested that the EE varies from physiological ejaculation [2]. Thus, the electrical stimuli during the EE artificially stimulate the accessory glands causing an increase of volume [20], which results in greater amount of seminal plasma. In addition, differences on seminal plasma composition have been demonstrated between semen obtained by AV and EE in rams [18] and it could affect to sperm membranes being spermatozoa more or less vulnerable to cryopreservation procedure [2]. One of the few studies which compared the effect of semen collection method (AV *vs.* EE) on sperm cryopreservation in goats reported lower quality after thawing for samples obtained by EE, suggesting a detrimental effect of seminal plasma on cryopreservation [16]. This could be due to the presence in the seminal plasma of a phospholipase secreted by bulbourethral gland which catalyzes the

hydrolysis of lipids in the egg yolk to fatty acids and lysophospholipids which are toxic to buck spermatozoa [14,27] and this effect may be more marked in samples collected by EE. Hence, sperm samples collected by EE could require different freezing extenders and protocols to carry out a successful cryopreservation.

Although the sperm cryopreservation using different freezing extenders for samples from different origin has been studied for buck semen [16], few studies have been carried out on the effect of modifying other factors involved during the cryopreservation procedure. In order to avoid the negative effect of seminal plasma in the buck sperm cryopreservation, most of studies made changes on the concentration of egg yolk contained in the freezing extender with results different, although most of them were conducted in samples collected by AV and few studies used the electroejaculation as method of semen collection [1,3,6,25]. In addition, other factors such as reducing the period of contact between spermatozoa and seminal plasma during the cooling and equilibration steps could report a beneficial effect on sperm cryopreservation for samples collected by EE.

With this background, the objective of this study was to examine the effect of different factors involved during the cryopreservation process, namely, egg yolk concentration, temperature of glycerol addition, cooling rate and equilibration time in samples collected by EE from Blanca-Celtibérica bucks to achieve samples with similar sperm quality than those obtained by AV. In addition to this, a heterologous *in vitro* fertilization test was carried out to evaluate the fertility of the control (collected by AV) and EE samples frozen with the best cryopreservation protocol.

Material and Methods

Animals and reagents

Animal handling was performed in accordance with Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63. Six males of Blanca-Celtibérica goat breed (age > 1.5 years) were used. All males were maintained and managed at the Regional Center of Animal Selection and Reproduction (CERSYRA) located in Valdepeñas (Spain). The thawing procedure was conducted at laboratories from SaBio group in Albacete (Spain).

Chemicals were of reagent grade and were purchased from Sigma (Madrid, Spain). Biladyl® was purchased from Minitüb (Tiefenbach, Alemania). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) which was purchased from Sigma. Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA).

Semen collection

All experiments were carried out during breeding season, from September to December. For each male, the collection of ejaculates was performed using AV and EE, both on the same day. Males were trained to conduct collections by AV and ejaculates were routinely collected once per week. The procedure of EE was carried out using the protocol described by Garde et al. [10]. Males were anesthetized with xylacine (0.2mg/kg Rompun® 2% i.m.; Bayer S.A., Barcelona, Spain), the rectum was cleaned of faeces and the prepucial area was shaved and washed with physiologic saline serum. A three electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The EE regime consisted

of consecutive series of 5-sec pulses of similar voltage, each separated by 5-sec break. Each series consisted of a total of four pulses. The initial voltage was 1 V which was increased in each series until a maximum of 5 V. Urine contamination was tested, and ejaculates with urine were rejected. Three ejaculates per male and collection method were obtained for all experiments.

Evaluation of ejaculates

Immediately after the collection of ejaculates, the volume of ejaculate (measured in a conical graduated tube), sperm concentration (by spectrophotometer) and wave motion (scored on a scale of 0 to 5) were evaluated. Spermatozoa total number (STN) was calculated with the volume and concentration (Volume × Concentration). The proportion of motile spermatozoa (SM) was evaluated subjectively in aliquots of semen diluted (1:200) in a phosphate buffer saline (PBS) and incubated for 5 min at 37 ºC, using a phase-contrast microscope (× 100).

Semen cryopreservation

Sperm samples were collected for each male by AV (control samples) and by EE from six males of Blanca Celtibérica breed in each experiment. For all experiments, the commercial extender Biladyl® was used. Egg yolk added to the extender was clarified as described by Holt et al. [12]. Control samples were frozen by the standard conditions usually used for buck semen cryopreservation [5]. These conditions were: dilution in extenders containing 20% egg yolk, cooling to 5 ºC for 90 min, addition of glycerolated fraction of extender at 5 ºC and equilibration time for 2 hour.

Experiment 1: Effect of the concentration of egg yolk contained in freezing extender on sperm quality at thawing

Each ejaculate was divided into 4 aliquots and diluted with four extenders containing different egg yolk concentration: 0%, 1.5%, 10% and 20%. The dilution was performed by two-step method. Each aliquot was diluted with the Fraction A of diluent at 30 ºC and cooled to 5 ºC for 90 min. Then, it was further diluted (v:v) with Fraction B containing glycerol (7%) and maintained at 5 ºC for 2 h more before freezing. At the end of this time, the diluted semen was loaded into 0.25 mL plastic straws and frozen in nitrogen vapours for 10 min by placing the straws 4 cm above the surface of liquid nitrogen. The straws were subsequently plunged into liquid nitrogen and stored. Sperm samples were frozen at a final concentration of 100-200 × 10^6 spermatozoa/mL.

Since results from this experiment showed Biladyl® with 20% egg yolk as the best extender to buck sperm cryopreservation, it was used in Experiments 2 and 3.

Experiment 2: Effect of cooling rate and temperature of glycerol addition on sperm quality at thawing

In the Figure 1 is showed the experimental design for experiment 2. Briefly, each ejaculate was diluted with the non-glycerolated fraction of Biladyl® (Fraction A) with 20% egg yolk at 30 ºC and divided into four aliquots in order to evaluate two cooling rates: a *slow cooling rate* in which temperature dropped from 30 ºC to 5 ºC in 90 min and a *fast cooling rate* in which temperature dropped from 30 ºC to 5 ºC in 10 min, and two temperatures of glycerol addition (Fraction B) at 30 ºC and at 5 ºC. Samples in which the glycerolated fraction was added at 30 ºC were cooled to 5 ºC by means of fast and slow cooling rates, respectively. For samples which were cooled to 5 ºC before adding glycerol, using both fast and slow cooling, the glycerol was added at 5 ºC after finishing the cooling time. All aliquots were maintained at 5 ºC for 2 hours more (equilibration time) and then the diluted semen was loaded into 0.25 mL plastic straws

and frozen over nitrogen vapours as described in Experiment 1. Sperm samples were frozen at a final concentration of 100-200 × 10^6 spermatozoa/mL.

Experiment 3: Effect of equilibration time on sperm quality at thawing

Since results in Experiment 2 showed that slow cooling rate reported better results at thawing, it was used in this experiment. Regarding temperature of glycerol addition, we used the temperature of 5 ºC for convenience, due to this temperature was used for the control samples and there were no differences between adding glycerol at 30 ºC or 5 ºC for samples collected by EE.

Each ejaculate was diluted by the two-step method with Fraction A of Biladyl® with 20% egg yolk at 30 ºC and cooled to 5 ºC for 90 min. Then, it was further diluted with Fraction B containing glycerol at 5 ºC and the diluted ejaculates were divided into 4 aliquots and maintained at 5 ºC for 0, 1, 2 and 3 h (equilibration time), respectively. After equilibration time, samples were loaded into 0.25 mL plastic straws and frozen over nitrogen vapours as described in Experiment 1. Sperm samples were frozen at a final concentration of 100-200 × 10^6 spermatozoa/mL.

Thawed semen evaluation

Thawing was performed by placing the straws in a water bath at 37 ºC for 30 sec, and then emptying the content into dry tubes and incubating for 5 min at the same temperature.

For thawed sperm samples, samples were fixed in buffered 2% glutaraldehyde solution to assess NAR by observing spermatozoa using phase-contrast microscopy (400×). Motility was evaluated by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA® 2002, Microptic, Barcelona, Spain) with the following software settings adjusted to goat spermatozoa: 25 frames/s, 20–70 μm^2 for head area,

183 velocity limit for slow sperm: 30 μm/s, velocity limit for medium sperm: 45 μm/s,
184 velocity limit for fast sperm: 75 μm/s and minimal straightness for progressive
185 spermatozoa: 80%. Sperm samples were diluted (v:v) with TALP-HEPES medium (87
186 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 40 mM
187 HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 μg/mL kanamycin, 10
188 μg/mL phenol red, and 6 mg/mL bovine serum albumin (BSA) (pH 7.5)) and 5 μL were
189 put on a Makler chamber. At least 200 spermatozoa were counted with SCA® using a
190 phase contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan) at ×100
191 magnification and the following motility parameters were assessed: percentage of
192 motile spermatozoa (TM; %), percentage of progressive motile spermatozoa (PM; %),
193 straight line velocity (VSL; μm/sec), linearity index (LIN; %), and amplitude of the
194 lateral displacement of the sperm head (ALH; μm).

195 Also, aliquots of thawed semen were used to carry out flow cytometry analyses.
196 It was assessed the membrane stability with YO-PRO-1, the mitochondrial membrane
197 potential with Mitotracker Deep Red and the viability with Propidium Iodide (PI) [19].
198 A staining solution using TALP-HEPES was prepared by adding 50 nM YO-PRO-1
199 (stock: 100 μM in DMSO) and 15 μM propidium iodide (stock: 7.5 mM in milli-Q
200 water). We diluted 20 μL of sample in 0.5 mL of staining solution in polypropylene
201 tubes for flow cytometry. The tubes were kept in dark for 15 min. Mitochondrial
202 membrane potential of the samples was evaluated with 0.1 μM YO-PRO-1 and 100 nM
203 Mitotracker Deep Red solution (stock: 1 mM in DMSO) in TALP-HEPES. Sperm
204 samples were diluted to 1×10^6 spermatozoa/mL, and 300 μL were transferred to a
205 polypropylene tube and left in the dark for 30 min. After incubation times, sperm
206 samples were analyzed using a Cytomics FC500 flow cytometer (Beckman coulter, Inc.

USA) and the following parameters were taken into account: YO-PRO-1-/PI- spermatozoa were considered as intact cells (indicating live spermatozoa with intact plasmalemma), YO-PRO-1+/PI– as apoptotic spermatozoa (indicating live spermatozoa with altered plasmalemma) and Mitotracker+/YO-PRO-1- as viable spermatozoa with high mitochondrial membrane potential.

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics, Inc., Brookings, SD, USA) [8]. This technique is based on the susceptibility of the sperm DNA to acid-induced denaturation *in situ* and the metachromatic staining Acridine Orange (AO). This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denaturated). Thawed spermatozoa were diluted with TNE buffer to 2×10^6 cells/mL. Samples were flash frozen in LN_2 and stored at -80 ºC until analysis. We calculated the DNA fragmentation index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red + green). High values of DFI indicate chromatin abnormalities. The %DFI was calculated as the percentage of spermatozoa with DFI > 25.

In vitro fertilization test (IVF)

Control samples (those collected by AV) and samples collected by EE and with best sperm quality after different modifications of cryopreservation protocol (those freezing with 20% egg yolk, temperature of glycerol addition 30 °C or 5 °C, slow cooling rate and long equilibration time) were also used to perform a heterologous *in vitro fertilization test* (IVF) to assess the fertilization ability. A pool of three males with similar sperm quality belong to the same treatment (electroejaculation or artificial vagina) was used for IVF.

Heterologous IVF was carried out three times for each treatment and thirty oocytes per well and replicate were used (*n*=180). Ovaries were collected at slaughterhouse from prepubertal sheep and transported to our laboratory in saline solution (25-30 ºC) between 1-2 h after removal. Ovaries were sliced using a micro-blade and the follicle content was released in TCM 199 medium supplemented with HEPES (2.38 mg/mL), heparin (2µL/mL) and gentamycin (40µg/mL). Cumulus oocyte complexes (COC) were washed in TCM 199-gentamycin (40 µg/mL), and those dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected and randomly placed in four-well plates containing 500 µL of TCM 199 supplemented with cysteamine (100 µM), FSH/LH (10 µg/mL), FCS (10%) and gentamycin (40 µg/mL), and matured at 38.5 ºC in 5% CO_2. After 24 h, COC were washed in fertilization medium (synthetic oviduct fluid (SOF) supplemented with gentamycin (40 µg/mL), 20% of oestrous sheep serum and heparin (1 µL/mL)) and cumulus cells were removed by gentle pipetting. Oocytes were transferred into four-well plates containing 450 µL of fertilization media under mineral oil.

Thawed spermatozoa were selected on a Percoll® discontinuous density gradient (45/90) and were capacitated in the fertilization medium for 1 h. Finally, spermatozoa were co-incubated with oocytes at a final concentration of 1×10^6 spermatozoa/mL for 18 h at 38.5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere. Presumptive zygotes were cultured at 18 h of insemination in SOF supplemented with BSA [11] for 48 h at 38.5 ºC in 5% CO_2, 5% O_2 and 90% N_2.

Fertility (cleavage rate) was assessed at 48 h post insemination by phase-contrast microscopy.

Statistical analysis

The R program (R Core team, 2012) was used to perform statistical analysis. Numeric results are expressed as the mean ± standard error of the mean (SEM). Data were considered statistically significant when $P \leq 0.05$.

The mixed effects models used in the analyses were the following:

Exp 1: $y = EY_{concentration} + male + replicate(male) + \varepsilon$

Exp 2: $y = COOLING_{rate} + T^{a}{}_{glicerol} + COOLING_{rate} \times T^{a}{}_{glicerol} + male + replicate(male) + \varepsilon$

Exp 3: $y = EQ_{time} + male + replicate(male)$

where,

y represents the different sperm quality parameters at thawing,

$\boldsymbol{EY_{concentration}}$ is the egg yolk concentration (0, 1.5, 10 and 20 %),

$\boldsymbol{COOLING_{rate}}$ is the cooling rate (slow=90 min, *vs.* fast=10 min),

$\boldsymbol{T^{a}_{glycerol}}$ is the temperature of glycerol addition (30 ºC *vs.* 5 ºC),

$\boldsymbol{COOLING_{rate} \times T^{a}_{glycerol}}$ is the interaction between cooling rate and temperature of glycerol addition,

$\boldsymbol{EQ_{time}}$ is the length of equilibration step (0, 1, 2 and 3 hours),

male is the effect of male (n=6), treated as random, and

replicate(male) is the effect of replicate for each experiment (n=3) within male, treated as random.

When necessary, multiple comparisons were performed using the Bonferroni adjustment.

A GLM (General Lineal Model) ANOVA tested the effect of collection method (electroejaculation *vs.* artificial vagina) on *in vitro* fertility.

Results

Sperm characteristics for ejaculates obtained by AV and EE

Ejaculates collected by AV and EE showed great values of sperm viability, motility and NAR. Differences ($P \leq 0.05$) between collection methods were observed for sperm concentration, STN and SM, showing lower values for samples obtained by EE, except for SM which was higher for this kind of samples (Table 1).

Experiment 1: Effect of the concentration of egg yolk contained in freezing extender on sperm quality at thawing

Control samples showed higher values ($P \leq 0.05$) for TM and PM compared to EE samples frozen with the different extenders. However, there were no differences ($P > 0.05$) for the other sperm parameters between control and EE samples frozen with 10 or 20% egg yolk except for Mitotracker+/YOPRO- with greater values for AV samples compared to EE samples frozen with 10% egg yolk (Tables 2 and 3). Nevertheless, those EE samples frozen with 0 or 1.5% egg yolk displayed lower values ($P \leq 0.05$) for the most sperm parameters assessed in relation to those frozen with 10 or 20% egg yolk and control samples. (Tables 2 and 3).

Experiment 2: Effect of cooling rate and temperature of glycerol addition on sperm quality at thawing

Regarding the cooling procedure, control samples showed higher values ($P \leq 0.05$) for TM compared to EE samples and no differences ($P > 0.05$) for other parameters were found between control samples and EE samples cooled slowly (Tables 2 and 3). There were no differences ($P > 0.05$) for the most sperm parameters for EE samples cooled slowly or fast except for TM with higher values ($P > 0.05$) for those processed slowly (Table 2).

In relation to the temperature of glycerol addition, control samples showed higher TM in relation to those collected by EE ($P \leq 0.05$). No differences ($P > 0.05$) between both temperatures of glycerol addition were found on sperm quality after thawing in samples obtained by EE.

The interaction glycerol temperature × cooling rate was no significant ($P > 0.05$).

Experiment 3: Effect of equilibration time on sperm quality at thawing

No differences ($P > 0.05$) were observed between control samples and samples collected by EE and equilibrated for 3 h, except for TM with higher values for control samples.

For sperm samples collected by EE, the equilibration time for 3 h provided better results on sperm quality after cryopreservation compared to 0 and 1 h. Thus, YO-PRO-1/PI- and Mitotracker+/YO-PRO-1- were higher ($P \leq 0.05$) when samples were equilibrated for 3 h. There were no differences between equilibration times for TM, NAR and velocity parameters and %DFI (Tables 2 and 3).

In vitro fertilization test

Sperm samples collected by EE and frozen with the best sperm cryopreservation protocol (20% egg yolk, slow cooling rate, glycerol addition at 5 ºC and equilibration for 3h) provided higher fertility ($P \leq 0.05$) than control samples, (35 ± 1.81 % *vs*. 28 ± 1.81%) (Figure 2).

Discussion

The correct design and optimization of the sperm cryopreservation protocol is a key step for the creation and maintenance of a cryobank. Semen collection is performed

traditionally by artificial vaginal (AV), although alternative methods exist. The electroejaculation (EE) is a substitute to collect semen from untrained or wild males whose training to AV is not possible, being a suitable method for Blanca-Celtibérica bucks due to its production system. However, this collection method could lead to different ejaculates from those obtained by physiological way and could influence on the response of spermatozoa to different procedures such as cryopreservation.

The aim of this study was to assess modifications in the cryopreservation protocol in Blanca-Celtibérica buck semen collected by EE for improving sperm quality after thawing, achieving samples with similar quality to those obtained with AV. The collection method had an effect on sperm production parameters such as the concentration and STN, with higher values for AV samples. These results were similar to those found previously by our group [16] and in the same line that those reported by Memon et al. [21] and Marco-Jiménez et al. [17,18] in bucks and rams, respectively. During the EE procedure, sexual glands are artificially stimulated and the volume of seminal plasma varies in relation to physiological ejaculate [17,18]. Although initially no differences were found in sperm quality for the most sperm parameters assessed in ejaculates collected by AV and EE, after sperm cryopreservation the AV samples showed higher values for motility parameters than those obtained by EE. However, there were no differences between AV samples and those from EE frozen with the best cryopreservation protocol. Our results agree with those reported by Álvarez et al. [2] who observed lower values of motility for EE samples and similar values for the percentage of normal acrosomes and viability compared to AV samples. Nevertheless, in a study conducted previously by our group, we found a worse sperm quality for the most of sperm parameters assessed in samples collected by EE compared to those

obtained by AV [16]. This fact could be due to the individual variability observed in the resistance to cryopreservation for EE samples. Thus, some males showed frequently low values of sperm quality because of freezing extender is coagulated. However, the same males displayed acceptable values of sperm quality when samples were collected by AV. A specific problem for buck semen cryopreservation has been described when extenders containing egg yolk are used due to the presence of a phospholipase enzyme in the seminal plasma [14,27]. Seminal plasma from EE samples have a different composition from that collected by AV [17,18], affecting possibly the response of spermatozoa to cryopreservation procedure and this effect may be more noticeable in some males.

The best cryopreservation protocol reported for EE samples involved changes in the concentration of egg yolk, cooling rate and equilibration time. Regarding to studies about egg yolk, 20% rendered the best sperm quality being our results similar to a study conducted by Daskin et al. [6], although other authors found that lower egg yolk concentrations preserved better the sperm quality during cryopreservation than the greater concentrations [26]. This could be due to differences in the cryopreservation extender, in the species or even to the season of semen collection. Besides, it is important to highlight that the egg yolk used in our experiments was clarified [12] and this type of egg yolk could provide lesser sperm toxicity than whole egg yolk during the freezing-thawing process.

In relation to cooling rate, our results suggested that slow cooling was better and agree with the study carried out in bucks by Memon et al. [22], although in that study the semen was collected by AV. However, for other species a fast cooling rate yield a higher sperm quality [9] which could be due to the different origin of spermatozoa. It is

known that a too rapid cooling of ungulates semen from 30 to 0 ºC induces a lethal stress on spermatozoa proportional to the rate of cooling, the temperature interval and the temperature range [28]. In addition, it would be possible to add the glycerol at 30 °C since there were no differences between adding glycerol at 30 or 5 °C, making easier the work in field conditions. These results agree with those from Coloma et al. [4] in bucks, who reported no effect of the glycerolization temperature.

Finally, the effect of different times of equilibration (0, 1, 2 and 3 h) at 5 ºC was studied in this work. The best sperm quality was obtained when samples were equilibrated for 3 h in relation to shorter times. Our results agree with Deka and Rao [7] who found greater progressive motility when the equilibration period increased from 1 to 5 h in samples collected by AV. On the other hand, some authors have frozen buck semen collected by AV without equilibration time with good results [23,24]. The exposure of spermatozoa to subphysiological temperatures prior freezing can induce alterations in the organization of the lipid bilayer of the plasma membrane, particularly in species with high concentrations of polyunsaturated fatty acids [13]. The better results obtained when longer equilibration periods are used could be due to an improvement on the organization of the cell membrane during this transition phase, thereby minimizing cryoinjury during semen processing.

The fertility assessed by IVF for those samples collected by EE and cryopreserved with the best protocol was higher than for AV samples, although the motility values were higher for the latter. Although this fact seems surprising, we have to consider that spermatozoa were selected by means of density gradients for using them in IVF test. Jiménez-Rabadán et al. [15] showed that sperm quality of thawed semen improved after selecting by single layer centrifugation (SLC). Thereby, the differences

observed in the motility parameters between AV and EE samples after cryopreservation might disappear, showing similar values and comparable fertility rates. In addition, Álvarez et al. [2] found acceptable fertility values for ram sperm samples collected by EE being similar to those obtained by AV, although AV samples showed higher values of motility.

In conclusion, the protocol which renders best sperm quality after cryopreservation for samples collected by EE was that which included a 20% of egg yolk, with a slow cooling rate, with glycerol added at 30 ºC or 5ºC and an equilibration time equals to 3 h. Under such conditions, sperm samples collected by EE showed a sperm quality at thawing quite similar to those collected by AV and cryopreserved using the standard protocol.

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506 Table 1. Effect of semen collection method on fresh sperm characteristics in Blanca-Celtibérica bucks.
507

	Sperm characteristics					
	Volume (mL)	**Concentration (x 10^6 spz/mL)**	**STN (x 10^6 spz)**	**SM (%)**	**NAR (%)**	**Viability (%)**
Semen Collection Method						
AV	1.16 ± 0.12	2845.42 ± 142.64[a]	3328.03 ± 319.27[a]	71.92 ± 2.09[b]	95.21 ± 0.75	79.02 ± 3.03
EE	1.15 ± 0.13	1334.35 ± 143.35[b]	1423.36 ± 322.05[b]	77.52 ± 2.12[a]	95.80 ± 0.76	79.70 ± 0.848

508 Data are means ± SEM. Different superscripts within a column differ significantly ($P \leq 0.05$). AV: artificial vagina; EE: electroejaculation; STN: spermatozoa
509 total number; SM: sperm motility; NAR: spermatozoa with intact acrosomes.
510

Table 2. Effect of concentration of egg yolk, cooling rate, temperature of glycerol addition and equilibration time on motility parameters and acrosome integrity after thawing in cryopreserved Blanca-Celtibérica buck ejaculates obtained by EE.

	Sperm parameters					
	TM (%)	**PM (%)**	**VSL (µm/sec)**	**LIN (%)**	**ALH (µm)**	**NAR (%)**
Egg yolk concentration						
0%	3.52 ± 4.36^{c}	1.68 ± 2.12^{c}	77.74 ± 8.77	65.00 ± 3.86	2.89 ±0.27	67.75 ± 3.62^{b}
1.5 %	10.59 ± 4.55^{c}	5.32 ± 2.21^{c}	80.04 ± 5.47	63.19 ± 2.49	3.18 ± 0.18	70.55 ± 3.80^{ab}
10 %	29.12 ± 4.36^{b}	14.08 ± 2.12^{b}	83.37 ± 4.92	60.28 ± 2.28	3.51 ± 0.16	74.74 ± 3.80^{ab}
20 %	35.71 ± 4.36^{b}	16.54 ± 2.12^{b}	84.11 ± 4.92	61.53 ± 2.27	3.46 ± 0.16	77.13 ± 3.71^{ab}
Control samples	61.43 ± 4.55^{a}	27.15 ± 2.21^{a}	74.01 ± 4.70	58.53 ± 2.18	2.98 ± 0.16	80.36 ± 3.80^{a}
Cooling Rate						
Slow (90 minutes)	52.71 ± 5.28^{b}	21.64 ± 2.60^{ab}	75.03 ± 4.87	57.33 ± 1.80	3.44 ± 0.12^{ab}	81.18 ± 2.59
Fast (10 minutes)	38.36 ± 5.51^{c}	18.27 ± 2.72^{b}	71.49 ± 5.01	55.49 ± 1.89	3.73 ± 0.13^{a}	78.56 ± 2.76
Control samples	69.96 ± 5.93^{a}	27.19 ± 2.94^{a}	71.86 ± 5.06	54.45 ± 1.94	3.33 ± 0.13^{b}	78.86 ± 2.80
Glycerol Temperature						
30 ºC	46.46 ± 5.32^{b}	21.17 ± 2.62^{ab}	74.10 ± 4.87	57.49 ± 1.80	3.49 ± 012^{ab}	80.37 ± 2.63
5 ºC	44.62 ± 5.43^{b}	18.74 ± 2.68^{b}	72.42 ± 4.99	55.33 ± 1.88	3.67 ± 0.13^{a}	79.37 ± 2.67
Control samples	69.96 ± 5.93^{a}	27.19 ± 2.94^{a}	71.86 ± 5.06	54.45 ± 1.94	3.33 ± 0.13^{b}	78.86 ± 2.80
Equilibration Time						
0 h	16.51 ± 5.84^{b}	7.88 ± 3.31^{c}	70.55 ± 9.14	58.20 ± 4.03	3.49 ± 0.21	60.98 ± 4.79
1 h	17.60 ± 5.84^{b}	9.38 ± 3.31^{bc}	74.80 ± 9.40	57.51 ± 4.19	3.29 ± 0.22	64.30 ± 4.29
2 h	35.48 ± 6.13^{ab}	18.58 ±3.46^{ab}	76.93 ± 9.20	59.26 ± 4.04	3.17 ± 0.21	58.73±4.52
3 h	29.79 ± 6.30^{b}	15.48 ± 3.54^{ac}	83.54 ± 9.64	59.79 ± 4.35	3.25 ± 0.22	73.23 ± 4.79
Control samples	49.75 ± 5.49^{a}	22.65 ± 3.12^{a}	76.52 ± 8.12	58.40 ± 3.32	3.02 ± 0.18	72.50 ± 3.92

Data are means ± SEM. For each factor, different superscripts within a column differ significantly ($P \leq 0.05$). TM: percentage of motile spermatozoa; PM: percentage of progressive motile spermatozoa; VSL: straight line velocity; LIN: linearity index; ALH: amplitude of lateral head displacement; NAR: percentage of spermatozoa with intact acrosome; Control samples: samples collected by AV and cryopreserved with 20% egg yolk, slowly cooled, glycerol added at 5 ºC, and equilibrated time for 2 h.

Table 3. Effect of concentration of egg yolk, cooling rate, temperature of glycerol addition and equilibration time on sperm parameters evaluated by flow cytometry after thawing in cryopreserved Blanca-Celtibérica buck ejaculates obtained by EE.

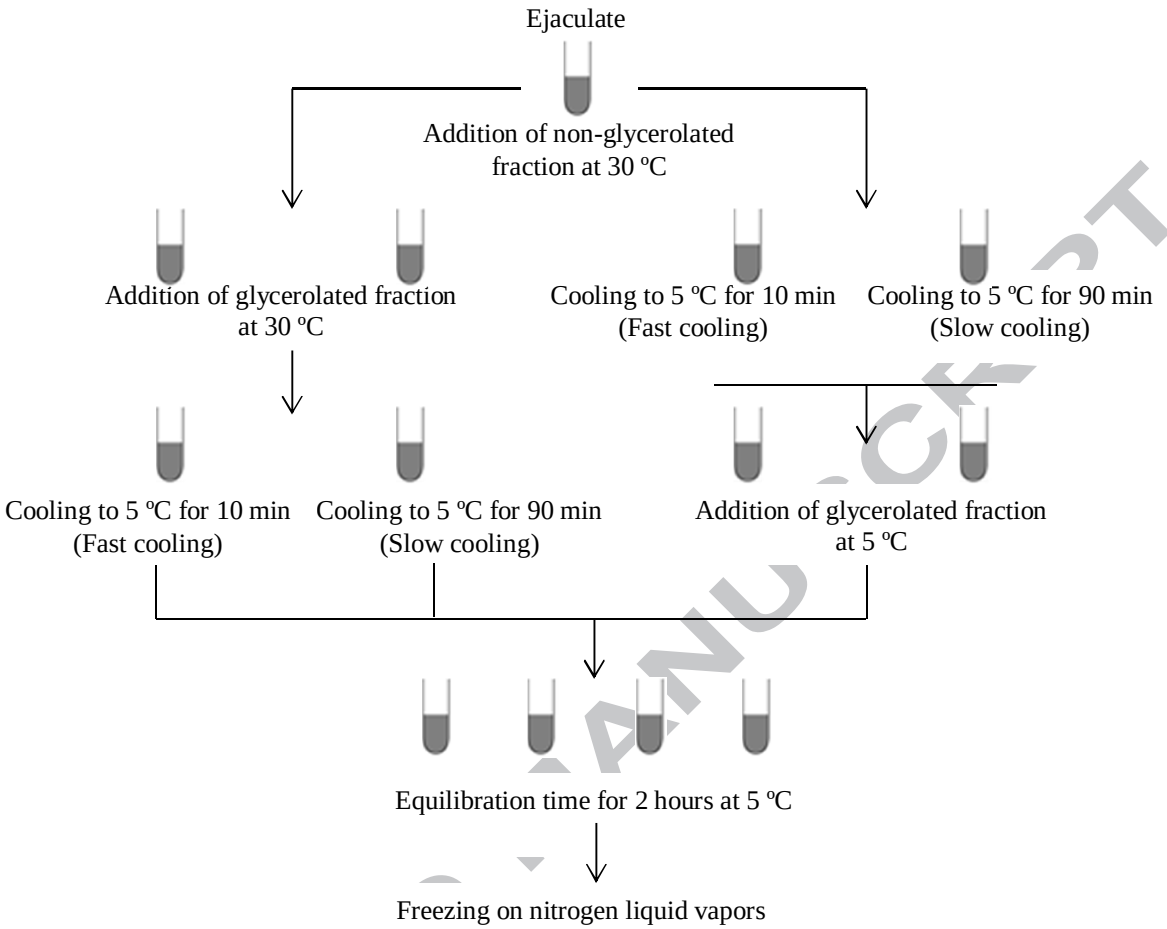
	Sperm parameters			
	YOPRO-/PI- (%)	**YOPRO+/PI- (%)**	**Mitotracker+/YOPRO- (%)**	**DFI (%)**
Egg yolk concentration				
0%	6.22 ± 2.30^{c}	1.75 ± 0.75^{c}	2.79 ± 3.36^{c}	1.52 ± 0.38
1.5 %	18.54 ± 2.40^{b}	4.89 ± 0.79^{b}	13.04 ± 3.49^{c}	1.91 ± 0.39
10 %	30.80 ± 2.30^{a}	7.52 ± 0.75^{ab}	30.54 ± 3.36^{b}	2.21 ± 0.38
20 %	37.60 ± 2.30^{a}	8.53 ± 0.75^{a}	36.35 ± 3.36^{ab}	2.77 ± 0.39
Control samples	39.52 ± 2.40^{a}	9.12 ± 0.79^{a}	45.02 ± 3.49^{a}	1.59 ± 0.39
Cooling Rate				
Slow (90 minutes)	30.60 ± 2.37	11.84 ± 1.09	25.25 ± 3.48^{ab}	1.24 ± 3.37
Fast (10 minutes)	34.46 ± 2.61	10.04 ± 1.13	18.49 ± 3.68^{b}	1.06 ± 3.63
Control samples	33.82 ± 3.14	11.55 ± 1.19	32.37 ± 4.01^{a}	1.52 ± 3.63
Glycerol Temperature				
30 ºC	31.73 ± 2.45	11.13 ± 1.10	23.37 ± 3.53^{ab}	1.26 ± 3.42
5 ºC	33.33 ± 2.53	10.74 ± 1.12	20.37 ± 3.60^{b}	1.05 ± 3.56
Control samples	33.82 ± 3.14	11.55 ± 1.19	32.37 ± 4.01^{a}	1.52 ± 3.63
Equilibration Time				
0 h	17.23 ± 3.23^{b}	7.20 ± 1.37^{b}	7.74 ± 2.92^{c}	1.85 ± 0.47
1 h	19.00 ± 3.23^{b}	9.15 ± 1.37^{ab}	9.42 ± 2.92^{bc}	1.98 ± 0.47
2 h	24.96 ± 3.42^{ab}	10.17 ± 1.43^{ab}	11.94 ± 2.98^{bc}	2.03 ± 0.49
3 h	32.90 ± 3.53^{a}	12.21 ± 1.47^{a}	14.11 ± 3.01^{ab}	1.90 ± 0.50
Control samples	30.00 ± 3.01^{a}	11.84 ± 1.29^{a}	19.15 ± 2.82^{a}	1.86 ± 0.44

Data are means ± SEM. For each factor, different superscripts within a column differ significantly ($P \leq 0.05$). YOPRO-/PI-: intact spermatozoa; YOPRO+/PI-: apoptotic spermatozoa; Mitotracker+/YOPRO-: viable spermatozoa with high mitochondrial membrane potential; DFI: DNA fragmentation index; Control samples: samples collected by AV and cryopreserved with 20% egg yolk, slowly cooled, glycerol added at 5 ºC, and equilibrated time for 2 h

Figure 1. Design of the Experiment 2.

Figure 2. *In vitro* fertility for sperm samples collected by AV, control samples, and EE, those frozen with the best cryopreservation protocol.

Data are means ± SEM. Different superscripts differ significantly ($P \leq 0.05$). Control: samples collected by AV (artificial vagina) and frozen by standard cryopreservation protocol: 20% egg yolk, slow cooling to 5 ºC, glycerol addition at 5 ºC and equilibration time for 2 h; EE: samples collected by electroejaculation and frozen with 20% egg yolk, slow cooling to 5 ºC, glycerol addition at 5 ºC and equilibration time for 3h.



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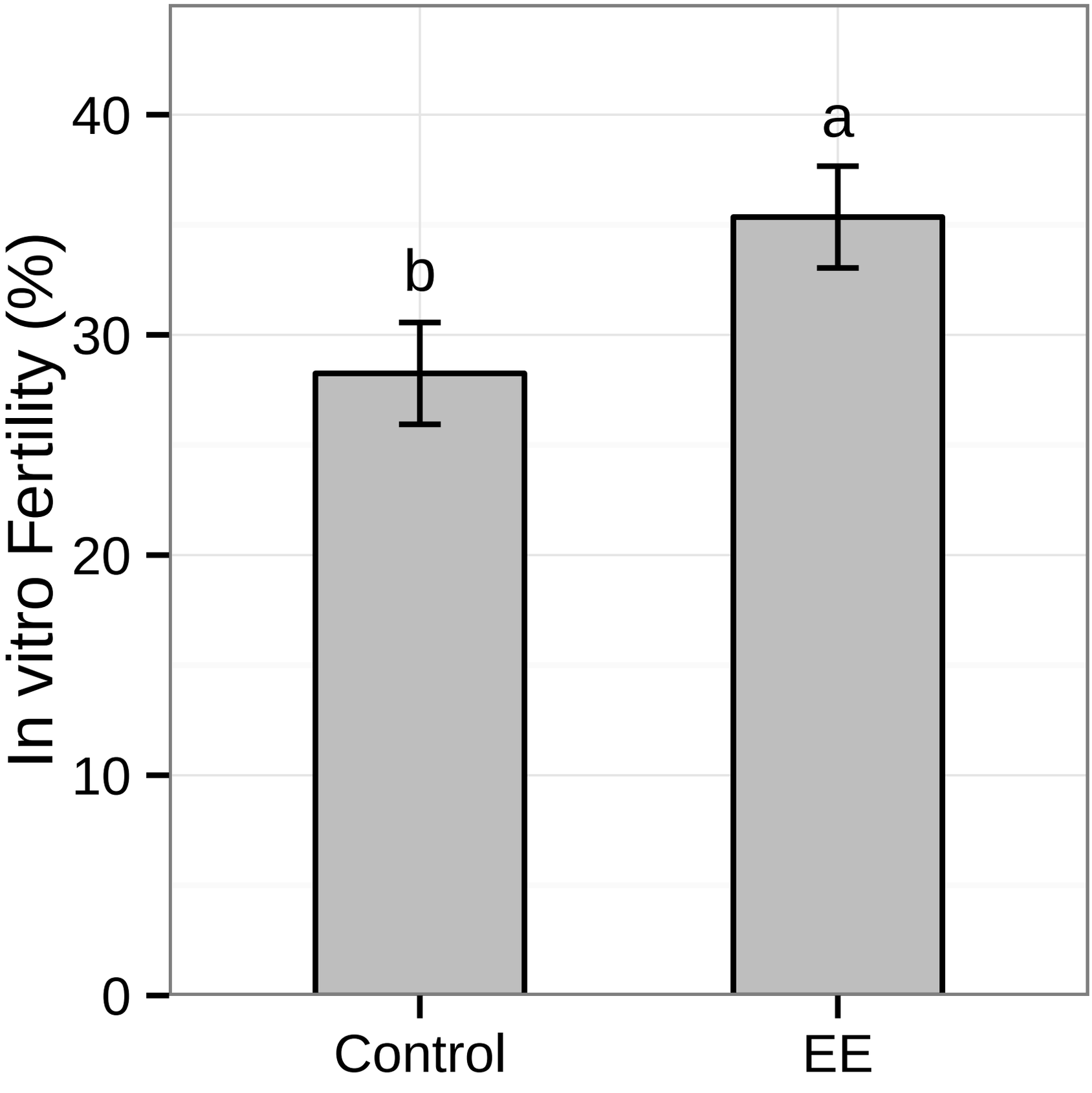
In vitro Fertility (%)
40
30
20
10
0
b
a
Control
EE