UNIVERSITAT POLITÈCNICA DE VALÈNCIA

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Dpto. de Ciencia Animal

Efecto del plasma seminal y el proceso de congelación sobre la calidad espermática en caprino

Trabajo Fin de Máster

Máster Universitario en Producción Animal

AUTOR/A: Esteve Ambrosio, Inés Carolina

Tutor/a: Vicente Antón, José Salvador

Cotutor/a externo: MOCE CERVERA, EVA TERESA

CURSO ACADÉMICO: 2022/2023

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Máster en Producción Animal

Efecto del plasma seminal y el proceso de congelación sobre la calidad espermática en caprino

Effect of seminal plasma and the freezing process on goat bucks sperm quality

Trabajo Fin de Máster

Valencia, Julio 2023

Inés Carolina Esteve Ambrosio

Director/es
José Salvador Vicente Antón
Eva Mocé Cervera
Ernesto A. Gómez Blasco


BY NC ND

EFFECT OF SEMINAL PLASMA AND THE FREEZING PROCESS ON GOAT BUCKS SPERM QUALITY

ABSTRACT

In goat bucks the seminal plasma (SP) must be removed before freezing for obtaining surviving the process when egg yolk- or skimmed milk (SM)-based extenders are used. It is clear that SP is prejudicial during the freezing-thawing process in this species but the stage at which SP is lethal for the sperm remains unelucidated. For this reason, the objective of this study was to study the effect of seminal plasma on goat buck sperm quality in each of the stages of the semen freezing process (F:after centrifugation and addition of the first diluent; R: after reaching 4°C; G: after addition of the extender containing glycerol; E: after 90 min of equilibration with glycerol at 4°C; T: after freezing-thawing). Twenty-one ejaculates from seven goat bucks from Murciano-Granadina breed were used in the study. Each of the ejaculates was split into two samples: one of them was processed with SP (SP+) and in the other one the SP was removed (SP-) before freezing. Samples were frozen with a SM-glycerol extender (SM2) and sperm quality (motility and sperm plasma membrane integrity (PMI), acrosomal integrity and mitochondrial functionality) was evaluated at each of the points of the freezing protocol (F, R, G, E and T). The results showed significant differences ($p<0.05$) in all the quality parameters analyzed, varying among the different stages in terms of significance. The analyses determined that the quality of samples processed without SP was superior to samples processed with SP. The most affected stages were the addition of glycerol and thawing. For the glycerol stage, values in samples with SP+ were 50 % total motile and 34 % live with intact acrosome versus 70 % and 56 %, respectively. In the case of the thawing stage it was more evident, values in samples SP+ were 7 % total motile and 4 % live with intact acrosome versus 40 % and 28 %, to samples SP-. In conclusion, the SP deteriorates the spermatozoa through all the steps of the freezing protocol. However, the stage that affected the most the sperm quality was the freezing-thawing.

Key words: Sperm; Quality; Seminal plasma; Freezing; goat

Student: Inés Carolina Esteve Ambrosio

Supervisors: José Salvador Vicente Antón, Eva Mocé Cervera and Ernesto A. Gómez Blasco

Valencia, July 2023

EFECTO DEL PLASMA SEMINAL Y EL PROCESO DE CONGELACIÓN SOBRE LA CALIDAD ESPERMÁTICA EN CAPRINO

RESUMEN

En los machos cabríos, el plasma seminal (SP) debe eliminarse antes de la congelación para conseguir que sobreviva al proceso cuando se utilizan diluyentes a base de yema de huevo o leche desnatada (SM). Está claro que el SP es perjudicial durante el proceso de congelación-descongelación en esta especie, pero la etapa en la que el SP es letal para los espermatozoides sigue siendo desconocida. Por esta razón, el objetivo de este trabajo fue estudiar el efecto del plasma seminal sobre la calidad espermática del macho cabrío en cada una de las etapas del proceso de congelación del semen (F: tras la centrifugación y adición del primer diluyente; R: tras alcanzar los 4°C; G: tras la adición del diluyente conteniendo glicerol; E: tras 90 min de equilibrio con glicerol a 4°C; T: tras la congelación-descongelación). En el estudio se utilizaron 21 eyaculados de siete machos cabríos de raza Murciano-Granadina. Cada uno de los eyaculados se dividió en dos muestras: una de ellas se procesó con SP (SP+) y en la otra se eliminó el SP (SP-) antes de la congelación. Las muestras se congelaron con un diluyente SM-glicerol (SM2) y se evaluó la calidad espermática (motilidad e integridad de la membrana plasmática espermática (PMI), integridad acrosomal y funcionalidad mitocondrial) en cada uno de los puntos del protocolo de congelación (F, R, G, E y T). Los resultados mostraron diferencias significativas ($p<0,05$) en todos los parámetros de calidad analizados, variando entre las distintas etapas en términos de significación. Los análisis determinaron que la calidad de las muestras procesadas sin SP fue superior a las procesadas con SP. Las etapas más afectadas fueron la adición de glicerol y la descongelación. Para la etapa de glicerol, los valores en las muestras con SP+ fueron de 50 % de móviles totales y 34 % de vivos con acrosoma intacto, frente a 70 % y 56 %, respectivamente. En el caso de la etapa de descongelación fue más evidente, los valores en las muestras SP+ fueron 7 % de móviles totales y 4 % de vivos con acrosoma intacto versus 40 % y 28 %, a las muestras SP-. En conclusión, el SP deteriora los espermatozoides a lo largo de todas las etapas del protocolo de congelación. Sin embargo, la etapa que más afectó a la calidad espermática fue la de congelación-descongelación.

Palabras clave: Esperma; Calidad; Plasma seminal; Congelación; caprino

Alumna: Inés Carolina Esteve Ambrosio

Tutores/as: José Salvador Vicente Antón, Eva Mocé Cervera y Ernesto A. Gómez Blasco

Valencia, Julio de 2023

GENERAL INDEX

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

AI: acrosome intact sperm

AI: Artificial insemination

ALH: amplitude of the lateral movement of the head

ALP: alkaline phosphatase

AR: acrosome reacted sperm

AST: aspartate amino transferase

BCF: beat cross frequency

BSA: bovine serum albumin

D30: dilution to concentration of 30 x 106 sperm/mL with TCG

DMM: dead with high mitochondrial membrane potential

DMM: dead with low mitochondrial membrane potential

E: equilibrated

EY: egg yolk

F: Fresh

FITC-PNA: fluorescein isothiocyanate-conjugated peanut agglutinin

G: glycerol

H: Hoechst

LDH: lactate dehydrogenase

LIN: linearity

LMB: live with high mitochondrial membrane potential

LMM: live with low mitochondrial membrane potential

M: Mitotracker

MAI: dead acrosome intact sperm

MAR: dead acrosome reacted sperm

MITOK: sperm with a high mitochondria membrane potential

MMP: mitochondria membrane potential

PI: propidium iodide

PM: progressively motile

PMI: Plasma membrane integrity

R: refrigerated

ROS: exogenous oxidative stress

skimmed milk-based (SM)

SM: skimmed milk

SM1: diluent was made with skimmed milk

SM2: was made with SM1 to which 28 % of glycerol (v:v)

SP: seminal plasma

STR: straightness index

T: thawing

TCG: Tris-citrate-glucose

TM: total motile

VAI: live acrosome intact sperm

VAIMITOK: live acrosome intact sperm and high mitochondrial membrane potential

VAP: average path velocity

VAR: live acrosome reacted sperm

VCL: curvilinear velocity

VSL: straight line velocity

WOB: wobble

Este trabajo va dedicado a mi pequeño y fiel amigo Aslan,
Me enseñaste que la fuerza está dentro de cada uno,
que la palabra rendirse no está en nuestro diccionario
y que nunca se deja de luchar
Estuviste, estás y estarás siempre conmigo.
Te quiero

ACKNOWLEDGMENTS

Funded by INIA-ERDF (RTA2017-00049-C02-01), GVA-IVIA susceptible of funding by UE through OP ERDF of the Comunitat Valenciana 2021-2027 (52201-K) and AMURVAL (71714).

This project is subjected to the following expressions of interest: AGROALNEXT/2022/063 and AGROALNEXT/2022/062.

1. Introduction

Artificial insemination (AI) is an assisted reproductive technique and is a fundamental tool in goat breeding programs (López-Gatius, 2012). With this technique it is possible to spread the genetic response to the commercial herds (Mocé et al., 2019). Although AI can be performed with cryopreserved sperm, refrigerated sperm is the type of semen of preference in dairy goat farms (Mocé et al, 2020). However, cryopreserved sperm is a key pillar for gene conservation, maintenance of endangered species and distribution of genes without limitation of distance and time (Salomon and Maxwell, 2000).

The process for sperm preservation once ejaculates are extracted and until the semen dosas are used for AI is complex. There must be a balance between the spermatozoa and the surrounding environment to obtain sperm of good quality for AI. Moreover, knowledge of sperm physiology is essential, because during the cryopreservation process sperm are exposed to changing conditions of temperature and osmolarity according to the protocol stage (fresh, refrigeration, glycerol addition, equilibration, freezing and thawing; Purdy, 2006).

Ejaculates are a mixture of spermatozoa and seminal plasma (SP; Cortés, 2003). This SP is composed of fluids from various male accessory glands and from cauda epididymis (Pellicer et al., 1997). The SP include amino acids, proteins, ions, electrolytes, metabolites like nucleosides, lipids, monosaccharides, minerals, enzymes and steroid hormones (Jia et al., 2021) This secretion facilitates the survival of spermatozoa both *in vitro* and *in vivo* (Leahy et al., 2019). Moreover, SP participates in different cell signaling processes and provides energy to sperm, due to the variability of sugars and lipids it contains. In fact, a relationship between fertility and the sugars that the SP carries has been observed (Lahnsteiner et al., 1993). Other functions that are attributed to plasma factors are the regulation of capacitation, stimulation of motility, influence on semen storage in the female tract, as well as its participation in modulating the female's immune system for sperm tolerance (Leahy et al., 2019).

Thus, SP contains enzymes such as lactate dehydrogenase (LDH), aspartate amino transferase (AST) and alkaline phosphatase (ALP) that are essential for metabolic processes, since they supply energy for sperm motility, survival and fertility (Rodríguez et al., 2016). Different SP ions play also important roles in sperm survival

and function. For example, zinc is a modulator of chromatin stability and mitochondrial function, bicarbonate modulates sperm motility and induces plasma membrane destabilization and calcium is pivotal for triggering the acrosome reaction. Most of the ions are also related to fertility parameters but these are linked to a large protein content (Rodríguez-Martínez et al., 2021). Proteins are also found in SP, being one of the most important components in boars where proteomic studies have already detected more than 500 proteins in this fluid. Proteins modulate sperm functional capacity and protect them in their transit through the sow genital tract, helping to regulate the temporal kinetics of ovulation and at the same time the development of the corpus luteum. In addition, together with other SP components, proteins participate in the establishment of successful fertilization and gestation. The relationship between SP proteins and female fertility has been demonstrated in several species (Parrilla et al., 2020; De Lazari et al., 2018). Thus, SP participates in the regulation of the immune system and seems to be an important factor in the regulation of mating-induced endometritis in equine (Lozano et al., 2011) and positive effect of SP during estrus and on processes related to preimplantation embryo development and implantation has been found in rodents (Parrilla et al., 2020).

Despite its important roles in sperm physiology and in endometrial and embryo gene expression modulation (Parrilla et al., 2020), the presence of SP in goats is deleterious for frozen sperm (Corteel and Baril, 1974) up to a point that SP removal is mandatory for obtaining sperm surviving the freezing-thawing processes. Diluents commonly used for freezing goat sperm contain skimmed milk (SM) or egg yolk (EY), and some of their components are substrates for enzymes (BUSqp60 and egg yolk-coagulating enzyme) secreted by the bulbourethral gland. The enzymatic reactions triggered by these lipases release metabolites that are toxic for the sperm (i.e., oleic acid coming from the degradation of residual triglycerides from SM), that induce the acrosome reaction and subsequent cell death when spermatozoa are incubated in milk medium at 37 °C (Leboeuf et al., 2000; Purdy, 2006). In contrast, the SP is not detrimental for liquid stored goat buck sperm (Leboeuf et al., 2000), which seems puzzling, because the diluents are like those used for freezing. Indeed, refrigerated goat buck seminal doses contain the seminal plasma and the fertility does not decrease if these doses are used the same day they are produced (Leboeuf et al., 2000).

Due to the detrimental effect of SP, the protocol for freezing goat buck sperm usually includes a step where SP is removed, before the addition of the freezing diluent

(Cortés, 2003). Sperm washing have beneficial effect on goat buks sperm motility without a significant deleterious effect of centrifugation process (Salvador et al., 2006). The protocol for removing SP usually consists of two centrifugations. This washing step increases the processing time but also inflicts some detrimental actions on the sperm. Thus, mechanical forces of the centrifugation provoke sperm stress. In fact, some species such as rodents are very vulnerable to the semen washing stress due to their sperm morphology and in other species such as the stallion a reduction in the percentages of total motile sperm and in the sperm velocities have been observed as a result of the washing step (Marzano et al., 2020). Nevertheless, the sperm from other species such as red deer show more resistance to centrifugation although the risk of exogenous oxidative stress (ROS) increases during the SP elimination (Domínguez et al., 2009). Washing does not only eliminate detrimental compounds from goat buck SP (such as lipases) but also removes beneficial compounds like decapacitation factors or sperm metabolism stabilizers and as a result, the sperm are harmed (Cortés, 2003).

Although it is known that SP is detrimental for goat sperm freezing, it remains to be elucidated at what point of the freezing protocol the damage occurs.

2. Objective

Thus, the objective of this study was to study the effect of seminal plasma on goat buck sperm quality in each of the stages of the semen freezing process.

3. Materials and methods

3.1. Materials and preparation of diluents

All chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain), except for propidium iodide (PI) and Mitotracker deep red FM, which were purchased from Invitrogen (Barcelona, Spain). The diluents Tris-citrate-glucose (TCG) and TCG supplemented with bovine serum albumin (BSA; 0.3 %; w/v) were used to perform the analyses for sperm quality evaluation following the protocols described in Mocé et al. (2020). For freezing two skimmed milk-based (SM) diluents were used. The first diluent (SM1) was made with skimmed milk (Central Lechera Asturiana; Oviedo, Spain) and 0.2 % (w:v) of D (+)- glucose. The second diluent (SM2) was made with SM1 to which 28 % of glycerol (v:v) was added. The sodium chloride solution (0.9 %; w/v) was used for determination of sperm concentration.

3.2. Animals

This study was carried out in the Centro de Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA; Segorbe, Castellón, Spain) and seven adult Murciano-Granadina goat bucks were used as semen donors. The goat bucks were housed in pens with availability to water and straw ad libitum. Moreover, they were supplied with 1 kg/day of concentrated feed (17 % crude protein, 11.6 % crude fiber and 4.5 % crude oils and fat) per male. The protocols for semen collection, care and animals housing complied with European regulations for the care and use of animals for scientific purposes (RD 53/2013; BOE, 2020). According to the Spanish law, semen collection with an artificial vagina is considered as a routine agricultural practice and, for this reason, does not require ethical approval. In fact, the males were not considered to be experimental animals and no special ethical permission was required.

3.3. Semen collection

Semen extraction was made in the morning with artificial vagina following the protocol described in Silvestre et al. (2004). Semen was regularly collected (at least once/week) throughout the year. In total, 21 ejaculates were processed (three ejaculates/male). Tubes containing the ejaculates were immersed in a water bath at 25 °C until used. Volume and sperm concentration were measured according to the protocols described in Konyali et al. (2013).

3.4. Freeze-thaw protocol

Each ejaculate was split into two aliquots: one of them was frozen with SP and in the other one the SP was removed prior to freezing. The samples that retained the SP were kept at 22 °C until the SP was removed from the other samples. The SP was removed as described in Konyali et al. (2013). Briefly, each sample was diluted with TCG up to 10 mL, centrifuged for 15 min at 500 g at 22 °C and the supernatant was discarded. Then, the pellet was resuspended in 10 mL of TCG and centrifuged for a second time. After this, the supernatant was removed and the pellet was homogenized. Then, the volume and concentration were again evaluated.

The sperm concentration in each of the samples (with or without SP) was adjusted to 667 x 10^6 sperm/mL with SM1 at 22 °C. Then, the samples were refrigerated from 20 °C to 4 °C in a programmable water bath (Julabo GmbH, Seelbach, Germany) for 90 min at a rate of -0.18 °C/min.

After refrigeration the samples were transferred to a cold room (set at 4 °C) to continue with the protocol. First, a cocktail of antibiotics was added (comprised of 300 µg of spectinomycin, 250 µg of gentamycin, 150 µg of lincomycin and 50 µg of tylosin per mL of frozen semen; Minitub Ibérica S.L., Tarragona, Spain), according to the recommendations from the OIE (2023). Then, precooled SM2 was added (dilution 3:1 v:v) to the samples to obtain final concentrations of 500 x10^6 sperm/mL and 7 % glycerol (v:v). For avoiding osmotic shock, the volume was divided into three parts that were added at 10 min intervals to the samples. After the last addition the samples were equilibrated for 90 min at 4 °C during which the semen was loaded into 0.25 mL plastic straws (IMV Technologies, L'Aigle, France) that were sealed with polyvinyl alcohol (PVA, IMV Technologies, L'Aigle, France). After equilibration the straws were frozen in a programmable freezer (Minidigitcool, IMV Technologies, L'Aigle, France) at the following rates: -4 °C/min from 4 to -5 °C, -25 °C/min from -5 to -110 °C and -35 °C/min from -110 to -140 °C and later, they were plunged into liquid nitrogen for storage.

The straws were immersed in a water bath to 37.5 °C for 30 sec for thawing.

To perform the analyses in the laboratory, 40 µL aliquots were taken at these points of the freezing protocol: fresh (after SM1 addition), refrigeration (after chilling

to 4 °C), glycerol addition (after the SM2 addition), equilibration (after the 90 min equilibration with the glycerol at 4 ºC) and thawing (after freezing-thawing).

3.5. Evaluation of sperm quality

For determining the seminal quality, sperm motility and the sperm plasma membrane integrity (PMI), acrosomal integrity and mitochondrial functionality were evaluated at each of the points of the freezing-thawing protocol previously described. These analyses were performed according to the protocols described by Mocé et al. (2022a). All the analyses were performed at room temperature (~22 °C). The samples were first diluted to a concentration of 30 x 10^6 sperm/mL with TCG (D30). This dilution with TCG was progressively performed in the samples that had glycerol (after SM2 addition, equilibration and thawing). For this, a previous 1:3 dilution (v:v) was performed with TCG before the final dilution to 30 x 10^6 sperm/mL.

Motility was evaluated in samples adjusted to 6 x 10^6 sperm/mL with TCG-BSA and incubated at 37 °C for 10 min prior to analysis following the protocols described in Mocé et al. (2022a). Briefly, these analyses were performed with a 10x negative phase contrast objective under a Nikon Eclipse (IZASA, Barcelona, Spain). Subamples of 7.5 mL were placed inside a Makler chamber (Counting Chamber Makler, Sefi-Medical Instruments, Haifa, Israel) prewarmed at 37 °C on a thermal plate. The data from a minimum of 200 sperm from three different fields were collected. Individual sperm tracks were visually assessed to eliminate possible debris and wrong tracks. The quantity and quality of movement were determined in a CASA system (ISAS, version 1.0.17, Proiser, Valencia, Spain) that operated at 30 video frames per second (30 Hz). The particle area was set at 15-70 µm and the search radius at 12 µm. The following variables were obtained: percentages of total (TM; %) and progressively motile (PM; %) sperm, average path velocity (VAP; µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL; µm/s), straightness index (STR; VSL/VAP x 100; %), linearity (LIN; VSL/VCL x 100; %), wobble (WOB; VAP/VCL x 100; %), amplitude of the lateral movement of the head (ALH, µm) and beat cross frequency (BCF; Hz). The spermatozoa were classified as motile if their VAP > 10 µm/s and PM if they presented VAP > 75 µm/s and STR≥ 80 %.

Acrosomal membrane integrity, mitochondrial functionality and live sperm were evaluated with flow cytometry following the protocol described in Mocé et al. (2022a). These structures were evaluated with fluorescent stains and a CytoFLEX S flow

cytometer (Beckman Coulter Life Sciences, L´Hospitalet de Llobregat, Barcelona, Spain) equipped with three lasers (a 50-mW 488-nm blue laser, a 50-mW 638-nm red diode laser and an 80-mW 405-nm violet laser) and the CytExpert software (Beckman Coulter life sciences, L´Hospitalet de Llobregat, Barcelona, Spain). CytoFlex Daily QC fluorospheres were used for the daily verifications of the flow cytometer's optical alignment and fluidics system according to the manufacturer's instructions. Samples were treated by quadruple staining with Hoechst 33342, propidium iodide (PI), fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and Mitotracker deep red FM. Samples were stained for the flow cytometric analysis by transferring 0.1 mL aliquots with 3 x 10^6 sperm to tubes with 25 µL of TCG diluent, 5 µL of Hoechst (0.1 mg/mL stock solution in Milli-Q water) and 0.25 µL of Mitotracker (25 µM stock solution in DMSO). Samples were incubated for 20 min at room temperature in the dark. Then a solution containing 25 µL of TCG diluent with 0.25 µL of PI (1 mg/mL stock solution in Milli-Q water) and 0.5 µL of FITC-PNA (1 mg/mL stock solution in Milli-Q water) was added. Samples were incubated for another 10 min period before being diluted with 0.40 mL of TCG and analyzed. Hoechst was excited with the violet laser and its fluorescence was detected using a 450/45 nm avalanche photodiode (APD). Mitotracker was excited with the red laser and its fluorescence was detected employing a 660/20 nm APD. PI and FITC-PNA were excited with the blue laser. The red fluorescence of PI was detected using 690/50 nm APD and the green fluorescence of FITC-PNA was detected employing 525/40 nm APD. Next 50,000 events per sample were analyzed. The compensation between PI-FITC-PNA was 0.93% and was 1.39% between PI-Mitotracker. Non DNA-containing events (Hoechst-negative) were excluded and the sperm population was gated based on the expected forward and side scatter signals.

PI penetrated non-viable cells to distinguish three populations: live (LIVE; PI-; plasma membrane intact sperm; %), and plasma membrane damaged sperm (PI+; %) distinguishing two populations, one with high PI fluorescence intensity and another one with low PI fluorescence intensity (APOPTOTIC; apoptotic sperm; %). Only the sperm with damaged acrosomes stained with FITC-PNA and two populations were distinguished acrosome reacted sperm (AR; FITC-PNA+; %) and acrosome intact sperm (AI; FITC-PNA-; %). Finally, all the sperm stained with Mitotracker and two populations were distinguished: one with low intensity corresponding to the sperm with a low mitochondria membrane potential (MMP; %) and another with high intensity corresponding to the sperm with a high MMP (MITOK; %).

First spermatozoa were categorized according to stains PI and FITC-PNA as dead acrosome intact sperm (MAI; PI+/FITC-PNA-; %), dead acrosome reacted sperm (MAR; PI+/FITC-PNA+; %), live acrosome intact sperm (VAI; PI-/FITC-PNA-; %), live acrosome reacted sperm (VAR; PI-/FITC-PNA+; %). PI and Mitotracker were also plotted on another chart, and four sperm populations were obtained dead with low mitochondrial membrane potential (DMM; PI+/Mitotracker-; %), dead with high mitochondrial membrane potential, (DMB; PI+/Mitotracker+; %), live with low mitochondrial membrane potential, (LMM; PI-/Mitotracker-; %), live with high mitochondrial membrane potential, (LMB; PI-/Mitotracker+; %). In addition, and only for the population of live acrosome intact sperm (PI-/FITC-PNA-), the percentage of sperm with high mitochondrial membrane potential was calculated (VAIMITOK; PI-/FITC-PNA-/ MITOK; %).

In summary, the following variables were considered in the results: TM, PM, VSL, VAP, VCL, LIN, STR, WOB, ALH, BCF, AR, LIVE, APOPTOTIC, VAI, LMB, MITOK and VAIMITOK.

3.6. Statistical analyses

First, the variables considered for the experiment were subjected to two types of analyses (Kolmogorov-Smirnov test and Shapiro-Wilk test) to confirm if the data followed a normal distribution so they could be later analyzed with parametric tests. The following variables were confirmed as normal, since they complied with the two tests: LIVE, LMB and VAI. The remaining variables (TM, PM, VSL, VAP, VCL, LIN, STR, WOB, ALH, BCF, AR, APOPTOTIC, MITOK and VAIMITOK) were not considered normally distributed, so the use of non-parametric tests was recommended for further analysis.

For the variables normally distributed, the parametric ***Student t-test*** was used. For those variables not following normal probability distribution, the non-parametric ***Wilcoxon test*** was used. First, an analysis was performed to compare the two treatments (presence or absence of SP) for each of the variables in the 5 stages of the procedure. In addition, a second analysis was performed to compare between stages (fresh, F; refrigerated, R; glycerol, G; equilibrated, E; thawing, T) within treatment. Statistical analyses were run using SPSS® 27.0 (IBM Corporation, New York, NY, USA). The level of significance was set at $p<0.05$ in all tests.

4. Results

Figure 1 shows the results for the motility variables comparing between treatments (presence or absence of SP) in each of the stages of the freezing protocol. Significant differences were observed between treatments for total and progressively motile sperm in all the freezing stages. The samples in which the SP was removed always exhibited higher values ($p<0.05$) than the samples processed with SP. For VSL and VAP velocities significant differences were observed between treatments after refrigeration and glycerol addition, presenting higher values ($p<0.05$) the samples with SP than the samples deprived of SP (121 vs 130 μm/s for refrigerated and 117 vs 126 μm/s for glycerol, respectively). For VCL, the differences were observed after glycerol addition and after thawing. The values were higher in the glycerol stage for the samples processed with SP (138 μm/s), and lower after thawing (100 μm/s vs 117 μm/s for SP and without SP respectively). For the ratios, samples treated with SP exhibited higher values ($p<0.05$) for LIN (85.4 %) and for WOB (93.2 %) than samples without SP (82.2 % and 90.6 % for LIN and WOB) after refrigeration. These SP+ treated samples also presented higher WOB values after the addition glycerol and the thawing stages, compared to the SP deprived samples. The index STR was similar for both treatments.

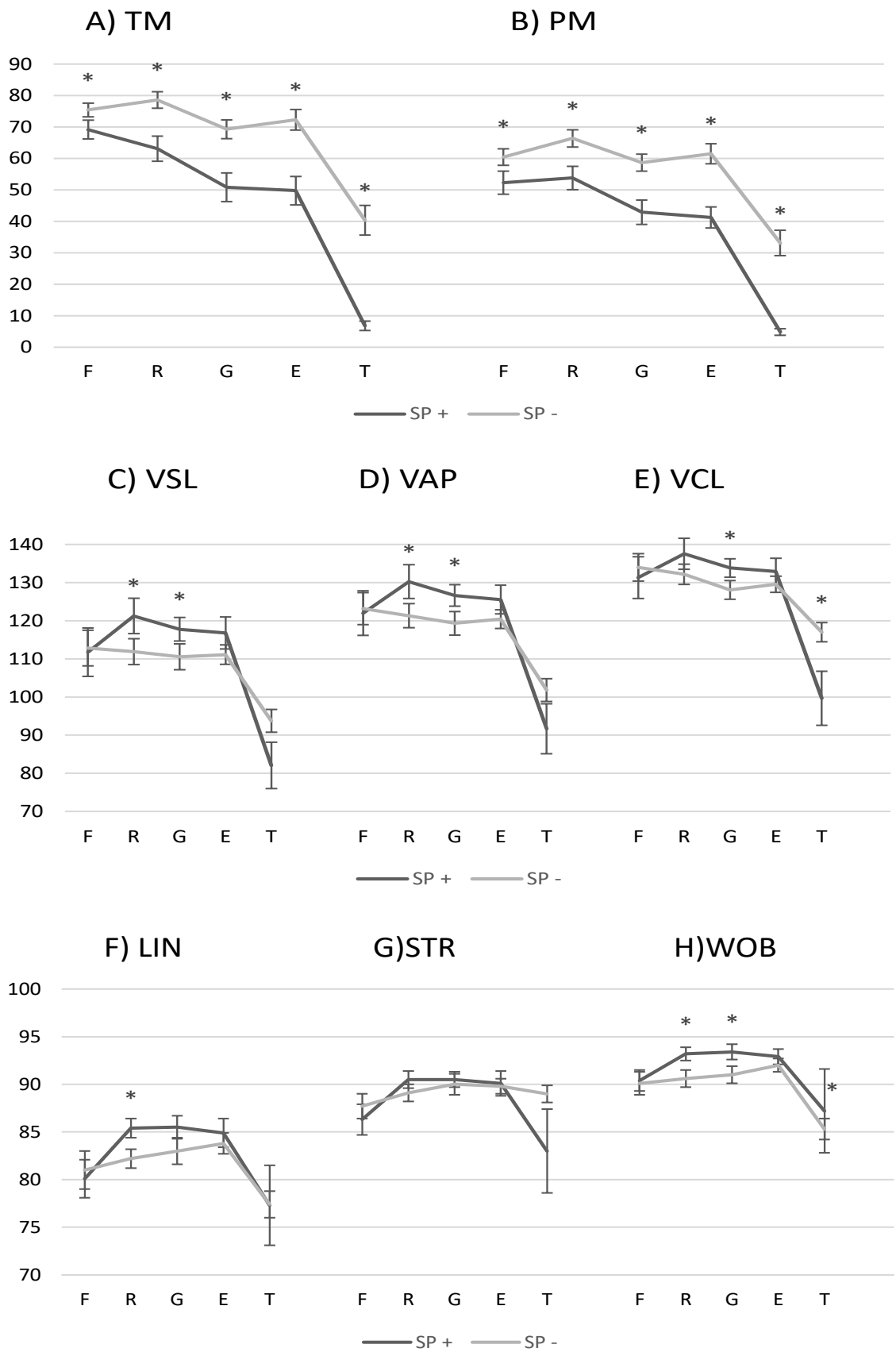

Figure 1. Goat bucks sperm quality (motility variables, n=21) in each stage of freezing protocol when samples where processed in presence (SP+) or in absence (SP-) of seminal plasma (SP). F: fresh (after centrifugation and addition of the first diluent); R: refrigerated (after reaching 4°C); G: glycerol (after addition of the extender containing glycerol); E: equilibrated (after 90 min of equilibration with glycerol at 4°C); T: thawed (after freezing-thawing); TM: total motile sperm (%); PM: progressive motile sperm (%); VSL: straight line velocity (μm/s); VAP: average path velocity (μm/s); VCL: curvilinear velocity (μm/s); LIN: linearity (%); STR: straightness index (%); WOB: wobble (%); * within a stage, indicates significant differences between treatments ($p<0.05$). All values are represented as means ± standard error (s.e.).

The results for ALH and BCF are exposed in Figure 2. As may be observed, samples in which the SP was removed exhibited higher ($p<0.05$) values for ALH in all the stages than the samples processed with SP. This difference between treatments was especially high after thawing (2.29 µm vs 1.7 µm for SP – and SP +, respectively). However, BCF only presented significant differences in the thawing stage, exhibiting higher values the samples treated without SP (11.5 Hz) than with SP (9.5 Hz). For the other stages, BCF did not present significant differences between treatments.

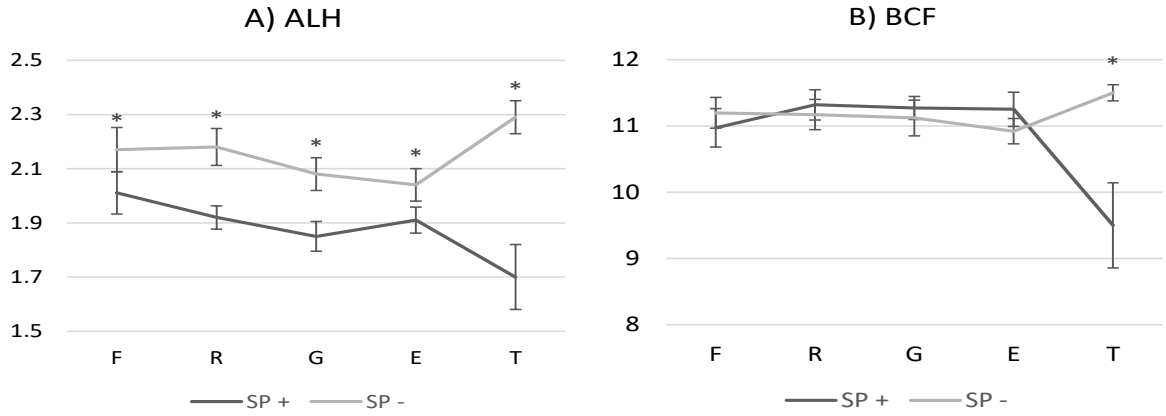

Figure 2. Goat bucks sperm quality (ALH and BCF, n=21) in each stage of a freezing protocol when samples where processed in presence (SP+) or in absence (SP-) of seminal plasma (SP). F: fresh (after centrifugation and addition of the first diluent); R: refrigerated (after reaching 4°C); G: glycerol (after addition of the extender containing glycerol); E: equilibrated (after 90 min of equilibration with glycerol at 4°C); T: thawed (after freezing-thawing); ALH: Amplitude of lateral head displacement (µm); BCF: Beat cross frequency (Hz); * within a stage, indicates significant differences between treatments ($p<0.05$). The values are represented as means ± standard error (s.e).

The results obtained for the variables analyzed in the flow cytometer are presented in Figure 3, where significant differences ($p<0.05$) were observed between treatments in all the stages of the freezing protocol for the variables VAI, LMB and apoptotic. The samples in which the seminal plasma remained exhibited lower values of VAI and LMB and higher values of apoptotic sperm than samples where the SP was eliminated. For the percentage of acrosome reacted sperm, the differences were observed in all stages except in fresh stage. In this case, the samples with SP exhibited higher percentages of acrosome reacted sperm than the samples without SP. For most of these variables, the largest difference between the treatments corresponded to the thawing stage.

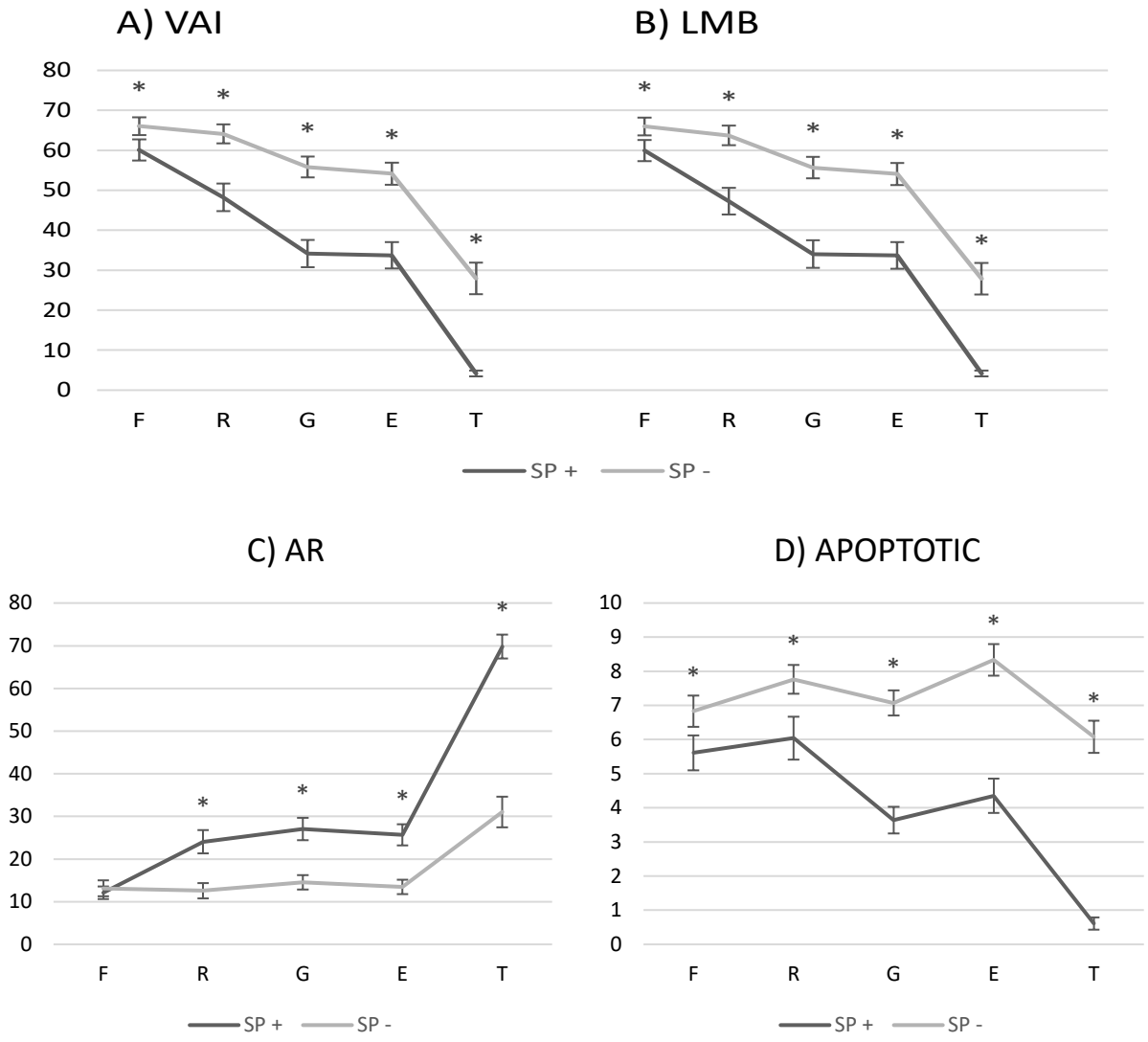

Figure 3. Goat bucks sperm quality (cytometer variables, n=21) in each stage of freezing protocol when samples where processed in presence (SP+) or in absence (SP-) of seminal plasma (SP). F: fresh (after centrifugation and addition of the first diluent); R: refrigerated (after reaching 4°C); G: glycerol (after addition of the extender containing glycerol); E: equilibrated (after 90 min of equilibration with glycerol at 4°C); T: thawed (after freezing-thawing); VAI: live with intact acrosome sperm (PI-/FITC-PNA-; %); LMB: sperm live with high mitochondrial membrane potential sperm (PI-/Mitotracker+; %); AR: reacted acrosome sperm (FITC-PNA+;%); APOPTOTIC: apoptotic sperm (PI+ with low intensity; %); * within a stage indicates significant differences between treatments ($p<0.05$). The values of all the variables are represented as means ± standard error (s.e.).

Table 1 shows the differences between successive stages of the freezing protocol in each of the treatments (SP – and SP +). In both treatments, most of the differences appeared between the equilibration and the thawing stages where differences were observed for most of the variables between these stages except for STR, WOB and ALH. On the contrary, the stages where less differences were observed corresponded

to glycerol addition and equilibrated stages. Here, the doses processed with SP exhibited differences only in the percentages of AR and apoptotic sperm. In samples frozen without SP, differences were observed between the same variables plus the TM sperm. The refrigeration (comparisons performed between the stages of fresh and refrigeration) was more noxious for samples processed with SP than for those processed without SP. Thus, more variables exhibited differences between the fresh and refrigerated sperm in presence than in absence of SP. The addition of glycerol affected mostly to the percentages of TM and PM sperm and to the cytometer variables in both treatments when comparisons were made between the refrigeration stage and after glycerol (SM2) addition.

Table 1. Effect on sperm quality between the different stages for each treatment, when the samples were processed in presence (SP+) or in absence (SP-) of seminal plasma (SP).

	SP +				SP -			
Parameters	F-R	R-G	G-E	E-T	F-R	R-G	G-E	E-T
TM (%)	6.1*	12.3*	1.1	43.0*	-3.2	9.3*	-3.0*	32.0*
PM (%)	-1.5	10.9*	1.7	36.4*	-5.9*	7.7*	-2.8	28.4*
VSL (µm/s)	-9.5*	3.5	1.0	34.7*	1.0	1.3	-0.57	17.4*
VAP (µm/s)	-8.3*	3.6	1.1	33.9*	1.9	2.0	-1.1	18.6*
VCL (µm/s)	-6.3	3.7	0.91	33.3*	1.8	4.1	-1.5	12.6*
LIN (%)	-5.3*	-0.10	0.60	7.6*	-1.2	-0.80	-0.80	6.4*
STR (%)	-4.2*	0.00	0.40	7.1	-1.4	-0.90	0.20	0.80
WOB (%)	-2.8*	-0.20	0.50	5.7	-0.50	-0.40	-1.0	6.7*
ALH (µm)	0.091	0.070	-0.060	0.21	0.010	0.10*	0.040	-0.25*
BCF (Hz)	-0.35	0.048	0.020	1.8*	0.026	0.051	0.20	-0.58*
VAI (%)	11.9*	14.1*	0.44	29.6*	1.9*	8.3*	1.7	26.2*
LMB (%)	12.7*	13.2*	0.35	29.5*	2.2*	8.1*	1.6	26.2*
AR (%)	-11.9*	-3.0*	1.4*	-44.1*	0.53	-2.0*	1.0*	-17.5*
APOPTOTIC (%)	-0.43	2.4*	-0.71*	3.7*	-0.93*	0.69	-1.3*	2.3*
LIVE (%)	11.9*	14.1*	0.43	29.6*	1.9*	8.3*	1.7	26.2*
MITOK (%)	11.6*	17.3*	-0.10	22.6*	0.19	9.1*	1.3	13.2*
VAIMITOK	1.2	-0.48	0.15	5.6*	0.55	-0.06*	-0.46	2.2*

F: fresh (after centrifugation and addition of the first diluent); R: refrigerated (after reaching 4°C); G: glycerol (after addition of the extender containing glycerol); E: equilibrated (after 90 min of equilibration with glycerol at 4°C); T: thawed (after freezing-thawing); TM: total motile (%); PM: progressive motile (%); VSL: straight line velocity (µm/s); VAP: average path velocity (µm/s); VCL: curvilinear velocity (µm/s); LIN: linearity (%); STR: straightness index (%); WOB: wobble (%); ALH: Amplitude of lateral head displacement (µm); BCF: Beat cross frequency (Hz); VAI: live with intact acrosome sperm (PI-/FITC-PNA-;%); LMB: sperm live with high mitochondrial membrane potential sperm (PI-/Mitotracker+;%); AR: reacted acrosome sperm (FITC-PNA+; %); MITOK: high mitochondrial membrane potential sperm (Mitotracker+ with high intensity;%); VAIMITOK: live with intact acrosome sperm and high mitochondrial membrane potential sperm (PI-/FITC-PNA-/MITOK; %); * indicates significant differences between successive stages of the same treatment ($p<0.05$). The values are represented as differences between stages for each measured parameter.

5. Discussion

Artificial insemination plays an important role in goat breeding programs by facilitating evaluation of the genetic potential of goat bucks in different herds as well as diffusion of the best genetics between herds, reducing sanitary risks. Frozen semen is an interesting product for using in AI, since the sperm fertilizing ability is kept for indefinite time and this allows the moments of semen extraction and insemination to be completely dissociated. Nevertheless, the use of refrigerated semen doses is preferred over cryopreserved semen doses in commercial herds, due to their higher fertility values. However, refrigerated goat buck sperm quickly deteriorates after preparation, which, in turn, jeopardizes its fertilizing ability.

In caprine, SP must be removed during the freezing process since there are interactions among some enzymes from the SP and the diluents used (skimmed milk or egg yolk; Leboeuf et al., 2000; Purdy, 2006). Therefore, the frozen-thawed sperm doses in this species are devoid of SP, which may have some implications in their future performance. Thus, the SP has several important functions within the ejaculate others than being a vehicle for transporting spermatozoa. It contains lipids, proteins, minerals, enzymes, among others, that modulate some sperm functions such as motility or metabolic processes. In addition, SP participates in the cell signaling process within the female reproductive tract, in capacitation and during the fertilization process (Lopera-Vázquez, 2009).

Although the SP of this species is considered an inadequate medium to maintain sperm viability after ejaculation (Pellicer et al., 1997) and their removal increases the percentage of viable cells and their motility during liquid storage (Leboeuf et al., 2000), the presence of SP in refrigerated doses is not as noxious as it is for frozen semen. Thus, the damaging effect that SP has on the sperm is puzzling since it is observed in frozen semen (up to a point that the presence of SP is incompatible with the sperm survival to the freezing-thawing process), but not in refrigerated semen where SP is noy removed. For the SP removal before freezing, the protocol most widely used consists of diluting the samples and centrifuge them to eliminate the supernatant that contains the SP (Corteel and Baril, 1974). The prejudicial effect of SP for frozen-thawed is clear, but it is not known at which point of the freezing protocol the SP is lethal for the sperm.

According to our results, the noxious effect of SP is already observed in fresh stage of the protocol (after centrifugation and addition of SM1; Figures 1, 2 and 3). At this point, significant differences for some of the quality variables (TM, PM, ALH, VAI, LMB and APOPTOTIC) were already observed between treatments. All of them were higher in the samples processed without SP than with SP, except for ALH. At this stage the only difference between treatments was the SP removal by means of centrifugation. These results confirm the reports from previous studies where the removal of SP improved sperm viability during sperm storage in the presence of skimmed milk diluent (Pellicer-Rubio et al., 1997). Our motility and cytometry values for fresh semen with SP fell within the range of the values published in other studies (between 63 and 80% of TM and 57 and 68% VAI) with the same breed (Mocé et al., 2020; Sadeghi et al., 2020; Mocé et al., 2022a; Mocé et al., 2022b; Mocé et al., 2023). The same processed without SP presented also similar values to those observed in previous studies with the same breed and extender (Mocé et al., 2018). In addition, since the removal of SP improved goat buck sperm in vitro quality parameters, our results suggest that the centrifugation used do not seem to be detrimental for the sperm in this species, at least for the quality parameters that have been evaluated.

Our results do not agree with those from other authors that pointed out that the removal of SP can deteriorated spermatozoa either due to the decrease of antioxidants present in SP of goat bucks (Da Silva et al., 2014), to the effect of the reactive oxygen species (ROS) produced by the non-functional sperm contained in the pellet formed during the centrifugation that damage other sperm cells (Santiago-Moreno et al., 2017) or to the mechanical actions of centrifugation and compaction to the bottom of the sperm that can reduce sperm quality (Sadeghi et al., 2020). In addition, some authors reported that the effects of SP removal are observed even when the doses are inseminated, since the SP removal increases the risk of developing antibodies after depositing the sperm in the uterus (Santiago-Moreno et al., 2017). Perhaps the differences between our results and those observed in other works are due to different conditions in centrifugation that werelower in our study and perhaps, induced less harm to the sperm (500g vs. 800 or 1000g; Sadeghi et al., 2020; Da Silva et al., 2014) or even to the diluent used during washing (TCG vs egg yolk based extender; Da Silva et al., 2014). Nevertheless, the percentage of apoptotic sperm that we observed in the samples without SP might be indicating some sublethal damage due to the centrifugation. Perhaps the forces exerted during centrifugation or packing of the sperm in the pellet have caused damage at the intra- and extracellular level, triggering

a destabilization of the membrane. Thus, SP removal may have caused ROS production that are known to accelerate the apoptosis process (Liu et al., 2019). These values of apoptotic sperm in the samples processed without SP were like those observed in samples with SP from ejaculates obtained in non-breeding season (Mocé et al., 2022a).

After reaching 4 °C (refrigeration) differences between treatments were also observed in all parameters except for VCL, STR and BCF. Again, the values observed for the samples processed without SP are indicative of samples presenting higher sperm quality, except for the percentage of apoptotic spermatozoa that was higher for this treatment (SP-). Our results are within the range of the values published in other studies with the same breed for the samples processed with SP (Mocé et al., 2020; Mocé et al., 2022b, Mocé et al., 2023). Refrigeration induced more damage to the sperm processed with SP (almost all parameters exhibited significant differences before and after cooling) than for the samples processed without SP (Table 1). The parameters that worsened the most were VAI, LMB, LIVE and MITOK which indicates that SP is especially noxious for the acrosome, plasma membrane and sperm mitochondria. It is surprising that the samples processed without SP retained almost the same quality after refrigeration than before refrigeration. In fact, the decrease in sperm quality after refrigeration is somehow expected, since when membranes are cooled, the phospholipids undergo a phase transition from a liquid state to the crystalline-gel state and the proteins cluster into the remaining liquid lipid domains which results in unstable membranes that have lost their functionality (reviewed by Mocé et al., 2010). Therefore, upon rewarming a decrease in the sperm quality is normal. Thus, refrigeration of goat semen at 4 °C had a negative effect on sperm quality, such as increased apoptotic sperm or decreased mitochondrial activity over time, among others (Liu et al., 2019). In other species, the decrease in temperature causes changes in the orientation of lipids in the plasma membrane, loss of motility, low energy production, and damage to the acrosome and plasma membrane, among others (Pérez et al., 2022). Although in swine the SP plays a essential role in the acquisition of sperm resistance to the chilling to 4 ºC (De Lazari et al., 2018), perhaps SP does not play this role in goat buck sperm.

This decrease in the quality of the sperm after refrigeration in samples processed with SP could be due to a direct effect of the SP. Perhaps the presence of the SP does not allow the spermatozoa to adapt correctly to the temperature changes produced. Nevertheless, it seems more likely that these damages will be due to a direct or indirect effect of the lipases from the SP since the enzymatic activity in

samples processed with SP cannot be discarded at these temperatures. Although the enzymatic activity is higher at temperatures close to body temperature, they still are functional even at subzero temperatures (More et al., 1995). Therefore, BUSgp60 could act directly on sperm membrane and cause the hydrolysis of phospholipids, galactolipids or triglycerides or could degrade the SM residual triglycerides and produce the oleic acid, that is toxic for the sperm (Pellicer-Rubio and Combarnous, 1998). Oleic acid can damage the sperm membrane by increasing its lipid fusogenic activity or promote premature acrosomal exocytosis and negative effects on motility regulation via protein kinase C activation (Pellicer-Rubio and Combarnous, 1998). These effects would explain the increase in the percentage of acrosome reacted sperm observed in our study for the samples processed with the SP. In addition, if the permeability of the cell is modified by the glycerol and the oleic acid contained in the SP also increases this permeability, the osmotic resistance limits may be exceeded, damaging the cell.

The glycerol addition step is an important part of the freezing procedure where the permeant cryoprotectant is added to the sperm. Again, differences were observed between samples processed with or without SP for all the variables except for LIN, STR and BCF. Nevertheless, the addition of glycerol exerted negative effects in both treatments, since a drop in both kinetic and cytometric parameters was observed with respect to the values presented after refrigeration, although the decrease was again more pronounced for the samples processed with SP. Our motility values are close to those obtained in kili goats, while viability is more similar to Angora or Saanen goats (Kulaksiz et al., 2013). The decrease in sperm quality after adding the glycerol is not surprising since the SM2 is hyperosmolar and will induce osmotic shock in the sperm. Glycerol is one of the most widely used cryoprotectants in diluents for cryopreservation of goat semen. However, it is sometimes toxic to spermatozoa (in higher percentages) and can induce osmotic damage. Although glycerol is permeant and can traverse the membrane it will traverse the membrane at a slower rate than the water so the sperm will suffer from volume excursion (reviewed by Mocé et al., 2010). The addition of glycerol by itself can already cause some structural damage and decreased sperm motility (Sundararaman and Edwin, 2008). However, its benefits in sperm cryopreservation surpass its disadvantages since glycerol contributes to the stabilization of lipid membranes, causing an increase in the fluidity of the sperm cell membrane under dehydrated conditions and therefore confer the sperm greater capacity to survive cryopreservation (Holt, 2000).

How is the SP acting in this stage of the protocol is unknown. The fact that the quality of the samples drops sharply after the addition of glycerol, could indicate a direct effect of SP on the sperm. Perhaps, this fluid causes alterations in membrane permeability and, thus, spermatozoa suffer more intensively the osmotic effects even when the SM2 was added in three parts separated for 10 min to minimize the osmotic damage. Thus, these samples could benefit from a more gradual addition of the SM2. Nevertheless, the action of BUSgp60 cannot be underestimated either and this is probably the causal agent behind all these effects. The oleic acid released from the enzymatic reaction triggered by BUSgp60 could be responsible for the alterations in the sperm membrane permeability. Thus, oleic acid has been demonstrated to inducepermeability changes in other cells or membranes (Wnag et al., 1994; Kurniaawan et al., 2017), and perhaps it could also have the same effect on sperm plasma membranes.

The differences between treatments remained in the equilibration where significant differences were observed in motilities (TM and PM), ALH and cytometry parameters. However, in both treatments this phase did not result in great differences with the previous stage (glycerol addition) presenting the samples similar values in the equilibration than the after addition of glycerol. This stage is necessary in the freezing process for changes to occur in the sperm membrane (Gao et al., 1997) and according to our results it does not add more damage or stress to the sperm.

After freezing-thawing the differences between treatments remained, with the exception of VSL, VAP, LIN and STR that were similar for both treatments. Freezing-thawing was the stage that resulted more lethal for the sperm irrespective of the presence or absence of SP. These results were expected since freeze-thawing induces sperm damage and cryopreserved semen is always of lower quality than fresh semen (Mocé, 2012). Also, the fact that samples processed without SP at the end of the procedure presented higher values than samples processed with SP was expected and corroborates the results from previous studies (Corteel and Baril, 1974). Our values of quality to samples processed with absence of SP were similar that observed Esteve, et al. (2022; 48 % TM and 35 % PM) and slightly lower for PMI (43 % PMI). Also, the motility values of samples without SP are similar to previous studies in billy goat, but our values of live spermatozoa were lower (Küçük et al., 2014). Both ALH and BCF increased after freezing for samples processed without SP. Apparently the kinetics of the sperm differ before and after freezing. In fact, some studies linked elevated values of these two parameters with premature sperm capacitation and an increase in

flagellum beating frequency and the amplitude of lateral head displacement is observed in hyperactivation motility patterns (Mortimer, 1997). Thus, the freeze-thaw process induces cryocapacitation rendering sperm with a short lifespan for use in vivo (Bailey et al., 2000).

The freezing-thawing stage was affected to a larger extent to the samples processed with SP. Comparing the stages of equilibration and after freezing-thawing, the relative drops (considering as total values, 100 %, those obtained in the equilibrated stage) were 86 % TM and 88 % for VAI and LMB for the samples with SP and these values were half (44 % and 48 %, respectively) for the samples processed without SP. The decrease in quality in samples processed without SP occurs more gradually. In fact, the values obtained coincide with those described in sheep (Cabrera et al., 2011), where there is a 50% decrease in sperm viability during the freezing process. This is mainly due to the effect of temperature and osmotic pressure that induce, changes in the morphological organization of sperm membranes, the cells, that finally lead to modifications in membrane permeability, lipid composition of sperm membranes and intracellular fluid.

The prejudicial effect of SP has been observed for many parameters at different stages of the freezing process. The differences between the initial and final stage can show us that the quality sperm deterioration during the procedure. Overall, the treatment of samples with SP from fresh to thawed stage has decreased 62% TM, 56 % VAI and LMB, while samples without SP decreased 35 % TM and 38 % VAI and LMB. Considering as total values (100 %) those obtained in the fresh stage, the relative drops for the samples with SP were 90% for TM and 93% for VAI and LMB. However, for the samples without of SP the drop is 46 % of TM and 58 % of VAI and LMB. The differences obtained between fresh-thawed and frozen semen without SP are similar to those obtained by Dorado et al. (2007) or Barbas et al. (2018), although they started from higher values (sperm motilities between 80 and 90% in fresh). Thus, the cryopreservation process for most species is lethal for about 50% of the spermatozoa in an ejaculate, but many of the survivors (about 90%), despite having intact membranes and being motile, have sublethal damage that renders them unable to fertilize oocytes (Holt, 2000). The variation in quality between stages for each parameter does not seem to be high. But this way it can be observed how the drop in sperm quality with the treatment of the doses with SP is double than of the samples without SP. Thus, SP is detrimental for goat buck sperm freezing but the stage where most damaging effects occur is during freeze-thawing.

How can the SP be acting at this stage is unknown, but the lipase activity at the freezing temperatures can be discarded since the metabolism is inhibited at these temperatures (Chian, 2010) and at -196 ºC there is not enough thermal energy for chemical reactions to take place, nor is there liquid water, which is essential for metabolic processes (Mazur, 1984). Therefore, it is more likely that the presence of SP (or, more likely, the oleic acid released during all the previous stages) is altering the membrane permeability which in turn will affect the sperm ability to adapt to the osmotic changes that occur during the cryopreservation process. Sperm cryopreservation is a complex process, and its major challenge is to cross the intermediate temperature zone between -15° C and -60° C. Sperm must pass through these temperatures twice, first during freezing and then during thawing. Some authors point out that sperm injury during cryopreservation is more related to the negative effect of thawing (Martínez et al., 2022). Yet, SP has not been proven to cause this damage to sperm and neither have any of its components. If the components of SP are considered, the glycoproteins it contains bind to the sperm membrane and have a stabilizing effect on the membrane structure, as well as contribute to the adaptation to changes in temperature gradients during freezing and thawing (Leahy et al 2019). However, and as previously stated, the effect of the oleic acid cannot be discarded and it has been proven to negatively alter the cell membranes.

There is usually a difference in extracellular solute concentration on thawing. Cryoprotectants such as glycerol are permeant, but they traverse the membrane at a slower rate than the water. Thus, in presence of cryoprotectants, the rate at which thawing takes place causes cell swelling as a result of water entry (Vargas, 2023). Perhaps in presence of SP (or oleic acid released from the residual SM triglycerides) this membrane permeability to the cryoprotectant is altered so the osmotic shock and the swelling is higher. If the sperm swell above their osmotic tolerance limits the response to osmotic stresses will be lethal (Gilmore et al., 1998).

The presence of oleic acid in the cells has been mentioned above. Among other functions, it is also responsible for the regulation of membrane ion channels. In the osmotic regulation of the cell there is an input of ions such as calcium and an excess of this acid can promote an acrosomal negative exocytosis. In addition, if the permeability of the cell is modified by the glycerol and the oleic acid contained in the SP also increases this permeability, the osmotic resistance limits may be exceeded, damaging the cell. (Pellicer-Rubio and Combarnous, 1998).

Finally, another possibility is that the presence of SP may have caused ROS production which in turn will lead to lipid membrane peroxidation and consequently sperm damage and death. Nevertheless, this possibility seems unlikely since SP contains enzymes and antioxidants that protect spermatozoa from elevated ROS values (Rodríguez Martínez et al., 2021).

6. Conclusion

In conclusion, the SP deteriorates the spermatozoa through all the steps of the freezing protocol. However, the stage that affected the most the sperm quality was the freezing-thawing.

7. References

Bailey, J.L., Bilodeau, J.F., Cormier, N. (2000).
Sperm cryopreservation in domestic animals: a damaging and capacitating phenomenon, J. Androl. 21; 1–7.
https://doi.org/10.1002/j.1939-4640.2000.tb03268.x.

Barbas, J.P., Leahy, T., Horta, A.E., García-Herreros, M.(2018).
Sperm kinematics and subpopulational responses during the cryopreservation process in caprine ejaculates. 0011-2240
https://doi.org/10.1016/j.cryobiol.2018.03.005

BOE. Real Decreto 53/2013, de 1 de febrero,
por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. BOE (2013). Available online at: https://www.boe.es/boe/dias/2013/02/08/pdfs/BOE-A-2013-1337.pdf (accessed march 2, 2023).

Cabrera, V. P., Ayulo, L. A., and Pantoja, A. C. (2011).
Effect of tris and citrate-quail egg yolk extenders on viability of ovine frozen semen in straws. Revista de Investigaciones Veterinarias del Perú (RIVEP), 22(2), 105-113.
http://www.scielo.org.pe/scielo.php?s...

Cervera, E. M., Granell, M. M., Bernácer, J., Palazón, S. À. L., Vicente, C., Cervera, M. L. M., García, R. L., and Blasco, E. Á. G. (2019).
La labor de los centros de sementales en el Programa de mejora genética de ganado caprino de la raza Murciano-Granadina: Reproducción y genética. Tierras. Ovino, 25, 34-42.
https://riunet.upv.es/handle/10251/163762

Chian, R.C. (2010).
"Cryobiology: an overview," Chian RC, Quinn P (eds.), Fertility cryopreservation, Cambridge: Cambridge University Press, pp. 1-9.

Corteel, J.M., and Baril, G. (1974).
Viabilité des spermatozoïdes de bouc conservés et congelés avec ou sans leur plasma séminal: Effet du glucose. Annales de Biologie Animale Biochimie Biophysique, 14(4B), 741-745.
https://doi.org/10.1051/rnd:19740502

Cortés Gallego, S. (2003). Efecto de la conservación sobre la fisiología espermática de semen caprino (tesis doctoral). Universidad Complutense de Madrid, Madrid, España.
https://eprints.ucm.es/4195/

De Lazari, F.L., Sontag, E.R., Schneider, A., et al. (2018).
Seminal plasma proteins and their relationship with sperm motility and morphology in boars. Andrologia. e13222.
https://doi.org/10.1111/and.13222

Domınguez-Rebolledo, A. E., Fernandez-Santos, M.R., Garcıa, O., Alvarez, Maroto-Morales, A., Garde, J.J. and Martınez-Pastor, F. (2009).
Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa, 72, 1073–1084
https://doi:10.1016/j.theriogenology.2009.06.027

Dorado, J., Rodrıguez, I., Hidalgo, M. (2007).
Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. 68; 168–177.
https://doi:10.1016/j.theriogenology.2007.04.048

Esteve, I. C., Perez-Fuentes, S., Cortina, M., Gomez, E. A., Moce, M. L., Moce, E. (2022).
Does frozen goat sperm spoil in semen cryobanks?. In reproduction in domestic animals (Vol. 57, pp. 118-118). 111 River st, Hoboken 07030-5774, nj USA: Wiley.

Gallardo Bolaños, J.M., Balao da Silva, C.M., Martín Muñoz, P., Morillo Rodríguez, A., Plaza Dávila, M., Rodríguez-Martínez, H., Aparicio, I.M., Tapia, J.A., Ortega Ferrusola, C., Peña, F.J. (2014).
Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction. 148:221–35.
https://doi.org/10.1530/REP-13-0191 PMID:24850868

Gao, D., Mazur, P., and Critser, J. K. (1997).
Fundamental Cryobiology of Mammalian Spermatozoa. En A. M. Karow and J. K. Critser (Eds.), Reproductive Tissue Banking (pp.263-328). Academic Press. https://doi.org/10.1016/B978-012399770-8/50007-1

Gilmore, J. A., Liu, J., Peter, A. T., and Critser, J. K. (1998).
Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. Biology of Reproduction, 58(1), 28-36. https://doi.org/10.1095/biolreprod58.1.28

Holt, W. V. (2000).
Basic aspects of frozen storage of semen. Animal Reproduction Science, 62(1), 3-22.
https://doi.org/10.1016/S0378-4320(00)00152-4

Jia, B., Liang, J., Lv, C., Memon, S., Fang, Y., Wu, G. and Quan, G. (2021).
The characteristics of proteome and metabolome associated with contrasting sperm motility in goat seminal plasma, 11:15562.
https://doi.org/10.1038/s41598-021-95138-9

Konyali, C., Tomás, C., Blanch, E., Gómez, E. A., Graham, J. K., and Mocé, E. (2013).
Optimizing conditions for treating goat semen with cholesterol-loaded cyclodextrins prior to freezing to improve cryosurvival. Cryobiology, 67(2), 124-131. https://doi.org/10.1016/j.cryobiol.2013.06.001

Küçük, N., Aksoy, M., Uçan, U., Ahmad, E., Naseer, Z., Ceylam, A., Serin, I. (2014).
Comparison of two different cryopreservation protocols for freezing goat semen. Cryobiology 68(3): 327-331.
https://doi.org/10.1016/j.cryobiol.2014.04.009

Kulaksiz, R., Ari, U. C., Daşkin, A., and Üner, A. G. (2013).
The effect of different glycerol concentrations on freezability of semen from Angora, Kilis and Saanen goats. Slovak Journal of Animal Science, 46(2), 39-44.

Kurniawan, J., Suga, K., and Kuhl, T. L. (2017).
Interaction forces and membrane charge tunability: Oleic acid containing membranes in different pH conditions. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1859(2), 211-217.

Lahnsteiner, F., Patzner, R., and Weismann, T. (1993).
Energy resources of spermatozoa of the rainbow trout Oncorhynchus mykiss (Pisces, Teleostei). Reproduction Nutrition Development, 33(4), 349-360. https://doi.org/10.1051/rnd:19930404

Leahy, T., Rickard, J. P., Bernecic, N. C., Druart, X. and de Graaf, S. P. (2019).
Ram seminal plasma and its functional proteomic assessment. Reproduction 157, R243–R256.
https://doi.org/10.1530/REP-18-0627

Leboeuf, B., Restall, B., and Salamon, S. (2000).
Production and storage of goat semen for artificial insemination. Animal Reproduction Science, 62(1), 113-141.
https://doi.org/10.1016/S0378-4320(00)00156-1

Liu, T.; Han, Y.; Zhou, T.; Zhang, R.; Chen, H.; Chen, S.; Zhao, H. (2019).
Mechanisms of ROS-induced mitochondria-dependent apoptosis underlying liquid storage of goat spermatozoa. Aging 2019, 11, 7880–7898.
https://doi.org/10.18632%2Faging.102295

Lopera Vázquez, R. (2009). Estudio de factores que influyen en la fecundación in vitro heteróloga entre espermatozoides de caprino y oocitos de bovino madurados in vitro (tesis de Máster). University Politecnic of Valencia, Valencia, Spain.
https://riunet.upv.es/bitstream/handle/10251/11873/Tesis%20Master%20RICAURTE%20LOPERA.pdf?sequence=1andisAllowed=y

López-Gatius, F., (2012).
Factors of a noninfectious nature affecting fertility after artificial insemination in lactating dairy cows. A review. Theriogenology, 77(6), 1029-1041.
https://doi.org/10.1016/j.theriogenology.2011.10.014

Lozano Benito D. 1, Gil Huerta L.2, Álvarez San Martín C.3, 2011.
Efecto de la adición de plasma seminal en el semen equino descongelado. Sanid. mil. 2011; 67 (3): 284-290; ISSN: 1887-8571
https://dx.doi.org/10.4321/S1887-85712011000400005

Martínez Durán, J. A., Duverger Tellez, O., Interian Alvarez, L., Denis García, R., López Abreu, Z., Quintana Utra, M. D., and Palacios Espinosa, A. (2022).
Efecto del protector de membrana y la solución descongelante en la calidad in vitro del semen caprino congelado en pastillas. Revista de Salud Animal, 44.

Marzano, G.; Moscatelli, N.; Di Giacomo, M.; Martino, N.A.; Lacalandra, G.M.; Dell'aquila, M.E.; Maruccio, G.; Primiceri, E.; Chiriacò, M.S.; Zara, V.; et al. (2020).
Centrifugation force and time alter CASA parameters and oxidative status of cryopreserved stallion sperm. Biology 2020, 9, 22.
https://doi.org/10.3390/biology9020022

Mazur, P. (1984).
Freezing of living cells: mechanisms and implications. Am J Physiol. 247(3 Pt 1):C125-42.
https://doi.org/10.1152/ajpcell.1984.247.3.C125

Mocé, E., Blanch, E., Tomas, C. and Graham, J.K. (2010).
Use of Cholesterol in Sperm Cryopreservation: Present Moment and Perspectives to Future. 45 (Suppl. 2), 57–66; ISSN 0936-6768
https://doi: 10.1111/j.1439-0531.2010.01635.x

Mocé, E. (2012).
Tecnología del semen. En Francisco Marco Jiménez y José Salvador Antón (Ed.), Fundamentos y técnicas de la reproducción: mejora genética animal y biotecnología de la reproducción (pp. 105-145). Universidad Politécnica de Valencia.
https://riu-net.upv.es/bitstream/handle/10251/72469/TOC_0443_12_01.pdf?sequence=5andisAllowed=y

Mocé, E., Martínez-Granell, M., Fajardo, A., Vázquez-Briz, S., Bernácer, J., Villalba, I., Gómez, E.A. (2018).
Umbrales mínimos de calidad en semen descongelado de la raza caprino Murciano Granadina: efecto sobre el porcentaje de eyaculados almacenados en banco de germoplasma. XI Congreso Ibérico sobre Recursos Genéticos Animales SERGA-SPREGA. Participation: Oral comunication. Publication: Libro de resúmenes, p. 79. Murcia (España). Date: 27-28 de september 2018.

Mocé, E., Lozano-Palazón, S.A., Martínez-Granell, M.M., Mocé, M.L., and Gómez, E.A. (2020).
Effect of the Refrigeration System on In Vitro Quality and In Vivo Fertility of Goat Buck Sperm. Animals 10, no. 12: 2399.
https://doi.org/10.3390/ani10122399

Mocé, M.L., Esteve, I.C., Pérez-Fuentes, S., Gómez, E.A. and Mocé, E. (2022a).
Microbiota in Goat Buck Ejaculates Differs Between Breeding and Non-breeding Seasons. Front. Vet. Sci. 9:867671.
https://doi: 10.3389/fvets.2022.867671

Mocé, E., Mocé, M.L., Lozano-Palazón, S.A., Bernácer, J., Martínez-Granell, M.M., Esteve, I.C., Bernat, F., Contreras, S.J., Villalba, I., Gómez, E.A. (2022b).
Fertility prediction in dairy goats from Murciano-Granadina breed: The role of sperm evaluation and female traits
https://doi.org/10.1016/j.animal.2022.100525

Mocé, M. L., Esteve, I. C., Gómez, E. A., Pérez-Fuentes, S., and Mocé, E. (2023).
Microbial composition of goat buck's ejaculates is modified by the process of preparing and storing refrigerated semen doses. Theriogenology. https://doi.org/10.1016/j.theriogenology.2023.06.013

More, N., Daniel, R. M., and Petach, H. H. (1995).
The effect of low temperatures on enzyme activity. Biochemical Journal, 305(1), 17-20. https://doi.org/10.1042/bj3050017

Mortimer, S. T. (1997).
A critical review of the physiological importance and analysis of sperm movement in mammals. Human Reproduction Update, 3(5), 403-439. https://doi.org/10.1093/humupd/3.5.403

OIE, (2023).
Collection and processing of bovine, small ruminant and porcine semen, in: World Organization for Animal Health (Ed), Terrestrial Animal Health Core, 2022 pp. 152-157. Accessed 10 May 2023 https://www.woah.org/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_coll503 _semen.pdf

Parrilla, I., Martinez, E.A., Gil, M.A., Cuello, C., Roca, J., Rodriguez-Martinez, H., Martinez, C.A. (2020).
Boar seminal plasma: current insights on its potential role for assisted reproductive technologies in swine. Anim Reprod. 17(3):e20200022. https://doi.org/10.1590/1984-3143-AR2020-0022

Pellicer-Rubio, M.-T., Magallon, T., and Combarnous, Y. (1997).
Deterioration of Goat Sperm Viability in Milk Extenders is due to a Bulbourethral 60-Kilodalton Glycoprotein with Triglyceride Lipase Activity. Biology of Reproduction, 57(5), 1023-1031. https://doi.org/10.1095/biolreprod57.5.1023

Pellicer-Rubio, M. T., and Combarnous, Y. (1998).
Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleic acid released by the bulbourethral lipase BUSgp60. Reproduction, 112(1), 95-105. https://doi.org/10.1530/jrf.0.1120095

Pérez, M., Bustamante, C., Coronel, I., Carretero, M. I., Manrique, Y., Condori, E., and Perez, U. (2022).
Criopreservación de espermatozoides de llama obtenidos del conducto deferente utilizando tres curvas de congelamiento. Revista de Investigaciones Veterinarias del Perú, 33(6). http://dx.doi.org/10.15381/rivep.v33i6.24099

Purdy, P. H. (2006).
A review on goat sperm cryopreservation. Small Ruminant Research, 63(3), 215-225. https://doi.org/10.1016/j.smallrumres.2005.02.015

Rodríguez, G. J., Barrios-Arpi, M., Huanca, L. W., Rodríguez, G. A., and Revuelta, L. (2016).
Niveles de glucosa y triglicéridos en plasma seminal y motilidad espermática en COBAYOS alimentadas con 10% más en energía digestible. Revista Complutense de Ciencias Veterinarias, 10(1), 75-82.
https://doi.org/10.5209/rev_RCCV.2016.v10.n1.52501

Rodriguez-Martinez, H.; Martinez, E.A.; Calvete, J.J.; Peña Vega, F.J.; Roca, J. (2021).
Seminal Plasma: Relevant for Fertility? Int. J. Mol. Sci. 2021, 22, 4368. https://doi.org/10.3390/ijms22094368

Sadeghi, S., Del Gallego, R., García-Colomer, B., Gómez, E. A., Yániz, J. L., Gosálvez, J., López-Fernández, C. and Silvestre, M.A. (2020).
Effect of Sperm Concentration and Storage.Temperature on Goat Spermatozoa during. Liquid Storage 9, 300;
https://doi:10.3390/biology9090300

Salamon, S., and Maxwell, W. M. C. (2000).
Storage of ram semen. Animal Reproduction Science, 62(1), 77-111.
https://doi.org/10.1016/S0378-4320(00)00155-X

Salvador, I., Yániz, J., Viudes-De-Castro, M. P., Gómez, E. A., and Silvestre, M. A. (2006).
Effect of solid storage on caprine semen conservation at 5 C. Theriogenology, 66(4), 974-981.
https://doi.org/10.1016/j.theriogenology.2006.02.042

Santiago-Moreno, J., Esteso, M.C., Castano, C., Toledano-Díaz, A., Delgadillo, J.A., Lopez-Sebastian, A. (2017).
Seminal plasma removal by density-gradient centrifugation is superior for goat sperm preservation compared with classical sperm washing. Animal Reproduction Science
http://dx.doi.org/10.1016/j.anireprosci.2017.04.002

Silvestre, M. A., Salvador, I., Sánchez, J. P., and Gómez, E. A. (2004).
Effect of changing female stimulus on intensive semen collection in young Murciano-Granadina male goats. Journal of Animal Science, 82(6), 1641-1645.
https://doi.org/10.2527/2004.8261641x

Sundararaman, M. N., and Edwin, M. J. (2008).
Changes in motility characteristics of goat spermatozoa during glycerol-equilibration and the relevance to cryopreservation. Asian Journal of Cell Biology, 3(1), 22-33.

Vargas T. (2023). Efecto de diferentes métodos de congelación y descongelación en la criopreservación de los espermatozoides de alpaca (Vicugna pacos)
Lima: Universidad Nacional Mayor de San Marcos, Facultad de Medicina Veterinaria, Escuela Profesional de Medicina Veterinaria.

Wang, L. Y., Ma, J. K., Pan, W. F., Toledo-Velasquez, D., Malanga, C. J., and Rojanasakul, Y. (1994).
Alveolar permeability enhancement by oleic acid and related fatty acids: evidence for a calcium-dependent mechanism. Pharmaceutical research, 11, 513-517. https://doi.org/10.1023/A:1018906330308