

## Chemically defined diluent based on Tris-casein in freezing goat semen

Diluidor quimicamente definido à base de Tris-caseína na congelação de sêmen caprino

Diluyente químicamente definido a base de Tris-caseína en la congelación de semen caprino

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### **Abstract**

The objective of the present work was to elaborate on a chemically defined extender, based on Tris-casein, and to evaluate its efficacy in the goat semen cryopreservation. For this purpose, three goats were used. Eight ejaculates were collected per male ( $n = 24$ ). After semen collection, the ejaculates were frozen in a conventional extender (Tris-egg yolk) – control group, as well as an extender based on Tris-casein, in different concentrations (CAS1: 0.125 g/l; CAS2: 0.25 g/l; CAS3: 0.5 g/l), combined with 5% of glycerol. After dilution,  $200 \times 10^6$  sperm/ml was frozen in an automated system and stored in liquid nitrogen ( $-196^\circ\text{C}$ ). After thawing ( $37^\circ\text{C}$ , 30 s), the samples were submitted to kinetic analysis (CASA) and functional analysis, through plasma membrane integrity, acrosome integrity and mitochondrial membrane potential. Regarding the kinetic parameters, plasma membrane integrity, acrosome integrity and mitochondrial membrane potential, there were no significant differences ( $P > 0.05$ ) between the groups evaluated, with the exception of flagellar beat cross frequency (BCF), where the CAS3 group had a higher value ( $P \leq 0.05$ ) than the control group. It can be concluded that the chemically defined extender based on Tris-casein presents a viable alternative to the conventional diluent used for freezing goat semen.

**Keywords:** Buck; Cryoprotectant; Egg yolk; Freezing.

### **Resumo**

O objetivo do presente trabalho foi elaborar um diluente quimicamente definido, à base de Tris-caseína, e avaliar sua eficácia no processo de criopreservação de sêmen caprino. Para isso, foram utilizados três caprinos. Oito ejaculados foram coletados por macho ( $n = 24$ ). Após a coleta do sêmen, os ejaculados foram congelados em diluente convencional (Tris-gema de ovo) – grupo controle, bem como em diluente à base de Tris-caseína, em diferentes concentrações (CAS1: 0,125 g/l; CAS2: 0,25 g/l ; CAS3: 0,5 g/l), combinado com 5% de glicerol. Após a diluição,  $200 \times 10^6$  espermatozoides/ml foram congelados em sistema automatizado e armazenados em nitrogênio líquido ( $-196^\circ\text{C}$ ). Após o descongelamento ( $37^\circ\text{C}$ , 30 s), as amostras foram submetidas à análise cinética (CASA), integridade de membrana plasmática, integridade de acrossoma e potencial de membrana mitocondrial. Em relação aos parâmetros cinéticos, integridade de membrana plasmática, integridade de acrossoma e potencial de membrana mitocondrial, não houve diferenças significativas ( $P > 0,05$ ) entre os grupos avaliados, com exceção da frequência de batimento flagelar cruzado (BCF), onde o grupo CAS3 apresentou maior valor ( $P \leq 0,05$ ) do que o grupo controle. Pode-se concluir que

o diluyente químicamente definido à base de Tris-caseína apresenta uma alternativa viável ao diluyente convencional utilizado para congelamento de sêmen caprino.

**Palavras-chave:** Bode; Crioprotetor; Gema de ovo; Congelação.

### Resumen

El objetivo del presente trabajo fue elaborar un diluyente químicamente definido, a base de Tris-caseína, y evaluar su eficacia en el proceso de criopreservación de semen caprino. Para este propósito, se utilizaron tres chivos. Se recogieron ocho eyaculados por macho (n = 24). Luego de la recolección del semen, los eyaculados fueron congelados en un diluyente convencional (Tris-yema de huevo) – grupo control, así como un diluyente a base de Tris-caseína, en diferentes concentraciones (CAS1: 0.125 g/L; CAS2: 0.25 g/L CAS3: 0,5 g/L), combinado con 5% de glicerol. Después de la dilución, se congelaron  $200 \times 10^6$  espermatozoides/mL en un sistema automatizado y se almacenaron en nitrógeno líquido ( $-196 \text{ }^\circ\text{C}$ ). Después de la descongelación ( $37 \text{ }^\circ\text{C}$ , 30 s), las muestras se sometieron a análisis cinético (CASA), integridad de la membrana plasmática, integridad del acrosoma y potencial de la membrana mitocondrial. En cuanto a los parámetros cinéticos, integridad de la membrana plasmática, integridad del acrosoma y potencial de la membrana mitocondrial, no hubo diferencias significativas ( $P > 0,05$ ) entre los grupos evaluados, con excepción de la frecuencia de cruce de latidos flagelares (FBC), donde el grupo CAS3 tuvo una mayor valor ( $P \leq 0,05$ ) que el grupo de control. Se puede concluir que el diluyente químicamente definido basado en Tris-caseína presenta una alternativa viable al diluyente convencional utilizado para congelar semen caprino.

**Palabras clave:** Chivo; Crioprotector; Yema de huevo; Congelación.

## 1. Introduction

Semen cryopreservation is an important tool for the conservation of genetic resources. However, freezing spermatozoa can cause damage to sperm cells, particularly during freezing, by determining lethal or sublethal sperm alterations at ultrastructural, biochemical and functional levels, which result in reduced sperm motility, viability and fertilizing capacity (Alcay et al., 2020).

One of the key points for the success of cryopreservation is related to the dilution medium. In general terms, a good extender must have carbohydrates in its composition as a source of energy; buffers for maintaining pH and osmolarity; antibiotics to inhibit microbial growth; non-penetrating cryoprotectants that, in addition to having a nutritional function, protect cells against cold shock, as they are refrigerated up to  $5 \text{ }^\circ\text{C}$ ; and penetrating cryoprotectants to protect sperm from the deleterious effects of freezing (Purdy, 2006; Castelo et al., 2008).

The most commonly used extenders for semen cryopreservation are based on animal products, such as egg yolk or milk. Concerns have been raised about their potential risk as vehicles for disease-causing pathogens and their and safety (Bousseau et al., 1998; Chelucci et al., 2015; Ansari et al., 2016). In addition, post-thawed semen quality can also be influenced by individual differences in the quality inherent to the egg yolk, due to the number of days after laying, the storage period and the difference between the birds' feeding (Del valle et al., 2012; Anzar et al., 2019).

In order to solve the obstacles described above and allow a more adequate and safe processing of semen, some alternatives for the substitution of skimmed milk and egg yolk have been reported, among which soy lecithin can be highlighted (Alcay et al., 2017; Mehdipour et al., 2017; Najafi et al., 2017; Araújo Silva et al., 2019), caseinate (Da Silva et al., 2020) and low-density lipoproteins (Luna-Orozco et al., 2019). Diluent based on these components have demonstrated a protective effect on sperm cells, a fact that makes them important alternatives for the formulation of seminal extenders (Alcay et al., 2017; Araújo Silva et al., 2019; Da Silva et al., 2020).

Among the components mentioned, casein has demonstrated a positive effect on the sperm cryopreservation process. As the main protein component, casein represents 75–80% of all milk proteins (Batellier et al., 2001; Pereira, 2014), with the ability to stabilize sperm membranes, preventing the loss of phospholipids, since they compete directly with proteins seminal plasma cells, responsible for membrane destabilization (Bergeron & Manjunath, 2006; Bergeron et al., 2007).

In addition, casein micelles have the property of interacting with calcium ions. These ions are responsible for inducing tyrosine phosphorylation to initiate the capacitation process, as well as improving the rate of sperm binding to the zona pellucida of oocytes, with a consequent increase in *in vitro* embryo production (Da silva et al., 2020; Diniz et al., 2020).

Thus, casein has been responsible for the protection of sperm, even at low concentrations in chilled (0.6%) or frozen (1.35%) semen (Lagares et al., 2012). However, studies are needed on its use in the process of freezing goat semen, in order to evaluate its effect and efficacy on sperm cells.

Therefore, the objective of this study was to develop a chemically defined extender based on casein for freezing goat semen, in order to allow adequate sperm conservation, eliminating the risks and sanitary barriers arising from the use of conventional extenders.

## 2. Methodology

Unless otherwise specified, all reagents used in the experiment were obtained from the Sigma-Aldrich Company (St Louis, MO, USA).

Three sexually mature Saanen goats, with a history of fertility and individually housed, belonging to the Department of Animal Science, Universidade Federal Rural de Pernambuco (UFRPE), Recife, Brazil (8° 03' 14" S and 34° 52' 52" W) were used. The animals were fed 400 g/animal/day of commercial concentrate, as well as Tifton hay, water and mineral salt *ad libitum*. The experimental protocol was approved by the Ethics Committee in Animal Experimentation of the URFPE, under process N°. 051/2019/CEUA.

Ejaculates were obtained by the artificial vagina method, using a female as a dummy. Eight ejaculates were collected per male, using two collections per week and a total of 24 ejaculates. After collection, the ejaculates were immediately submitted to macroscopic (volume, color, appearance and odor) and microscopic (mass movement, motility and vigor) analysis under a phase contrast microscope (Olympus, Japan; 100x). The ejaculates that presented minimum values of 70% of total motility were approved and destined for the formation of the pool. Then, the sperm concentration of the pool was determined, using a Neubauer chamber, and the motility was analyzed using the computerized sperm analysis system (CASA, SCA™; Microptics, S.L., Version 5.1. Barcelona, Spain). The semen pool was washed twice in Tris Buffer (3.605 g Tris; 2.024 g citric acid; 1.488 g fructose, 100 ml Milli-Q water, pH 6.8), by means of centrifugation (600 g/10 min), for the removal of seminal plasma. After this procedure, the pools that had minimum values of 70% of total motility were used.

A semen sample was diluted in Tris-egg yolk-based freezing medium (Tris 375 mM, citric acid 124 mM, fructose 41.6 mM, egg yolk 20%, penicillin 100 IU, streptomycin 50 mg, glycerol 5%, pH 7.2), constituting the control group (GC). The remaining samples were diluted in a chemically defined medium based on Tris (Tris 375 mM, citric acid 124 mM, fructose 41.6 mM, L-carnitine 2 mM, sodium pyruvate 1 mM, penicillin 100 IU, streptomycin 50 mg, glycerol 5%, pH 7.2), plus casein at different concentrations, constituting the experimental groups (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l).

All groups had the concentration standardized to  $200 \times 10^6$  sperm/ml. Then, the samples were stored in straws (0.25 ml) and frozen in an automated system (TK-3000®, TK freezing technology Ltda., Uberaba, Brazil), using the specific refrigeration curve for goat semen, with a reduction of 0.25 °C/min until reaching 5 °C, where they remained for 120 min for stabilization. The negative curve started with a reduction of 20 °C/min, until reaching -120 °C, when the straws were immersed and stored in liquid nitrogen (-196 °C).

After a minimum interval of 24 h of storage in liquid nitrogen, two straws per experimental group were thawed (37 °C for 30 s). For the evaluation of sperm kinetics, using the computerized system (CASA), aliquots (10 µl) of semen were diluted in Tris, obtaining a concentration of  $50 \times 10^6$  spermatozoa/ml, and incubated in a water bath (37 °C/5 min). Then, an aliquot

(2.5  $\mu$ l) of the diluted sample was placed on a slide and covered with a coverslip (18 x 18 mm), both preheated (37 °C), and evaluated under a phase contrast microscope (Eclipse 50i, Nikon, Japan, 100x). The images were captured using a video camera (Basler Vision Technologies TM A312FC, Germany). In each sample, five random fields were selected, with a record of at least 500 sperm cells.

The kinetic parameters evaluated using the SCA™ software, version 5.1 (Microptics, SL, Barcelona, Spain), were: total motility (TM, %), progressive motility (PM, %), rapid velocity (RAP, %), linearity (LIN, %), straightness (STR, %), oscillation index (WOB, %), curvilinear velocity (VCL,  $\mu$ m/s), straight-line velocity (VSL,  $\mu$ m/s), average trajectory velocity (VAP,  $\mu$ m/s), lateral displacement of the head (ALH,  $\mu$ m/s) and beat cross frequency (BCF, Hz). The CASA system values were measured with the following settings: temperature 37 °C; magnification, 100x; number of images, 25; images per second, 25; head area, 20–70  $\mu$ m<sup>2</sup>; VAP: slow 10  $\mu$ /s < medium 45  $\mu$ /s < fast 75  $\mu$ /s; progressivity, 80% STR; circular, 50% LIN.

The evaluation of plasma membrane integrity (iPM) was determined by the double staining method, using the combination of carboxyfluorescein diacetate (CFDA; 0.46 mg/ml in DMSO) and propidium iodide (PI; 0.5 mg/ml in PBS), as described by Araújo Silva et al. (2019). For each group, an aliquot of the sample (30  $\mu$ l) was stained with 5.0  $\mu$ l of CFDA and 5.0  $\mu$ l of PI and incubated for 10 min (25 °C). Using DBP 485/20 nm excitation and DBP 580–630 nm emission filters, 200 cells were evaluated per slide under an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) at 400x magnification. Spermatozoa stained only in green were considered as having intact membranes and those stained in red as having damaged membranes.

Acrosome integrity (iAC) was assessed using Peanut Agglutinin-conjugated fluorescein isothiocyanate dye (FITC-PNA; 100  $\mu$ g/ml in PBS) (Araújo Silva et al., 2019). Aliquots (10  $\mu$ l) of the sample were used to make a smear, which was air-dried, stained with 30  $\mu$ l of FITC-PNA and incubated in a humid chamber (4 °C for 15 minutes) in the dark. Then, the slides were immersed in PBS twice and air-dried naturally. Immediately before evaluation, 5.0  $\mu$ l of the solution (4.5 ml of glycerol, 0.5 ml of PBS and 5.0 mg of p-phenylenediamine) were placed on the slide and covered with a coverslip. A total of 200 sperm per slide were evaluated using BP 450–490 nm excitation and LP 515 nm emission filters, at 400x magnification. Spermatozoa stained in fluorescent green in the acrosome region were classified as having intact acrosomes, while those stained fluorescent green only in the equatorial region of the sperm head, or not stained, were classified as having damaged acrosomes.

Mitochondrial membrane potential (MMP) was evaluated using lipophilic cationic fluorochrome (JC-1; 0.15 mM in DMSO) (Araújo Silva et al., 2019). To this end, 30  $\mu$ l aliquots of the sample were stained with 5.0  $\mu$ l of JC-1 and incubated for 10 min (25 °C). In total, 200 sperm were evaluated using BP excitation 450–490 nm and LP 515 nm emission filters, at 400x magnification. Spermatozoa that showed an orange stained midpiece were classified as carriers of high MMP, while sperm stained green in the midpiece were classified as carriers of MMP.

For statistical analysis, experimental groups were tested for normality using the Shapiro–Wilk test. Then, the normally distributed variables were analyzed with one-sided analysis of variance (ANOVA), followed by Tukey’s test. Those not normally distributed were analyzed using the Kruskal–Walli’s test. Data were expressed as mean  $\pm$  SD (standard deviation) and were considered significant if  $P < 0.05$ . Statistical analyses were performed using GraphPad Prism8 (Version 8.0.1).

### 3. Results and Discussion

The results of the sperm kinetic analysis are shown in Table 1. Regarding the parameters of TM, PM, VAP, VSL, VCL, ALH, STR, LIN, RAP and WOB, no differences were observed ( $P > 0.05$ ) between the control group (Tris-egg yolk) and the other groups frozen in a chemically defined Tris-based medium, plus different concentrations of casein (CAS1, CAS2 and CAS3). However, there was a significant increase ( $P < 0.05$ ) in the BCF of sperm, when using the chemically defined extender based on Tris-casein, at a concentration of 0.5 g/l (CAS3), when compared to the control group (Tris-egg yolk).

**Table 1.** Kinematics (CASA) of goat sperm frozen in Tris-egg yolk or chemically defined Tris-based extender, combined with different casein concentrations (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l). Data are expressed as mean  $\pm$  standard deviation.

Parameters	Experimental Groups			
	CONTROL	CAS1	CAS2	CAS3
TM (%)	50.43 $\pm$ 12.89	36.11 $\pm$ 12.07	31.39 $\pm$ 11.17	33.09 $\pm$ 16.09
PM (%)	27.99 $\pm$ 8.51	19.06 $\pm$ 8.92	16.97 $\pm$ 6.79	18.54 $\pm$ 9.32
RAP (%)	10.96 $\pm$ 5.30	9.29 $\pm$ 5.77	6.21 $\pm$ 3.56	9.58 $\pm$ 5.45
LIN (%)	73.70 $\pm$ 13.96	67.56 $\pm$ 8.04	73.70 $\pm$ 4.01	66.47 $\pm$ 10.44
STR (%)	86.97 $\pm$ 7.83	86.59 $\pm$ 4.85	87.90 $\pm$ 2.21	87.80 $\pm$ 3.96
WOB (%)	87.03 $\pm$ 9.73	77.80 $\pm$ 5.84	83.39 $\pm$ 3.20	79.79 $\pm$ 4.90
VCL ( $\mu$ m/s)	66.26 $\pm$ 9.20	67.93 $\pm$ 12.25	61.30 $\pm$ 7.69	67.89 $\pm$ 10.36
VSL ( $\mu$ m/s)	47.97 $\pm$ 6.76	45.96 $\pm$ 10.14	46.28 $\pm$ 4.85	47.51 $\pm$ 7.83
VAP ( $\mu$ m/s)	55.11 $\pm$ 5.09	52.77 $\pm$ 9.74	51.01 $\pm$ 5.95	54.01 $\pm$ 7.96
ALH ( $\mu$ m)	1.90 $\pm$ 0.57	2.41 $\pm$ 0.43	2.03 $\pm$ 0.31	2.35 $\pm$ 0.55
BCF (Hz)	9.41 $\pm$ 2.17 <sup>b</sup>	11.74 $\pm$ 1.38 <sup>ab</sup>	10.44 $\pm$ 1.39 <sup>ab</sup>	12.97 $\pm$ 1.07 <sup>a</sup>

<sup>a,b</sup> different letters on the same line represent a statistical difference between treatments (P < 0.05). Source: Authors.

In relation to the parameters of plasma membrane integrity (iPM), acrosome integrity (iAC) and mitochondrial membrane potential (MMP) presented in Table 2, there was no significant difference (P > 0.05) in the values of these parameters (P > 0.05) in the different groups frozen with a chemically defined extender based on Tris, combined with casein at different concentrations (CAS1, CAS2 and CAS3), when compared to the control group (Tris-egg yolk).

**Table 2.** Percentage of goat sperm with intact plasma membrane and acrosome, and high mitochondrial membrane potential, after freezing in a Tris egg yolk-based extender (control) or chemically defined based on Tris-casein at different concentrations (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l). Data are expressed as mean  $\pm$  standard deviation.

Parameters	Experimental Groups			
	Control	CAS1	CAS2	CAS3
iPM (%)	38.07 $\pm$ 11.05	34.49 $\pm$ 9.45	23.57 $\pm$ 15.68	31.57 $\pm$ 12.54
iAC (%)	20.28 $\pm$ 5.23	25.14 $\pm$ 23.13	29.67 $\pm$ 10.51	22.42 $\pm$ 7.68
hMMP (%)	42.50 $\pm$ 17.49	45.07 $\pm$ 25.13	34.79 $\pm$ 26.63	21.50 $\pm$ 20.33

Source: Authors.

This study was carried out with the aim of developing and evaluating a Tris-casein-based extender obtained from bovine milk, in order to allow adequate conservation of goat spermatozoa, eliminating the biological risks and sanitary barriers arising from the use of conventional extenders (skimmed milk or Tris-egg yolk). The results obtained demonstrate that the Tris-casein-based extenders can be used for freezing goat semen, since they did not present a significant difference in the movement pattern and sperm kinetics when compared to the control group. However, the experimental group containing Tris-casein at a concentration of 0.5 g/l (CAS3) showed a significant increase only in the parameter BCF in relation to the control group.

Thus, the ability to protect sperm cells after the freezing process using a Tris-based extender, combined with casein at different concentrations (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l) is evident. The mechanism of protection of casein on gametes is not fully elucidated, but, as verified by some authors, it is believed that milk proteins protect spermatozoa through interaction with binder of sperm proteins (BSP), preventing loss of lipids through the sperm membrane, which can be harmful to sperm storage, similar to the already known protection of the Tris egg yolk-based extender (Bergeron & Manjunath, 2006; Igbokwe et al., 2019) and used in this study as a control group. However, in this study, the semen was centrifuged, removing the seminal plasma and consequently, the BSP before freezing the semen. Thus, it is unlikely that the mechanism by which caseins protect gametes involves only a direct interaction with seminal plasma proteins.

According to Da Silva et al. (2012) and Diniz et al. (2020) although the mechanism has not been fully elucidated, casein may increase sperm capacitation due to an increase in intracellular calcium. In addition, caseins can interact with the spermatid glycocalyx so that they can more easily expose the sperm/oocyte receptors or increase the sensitivity of these proteins to the oocyte and thereby increase the binding of sperm to the zona pellucida of the oocyte, which is desirable to obtain good fertilization.

Among the extenders based on milk protein, better results have been demonstrated with casein. In an experiment with pigs, casein reduced the damage generated by cryopreservation, retaining cholesterol, reducing caspase activity and maintaining DNA integrity (Da Silva et al., 2012). Additionally, *in vitro* fertilization studies, supplementation of the extender medium for semen cryopreservation with casein increased the embryo cleavage rate (Tatemoto et al., 2015).

It is noteworthy, however, that in the analysis of sperm kinetics of samples submitted to freezing/thawing, it was observed that the frequency of tail beating (BCF) of sperm diluted in Tris-casein at a concentration of 0.5 g/L (CAS3) was higher than that observed in gametes from the control group, consisting of Tris-egg yolk. This result may be favorable to fertilization since the migration and penetration of sperm into the cervical mucus are favored by high values of BCF and LIN (Mortimer, 2000). Furthermore, according to Dorado et al. (2010), the CASA results can be used as fertility indicators of post-

freezing goat semen, since mean values of BCF were significantly higher in semen samples that achieved successful pregnancy, when compared to those that did not achieve this result after artificial insemination (AI). Similarly, this parameter (BCF), along with ALH, LIN, VAP and VSL, was associated with high fertility semen in bulls (Farrell et al., 1998). In sheep, VAP, VCL and BCF have also been shown to have a high correlation with post-freezing sperm fertility (Del Olmo et al., 2013). Therefore, there is strong evidence that semen diluted in Tris-casein, at a concentration of 0.5 g/l, has higher fertility rates, when compared to semen frozen in an extender based on Tris-egg yolk (control group). However, further studies are needed.

The success of the semen freezing process can also be evaluated by the amount of sperm kept intact after thawing, demonstrating the protection exerted by the extender on the structure of the plasma membrane (Bergeron et al., 2007; Dias et al., 2009). Thus, sperm membrane integrity is a necessary requirement for post-thawing sperm survival (Watson et al., 2000). In the present study, the integrity of the plasma membrane was evaluated using fluorescent probes, where there was no significant difference between the Tris-egg yolk group (control) and the Tris-casein groups (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l).

The analysis of the acrosome integrity also showed no difference in the results obtained between the different groups frozen in a Tris egg yolk-based extender (control) or in Tris-casein (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l). These results demonstrated that the chemically defined extender based on casein can exert a protective function to the sperm membrane and the acrosome, in the same way as the conventional extender based on Tris-egg yolk.

Regarding the mitochondrial membrane potential, its association with fertility is uncertain. However, in most species, the use of fluorescent probes to assess mitochondrial potential is widespread, since there is a high affinity of this potential with energy capacity and cell motility (Vishwanath & Shannon, 2000; Roy & Atreja, 2008). In this study, there was also no significant difference in the percentage of sperm with high MMP in the control group (Tris-egg yolk) or in the groups frozen in a chemically defined extender based on Tris-casein (CAS1 = 0.125 g/l; CAS2 = 0.25 g/l; CAS3 = 0.5 g/l).

Therefore, when evaluating the results presented in this work, it is proven that the replacement of egg yolk by casein, in the composition of the caprine semen freezing extender, does not cause a reduction in the quality of kinetics, plasma membrane integrity, acrosome integrity and MMP of spermatozoa submitted to freezing and thawing. Therefore, these results are in agreement with previous data obtained with bovine (Batellie et al., 2001; Diniz et al., 2020), horse (Campos et al., 2020) and sheep (Salgado et al., 2020), demonstrating that casein can be as effective in preserving the fertility parameters of frozen sperm as conventional extenders that have egg yolk in their composition.

#### **4. Conclusion**

Based on the above, it can be concluded that the chemically defined extender based on Tris supplemented with casein (0.125 g/l, 0.25 g/l and 0.5 g/l) is a viable alternative to the conventional extender (Tris-egg yolk) used for freezing goat semen.

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