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# Effect of low-density lipoproteins and trehalose on the quality ofcryopreserved bovine semen

***Efecto de las lipoproteínas de baja densidad y la trehalosa en la calidad del semen bovino criopreservadoEfeito das lipoproteínas de baixa densidade e trealose na qualidade do sêmen bovino criopreservado***

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**Abstract**
**Background:** In artificial insemination, chicken egg yolk is added to bovine semen to protect it during the cryopreservation process, although it contains substances that can affect the microbiological quality and metabolism of sperm.
**Objective:** To evaluate post-thaw quality of bovine cryopreserved semen added with centrifuged and non-centrifuged egg yolk, low-density lipoproteins (LDL), and trehalose (T).
**Methods:** Ten ejaculates from five bulls were cryopreserved under the treatments T1: pure egg yolk (PEY) at 20% v/v, T2: centrifuged egg yolk (CEY) at 20% v/v, T3: LDL at 8% v/v, T4: T at 100 mM, and T5: T at 100 mM plus LDL at 8% v/v (TLDL). Spermatic motility and kinetics, functional membrane integrity (FMI), structural membrane integrity (SMI), sperm vitality (SV) and abnormal morphology (AM) were assessed using the Sperm Class Analyzer (SCA®) system, hypoosmotic test (HOST), SYBR/PI probes, and eosin–nigrosin staining, respectively. A completely randomized design was used. Normal distribution of the variables was validated through the Kolmogórov– Smirnov test. A generalized linear model was used to determine sources of variation. Means were compared using the Tukey test.
**Results:** Inclusion of CEY or LDL had a similar effect on sperm protection, and were superior for motility, kinetics and membrane integrity compared to the other treatments ($p<0.05$). CEY was superior for progressive motility ($p<0.05$). The cryoprotective action of LDL was similar to TLDL for motility and kinetics, SMI, SV, and AM ($p<0.05$). Inclusion of PEY and T resulted in the lowest semen quality ($p<0.05$). The use of T resulted in a reduction in FMI and SMI ($p<0.05$). No differences in AM between treatments were found ($p>0.05$).

**Conclusions:** Egg yolk can be replaced by centrifuged egg yolk or low-density lipoproteins in the freezing extender used for bovine semen used in artificial insemination.
**Keywords**: *bull; cryopreservation; cryoprotectant; egg yolk; extender; low-density lipoproteins; semen; trehalose.*

**Resumen**

**Antecedentes:** la yema de huevo de gallina se agrega al semen bovino usado en inseminación artificial para protegerlo durante el proceso de criopreservación, aunque ésta tiene sustancias que pueden afectar el metabolismo espermatico y la calidad microbiológica del semen.

**Objetivo:** evaluar la calidad post-descongelación del semen bovino criopreservado agregado con yema de huevo centrifugada y no centrifugada, lipoproteínas de baja densidad (LDL) y trehalosa (T).

**Métodos:** diez eyaculados de cinco toros se criopreservaron bajo los tratamientos, T1: yema de huevo pura (PEY) al 20% v/v, T2: yema de huevo centrifugada (CEY) al 20% v/v, T3: LDL al 8% v/v, T4: T a 100 mM, y T5: T a 100 mM más LDL al 8% v/v (TLDL). La movilidad y la cinética espermática, la integridad funcional de la membrana (FMI), la integridad estructural de la membrana (SMI), la vitalidad espermática (SV) y la morfología anormal (AM), se determinaron mediante el sistema Sperm Class Analyzer (SCA®), la prueba hipoosmótica (HOST), las sondas SYBR/PI y la tinción con eosina-nigrosina, respectivamente. Se utilizó un diseño completamente al azar. La normalidad de las variables se validó mediante la prueba de Kolmogórov-Smirnov. Se utilizó un modelo lineal generalizado para determinar las fuentes de variación. Las medias de los tratamientos se compararon mediante la prueba de Tukey.

**Resultados:** CEY y LDL tuvieron un efecto similar en la protección de los espermatozoides, siendo superiores a los demás tratamientos respecto a movilidad, cinética e integridad de la membrana ($p<0,05$). CEY fue superior para la movilidad progresiva ($p<0,05$). La acción crioprotectora de LDL fue similar a TLDL para movilidad y cinética, SMI, AM y SV ($p<0,05$). PEY y T resultaron en la más baja calidad seminal ($p<0,05$). El uso de T redujo la FMI y la SMI ($p<0,05$). No se encontraron diferencias en AM entre los tratamientos ($p>0,05$).

**Conclusiones:** la yema de huevo puede reemplazarse por yema de huevo centrifugada o por lipoproteinas de baja densidad en el diluyente de congelación usado para semen bovino destinado a inseminacion artificial.
**Palabras clave:** *bovino; criopreservación; crioprotecciòn; diluyente; inseminacion artificial; lipoproteínas de bajadensidad; motilidad; semen; toro; trehalosa; yema de huevo.*

**Resumo**

**Antecedentes:** a gema de ovo de galinha tem sido utilizada com a finalidade de proteger o sêmen bovino durante o processo de criopreservação, embora tenha substâncias que possam afetar o metabolismo dos espermatozóides e a qualidade microbiológica do sêmen utilizado para a inseminação artificial.

**Objetivo:** avaliar a qualidade pós-descongelamento do sêmen bovino criopreservado com gema de ovo centrifugada e não centrifugada, lipoproteínas de baixa densidade (LDL) e trealose (T).

**Métodos:** dez ejaculados de cinco touros foram criopreservados sob os tratamentos, T1: gema de ovo pura (PEY) 20% v/v, T2: gema de ovo centrifugada (CEY) 20% v/v, T3: LDL 8% v/v, T4: T 100 mM e T5: T 100 mM mais LDL 8% v/v (TLDL). Mobilidade e cinética espermática, integridade funcional da membrana (FMI), integridade estrutural da membrana (SMI), vitalidade espermática (SV) e morfologia anormal (AM) foram determinadas por o sistema Sperm Class Analyzer (SCA®), teste hiposmótico (HOST), coloração com SYBR/PI e eosina-nigrosina, respectivamente. Um design completamente

aleatoriedade foi usado. A normalidade das variáveis foi validada pelo teste de Kolmogorov-Smirnov. Um modelo linear generalizado foi utilizado para determinar as fontes de variação. As médias dos tratamentos foram comparadas pelo teste de Tukey.

**Resultados:** T2 (CEY) e T3 (LDL) tiveram efeito similar na proteção espermática, sendo superior aos demais tratamentos para mobilidade, cinética e integridade da membrana ($p<0,05$). T2 (CEY) foi superior para mobilidade progressiva ($p<0,05$). A ação crioprotetora de T3 (LDL) foi semelhante à T5 (TLDL) para motilidade e cinética, SMI, SV e AM ($p<0,05$). T1 (PEY) e T4 (T) tiveram a menor qualidade seminal ($p<0,05$). O uso de T4 (T) produziu uma redução na SMI e FMI ($p<0,05$). Não foram encontradas diferenças na AM entre os tratamentos ($p>0,05$).

**Conclusões:** a gema de ovo pode ser substituída por gema de ovo centrifugada ou lipoproteínas de baixa densidade no diluente de congelamento de sêmen bovino.

**Palavras-chave:** *criopreservação; crioprotetor; diluente; gema de ovo; lipoproteínas de baixa densidade; sêmen; touro;trealose.*

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# Introduction

Freezing semen enables commercialization on a global scale of sperm from animals with high genetic value (Moreno *et al.*, 2013) favoring the development of the livestock sector (Zhang *et al.*, 2015). However, spermatozoa are subjected to thermal, osmotic and oxidative stress throughout freezing and thawing processes, which results in plasma membrane alterations that compromise fertilization (Beran *et al.*, 2013; Sieme *et al.*, 2015). Additionally, studies on bovine semen have shown that sperm cryopreservation reduces motility, damages plasma and acrosomal membranes and decreases mitochondrial function (Celeghini *et al.*, 2008).

Since the beginning of bovine semen cryopreservation, egg yolk and milk-based extenders have been used to protect sperm from the detrimental effects of cooling and freezing (Layek *et al.*, 2016) despite they can transmit pathogens and interfere with microscopic observations (Amirat *et al.*, 2004). Additionally, egg yolk contains substances that hinder sperm metabolism and reduce motility, acrosomal integrity, and fertilizing ability (Moreno *et al.*, 2013). Anzar *et al.* (2019) hypothesized that egg yolk proteins mask the innate proteins of sperm plasma membrane, while semen dilution with protein-free extend would provide an opportunity to study mammalian sperm proteomics following cryopreservation. Forthese reasons, it is necessary to find alternativesto improve the quality of frozen semen.

Purified low-density lipoproteins (LDL) from egg yolk can improve motility of post-thaw bull semen, and its optimum concentration in freezing media is 8% (Moussa *et al.*, 2002). Some hypotheses regarding the protective mechanism of LDL include stabilization of membranes, replacement of membrane phospholipids lost during cryopreservation, or interaction with or binding to deleterious proteins present in bovine seminal plasma (Bergeron *et al.*, 2004; Akhter *et al.*, 2011) increasing membrane resistance against cold shock (Moussa *et al.*, 2002). This occurs because the major proteins of bull seminal plasma (BSP proteins: BSP-A1 / A2, BSP-A3, and BSP- 30-kDa) bind to sperm surface at ejaculation stimulating cholesterol and phospholipid efflux from the sperm membrane. Addition of LDL to the extender helps to prevent binding of BSP to sperm and lipid efflux from the sperm membrane resulting in increased semen

quality (Thérien *etal.*, 1999; Bergeron *et al.*, 2004).

Trehalose is another cryoprotectant utilized in freezing media (Hu *et al.*, 2010). Several studies have shown that trehalose positively affects post-thawing semen motility and acrosomal and plasma membrane integrity (Hu *et al.*, 2010). Viability and mitochondrial activity are increased, and DNA is kept intact when 100 mM trehalose is added to the extender (Öztürk *et al.*, 2017). These effects have been explained by trehalose`s ability to promote dehydration (Aisen *et al.*, 2005) or interact with membrane biomolecules (Bucak *et al.*, 2007). Oh *et al.* (2012) reported that semen cryopreserved with a tris-egg yolk extender containing 7% glycerol, 4% LDL, and 20 mM taurine, hypotaurine and trehalose improved viability, membrane integrity and acrosome integrity of bovine sperm, compared with a control tris-egg yolk extender. However, limited information is available on the use of LDL and trehalose combination in bovine semen. Therefore, the aim of this study was to evaluate post-thaw quality of bovine cryopreserved semen added with centrifuged and non-centrifuged egg yolk, low-density lipoproteins (LDL), and trehalose.

# Materials and Methods

## *Animals*

Ten ejaculates were selected from five healthy and sexually mature Holstein *bulls (Bos primigenius taurus*) located on farms in the Province of Antioquia, Colombia. The bulls were kept under controlled management and feeding conditions. The collection procedure was supervised by a veterinarian to avoid stress and discomfort for the animals. No tranquilizers, analgesics or anesthetics were necessary because these animals were trained for the procedure.

## *Treatments*

Commercial Triladyl® (Minitube, Tiefenbach, Germany) was used as the basic semen extender. It was prepared according to the manufacturer's specifications and was stored at 4 to 5 °C. Five treatments were developed by adding to the extender: T1: pure egg yolk (PEY) at 20% (Waheed *et al.*, 2012); T2: centrifuged egg yolk (CEY) at 20% v/v, prepared according to Nouri *et al.* (2013); T3: low-density lipoproteins (LDL) at 8% v/v, prepared according to Moussa *et al.* (2002); T4: trehalose (Merck, Darmstadt, Germany) at 100 mM (Uysal and Bucak, 2009); and T5: trehalose at 100 mM plus LDL at 8% v/v (TLDL).

## *Semen collection and dilutions*

Semen samples were collected once per week for a period of ten weeks using an electroejaculator (Electrojac 6, Neogen Corp., Lansing, MI, USA). First, the bull was mechanically immobilized in a bucking chute. Then, the hair of the prepuce was cut and urination was induced by a stimulating massage. Subsequently, the preputial orifice was washed with saline solution and dried with a paper towel. Following this, rectal evacuation was performed before inserting the probe and switching on the electroejaculation device, whose voltage was increased automatically (Baiee *et al.*, 2018). Subsequently, the ejaculate was collected in a sterile plastic sleeve and its color, volume, pH, sperm concentration, and mass motility were determined using a graduated glass collection tube, pH-indicator strips (Merck, Darmstadt, Germany), a specific spectrophotometer (Spermacue®, Minitube, St Louis, MO, USA) and a phase contrast microscope (100× magnification) (Domínguez *et al.*, 2008), respectively.

Finally, semen samples were diluted to a final concentration of $30 \times 10^6$ spermatozoa $mL^{-1}$, for each treatment (supplemented extender), and placed in an Equitainer® (Hamilton BioVet, Ipswich, MA, USA) at 4°C to be transported for 1 h to the Biotechnology laboratory of Politécnico Colombiano Jaime Isaza Cadavid (Municipality of Bello, Province of Antioquia, Colombia). The minimum requirements of semen quality to process the ejaculates were 70% normal morphology, 70% total motilityand a sperm concentration of $500 \times 10^6$ semen $mL^{-1}$ (Khumran *et al.*, 2015).

## *Semen freezing and thawing*

The extended semen samples were equilibrated at 4 °C for 1 hour in a refrigerator and loaded into 0.25 mL mini straws (IMV Technologies, L'Aigle, France), which were sealed with polyvinyl alcohol (Merck, Darmstadt, Germany) and placed on racks 4 cm above the surface of the liquid nitrogen vapor for 15 min. Subsequently, the straws were stored in liquid nitrogen ($LN_2$). After a week of storage in the $LN_2$ tank (−196 °C), cryopreserved samples were thawed by plunging two straws from each treatment into a water bath at 37 °C for 1 minute.

## *Sperm quality evaluation*

Evaluation of semen quality for all treatments was carried out after dilution and transport of samples (before equilibration time) and immediately after the freezing–thawing process (Khumran *et al.*, 2015). Post-thawing, a total of 100 semen straws (20 straws per treatment) were evaluated for seminal quality parameters.

*Motility and kinetics.* Sperm motility and kinetics were evaluated using the Sperm Class Analyzer (SCA®) system (Microptic S.L, Barcelona, Spain). A drop of thawed semen (7 µL) was placed on a prewarmed (37 °C) glass slide, and covered with a cover slip. A minimum of 500 spermatozoa were immediately analyzed (100×; Eclipse E200, Nikon Inc., Tokio, Japan) for total motility (TM), progressive motility (PM), average path velocity (VAP), straight linevelocity (VSL) and curvilinear velocity (VCL).

*Structural membrane integrity (SMI).* SMI was evaluated using the Live/Dead Sperm Viability kit (Molecular Probes Inc., Eugene, OR, USA). A drop of 50 µL of thawed semen was mixed with 0.3 µL of SYBR14 at a final concentration of 6 µM (green fluorescence) and incubated for 8 min at 35 °C. Following this, propidium iodide (PI; 0.48 mM) was added to the mixture and incubated for 8 min at 35 °C. Samples of 7 µL of thawed semen were placed on a glass slide, and covered with a cover slip. At least 200 spermatozoa were counted using an Eclipse E200 microscope with HBO fluorescence (Nikon Inc, Tokio, Japan). Percentages of live spermatozoa were established(Gamboa *et al.*, 2010).

*Sperm vitality (SV) and abnormal morphology (AM).* SV and AM were assessed using eosin– nigrosin stain. A drop of 20 µL of thawed semen was mixed with 20 µL of eosin–nigrosin (Merck, Darmstadt, Germany) on a slide for 15 seconds. The mixture was smeared using a glass slide and was dried on a thermal plate at 37 °C. At least 200 spermatozoa were evaluated for each test using a phase contrast microscope (Eclipse E200, Nikon, Tokio, Japan) at 400× and at 1000× with immersion oil. Spermatozoa with unstained heads were considered to be alive and spermatozoa with pink heads were classified as dead (Khumran *et al.*, 2015). At the same time, spermatozoa with normal or abnormal structure were classified accordingly (Nagy *et al.*, 2013).

*Functional membrane integrity (FMI).* FMI was evaluated by the hypoosmotic test (HOST) (Rekha *et al.*, 2016). For this, 50 µL of thawed semen was mixed with 200 µL of a pre-warmed (37 °C) hypoosmotic solution of 5.4% sucrose (100 mOsmol/l) in Falcon tubes, and incubated at 37 °C for 40 minutes. After that, 7 µL of the incubated solution was placed on a prewarmed (37 °C) glass slide, and

covered with a cover slip. A minimum of 200 spermatozoa were immediately analyzed in different fields (400×; Eclipse E200, Nikon Inc., Tokio, Japan). Reacting spermatozoa (rolled tails) were considered as sperm with intact membranes (Baiee *et al.*, 2018). The percentage of reacting spermatozoa was calculated.

### *Statistical analysis*

A completely randomized design was used. The normal distribution of the variables was validated through the Kolmogórov–Smirnov test to determine whether the analysis should be parametric or nonparametric. A generalized linear model (GLM) was used to evaluate the sources of variation. Fixed effects of treatment and the ejaculate nested within bull were included. In order to compare the adjusted means between treatments, a Tukey test was performed. All analyses were developed using SAS version 9.2 software (SAS Institute Inc., Cary, NC, USA; 2008).

## Results

A total of ten ejaculates were processed under the different treatments. Within the statistical models used, the fixed effects of treatment and the ejaculate nested within bull were significant for all variables in the extended and thawed semen ($p<0.05$). Motility and kinetics results of extended semen are presented in Table 1.

**Table 1.** Motility and kinetics of extended semen.

| Treatment | TM (%) | PM (%) | VCL (µm/s) | VSL (µm/s) | VAP (µm/s) |
|---|---|---|---|---|---|
| 1 (PEY) | 76.7±6.2$^{ab}$ | 41.7±7.4$^{b}$ | 50.3±4.3$^{c}$ | 19.2±1.4$^{b}$ | 30.5±2.1$^{c}$ |
| 2 (CEY) | 88.5±3.7$^{a}$ | 66.3±6.4$^{a}$ | 81.8±7.2$^{ab}$ | 38.8±5.2$^{a}$ | 54.9±6.5$^{ab}$ |
| 3 (LDL) | 84.8±4.4$^{a}$ | 69.0±5.8$^{a}$ | 88.2±6.9$^{a}$ | 38.4±5.1$^{a}$ | 57.0±5.7$^{a}$ |
| 4 (T) | 67.5±5.6$^{b}$ | 42.9±7.1$^{b}$ | 63.8±8.0$^{bc}$ | 24.8±5.3$^{ab}$ | 36.9±6.9$^{bc}$ |
| 5 (TLDL) | 89.0±3.1$^{a}$ | 70.5±5.3$^{a}$ | 90.9±8.8$^{a}$ | 34.0±3.6$^{ab}$ | 54.4±6.5$^{ab}$ |

TM: Total Motility. PM: Progressive Motility. VCL: Curvilinear Velocity. VSL: Straight-Line Velocity. VAP: Average Path Velocity. PEY: Pure Egg Yolk. CEY: Centrifuged Egg Yolk. LDL: Low-Density Lipoproteins. T: Trehalose. TLDL: Trehalose and LDL. The results are presented as mean ± standard error of mean (S.E.M). Different superscript letters ($^{a, b}$) within columns indicate significant differences ($p<0.05$).

Figure 1 presents the results for morphology, vitality, and functional and structural integrity of the plasma membrane of the extended semen for each treatment before freezing.

Motility and kinetics results of thawed semen are presented in Table 2.

Figure 2 presents morphology, vitality, and functional and structural integrity results of plasma membrane for thawed semen.

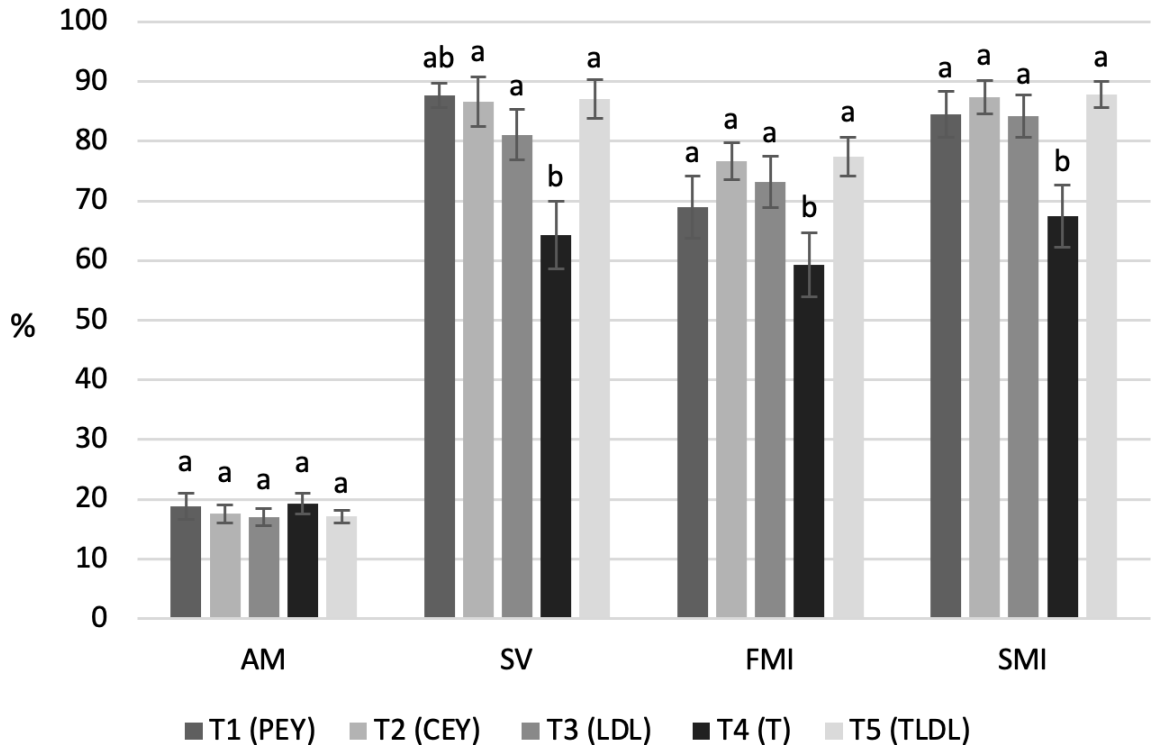


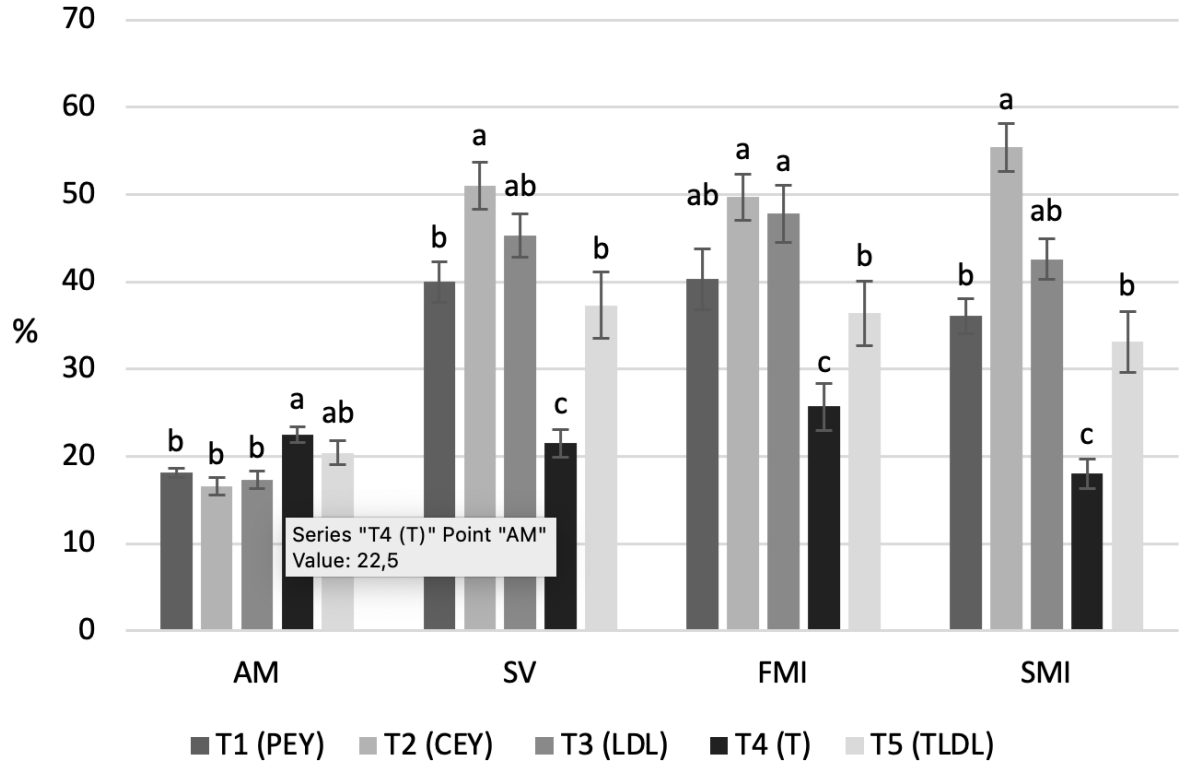

**Figure 1.** Morphology, vitality and membrane integrity of extended semen. AM: Abnormal Morphology. SV: Sperm Vitality. FMI: Functional Membrane Integrity. SMI: Structural Membrane Integrity. PEY: Pure Egg Yolk. CEY: Centrifuged Egg Yolk. LDL: Low-Density Lipoproteins. T: Trehalose. TLDL: Trehalose and LDL. The results are presented as mean ± standard errorof mean (S.E.M). Different letters (a, b; bars per treatment) indicate significant differences ($p<0.05$).

**Table 2.** Motility and kinetic parameters of thawed semen.

| Treatment | TM (%) | PM (%) | VCL (µm/s) | VSL (µm/s) | VAP (µm/s) |
|---|---|---|---|---|---|
| T1 (PEY) | $36.7\pm2.4^{b}$ | $9.9\pm1.1^{cd}$ | $32.4\pm1.2^{b}$ | $12.0\pm0.7^{c}$ | $19.8\pm1.0^{c}$ |
| T2 (CEY) | $55.4\pm3.6^{a}$ | $26.8\pm2.7^{a}$ | $49.9\pm2.4^{a}$ | $25.9\pm2.0^{a}$ | $35.2\pm2.3^{a}$ |
| T3 (LDL) | $44.9\pm2.4^{ab}$ | $17.3\pm1.5^{b}$ | $45.2\pm3.1^{a}$ | $20.0\pm2.9^{ab}$ | $29.4\pm3.5^{ab}$ |
| T4 (T) | $19.8\pm1.6^{c}$ | $2.7\pm0.3^{d}$ | $28.8\pm4.6^{b}$ | $5.1\pm0.6^{d}$ | $11.2\pm1.6^{d}$ |
| T5 (TLDL) | $36.6\pm3.9^{b}$ | $13.6\pm2.6^{bc}$ | $40.6\pm3.5^{ab}$ | $14.4\pm2.3^{bc}$ | $22.2\pm2.8^{bc}$ |

TM: Total Motility. PM: Progressive Motility. VCL: Curvilinear Velocity. VSL: Straight-Line Velocity. VAP: Average Path Velocity. PEY: Pure Egg Yolk. CEY: Centrifuged Egg Yolk. LDL: Low-Density Lipoproteins. T: Trehalose. TLDL: Trehaloseand LDL. The results are presented as mean ± standard error of mean (S.E.M). Different superscript letters ($^{a, b}$) within columns indicate significant differences between columns within a row ($p<0.05$).

**Figure 2.** Morphology, vitality and membrane integrity of thawed semen. AM: Abnormal Morphology.




SV: Sperm Vitality. FMI: Functional Membrane Integrity. SMI: Structural Membrane Integrity. PEY: Pure Egg Yolk. CEY: Centrifuged Egg Yolk. LDL: Low-Density Lipoproteins. T: Trehalose. TLDL: Trehalose and LDL. The results are presented as mean ± standard errorof mean (S.E.M). Different letters (a, b; bars per treatment) indicate significant differences ($p<0.05$).

# Discussion

In recent years, a demand for alternatives to conventional commercial semen extenders has arisen, since the risk of introducing exotic diseases through transport of products based on egg yolk has been recognized (Layek *et al.*, 2016). Egg yolk can also inhibit spermatozoa metabolism and reduce their functionality (Moreno *et al.*, 2013). Replacement of egg yolk with low-density lipoproteins (LDL) can improve post-thaw semen quality and conception rate (Anand *et al.*, 2017). In the present study, different egg yolk separation methods, such as centrifugation and purification, were used to conserve mainly the fraction rich in LDL and eliminate those components that could generate spermatozoa alterations. Additionally, trehalose was included as an alternative to improve semen cryopreservation due to reduction of the oxidative stress induced by freeze–thawing (Hu *et al.*, 2010).

Our results showed an effect of the additives on sperm motility and kinetics after semen dilution (Table 1). In general, CEY, LDL and TLDL favored sperm motility and speed, compared to PEY and trehalose. The addition of PEY -the most commonly used additive- reduced PM, VCL and VAP. Other studies have described that it is difficult to predict the effect of egg yolk, because there is great variability between batches due to its complex composition(Freschi *et al.*, 2011; Pillet *et al.*, 2011).

Integrity and functionality of the plasma membrane were negatively affected in the medium

supplemented only with trehalose (Figure 1), which confirms the importance of LDL to protect the plasma membrane even in the dilution stage and during refrigerated transport prior to freezing. It is likely that, according to the action mechanisms described for LDL, stabilization of the plasma membrane is the most relevant effect in this stage (Akhter *et al.*, 2011). No difference was observed between treatments with regard to sperm morphology in the extended semen, indicating that there were no alterations in morphology that could be mitigated differentiallyby any of the additives used.

For thawed semen, our results show that CEY protects motility and kinetics more efficiently than other treatments (Table 2), in addition to protecting the plasma membrane integrity (Figure 2). Although LDL had equivalent results to CEY for most parameters, this last treatment was superior with regard to progressive motility. A possible explanation is that CEY could have conserved important egg yolk components after centrifugation, which could have been eliminated during LDL purification. Egg yolk has antioxidants, such as vitamins E and B12, biotin and phosvitin (Seuss-Baum, 2007). These antioxidants have been associated with beneficial effects on cryopreserved semen of rams (Hamedani *et al.*, 2013), buffalo (Beheshti*et al.*, 2011), humans (Kalthur *et al.*, 2011), andbulls (Güngör *et al.*, 2019).

Among the CEY components, LDL lipids could prevent direct contact of sperm with ice crystals by forming a protective layer (Moussa *et al.*, 2002), while antioxidants could reduce lipid peroxidation of the plasma membrane (Surai *et al.*, 1998). However, according to Anzar *et al.* (2019), the exact sperm protection mechanism of egg yolk during the initial cooling phase is not fully understood; however, it is known that LDL sequesters the binder of sperm proteins (BSPs) in seminal plasma, which causes phospholipids and cholesterol efflux from the plasma membrane.

Current evidence suggests that LDL supplementation is one of the best options for sperm protection during cryopreservation (Peruma, 2018), and our results support this hypothesis (Table 2). It is possible that LDL was superior to PEY with regard to motility and kinetics because egg yolk contains high-density lipoproteins (HDL) that decrease sperm metabolism (Moreno *et al.*, 2013). Additionally, it is known that egg yolk generates microbiological risk and alterations of motility, acrosome integrity and oxidative phosphorylation of sperm, and complicates the biochemical, metabolic and microscopic evaluation of semen (Wall and Foote, 1999; Moussa *et al.*, 2002; Sariozkan *et al.*, 2010). Amirat *et al.* (2010) found that LDL (8%, v/v) has simmilar or superior cryoprotective effect on bull semen in comparison to egg yolk (20%, v/v). Given that our LDL results were lower for progressive motility compared with CEY supplementation (Table 2), it may be necessary to reconsider the optimal LDL percentage. Won-Mo Cho *et al.* (2012) found higher sperm qualityusing LDL at 4% v/v.

The cryoprotective effect of LDL was also observed in combination with trehalose (TLDL), which did not differ from PEY for any parameter (Table 2 and Figure 2), and showed lower results than LDL for FMI (Figure 2). According to these, trehalose addition would not be essential to improve seminal quality when supplementing LDL. However, it has been reported that it is possible to improve post-thaw quality of ovine and bovine sperm by using LDL and trehalose during freezing (Tonieto *et al.*, 2010; Shin-Ae *et al.*, 2012).

Trehalose has been proven to protect proteins and membranes from dehydration (Lins *et al.*, 2004). Although its mechanism of action has not yet been determined, it is thought that it covers layers of water; thus, preventing sperm exposure to ice crystals. This cryoprotective effect was not evident in our results; conversely, the trehalose treatment was the only one showing increased abnormal sperm (Table 2 and Figure 2) although it was used in optimal concentrations (Uysal and Bucak, 2009). The beneficial effects previously observed with the use of trehalose could be due to the simultaneous supplementation with egg yolk in the freezing medium (Hu *et al.*, 2010; Büyükleblebici *et al.*, 2014).

In conclusion, egg yolk could be replaced by centrifuged egg yolk or low-density lipoproteins in the freezing extender for bovine semen. However, since centrifuged egg yolk improves progressive motility of sperm and it is also easier to obtain, it could be considered as a better alternative to replace egg yolk in the freezing extender.

# Declarations


### *Funding*

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### *Conflicts of interest*

The authors declare they have no conflicts ofinterest with regard to the work presented in thisreport.

### *Author contributions*

Elizabeth Varela (EV), Alexandra Usuga (AU), Juan Duque (JD), Jorge Gómez (JG) and Giovanni Restrepo (GR) designed the study; JG administered the project; EV, AU and JD collected the data; GR did the statistical analysis; EV, AU and GR analyzed the data and carried out critical reading and paper edition.

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