ROGÉRIO AMORIM DOS REIS

COCONUT WATER-BASED EXTENDER FOR SEMINAL PRESERVATION IN SMALL RUMINANTS: A META-ANALYSIS STUDY

Dissertation submitted to the Animal Science Graduate Program of Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Advisor: Ciro Alexandre Alves Torres

Co-advisor: Bruna Waddington de Freitas

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ABSTRACT

REIS, Rogério Amorim dos, M.Sc., Universidade Federal de Viçosa, July 2021. **Coconut water-based extender for seminal preservation in small ruminants: a meta-analysis study.** Advisor: Ciro Alexandre Alves Torres. Co-advisor: Bruna Waddington de Freitas.

Semen extenders based on egg yolk and milk have been widely used in seminal preservation. However, they present health risks due to the transmission of pathogens. Therefore, new studies have emerged seeking for a diluent of vegetable origin, capable of guaranteeing sperm quality and eliminating the risk of contamination presented by that of animal origin. In light of the above, a meta-analytical review was conducted to determine the effect of coconut water as a seminal extender for small ruminants' sperm preservation. Studies that met the inclusion criteria were retrieved from PubMed, Science Direct, Scopus, and Web of Science. According to the selection criteria of this study, 88 independent comparisons were obtained from the nine studies included in the meta-analysis. The weighted mean difference (WMD) between treatments with coconut water (diluents including coconut water) and control treatments (diluents without the addition of coconut water) was evaluated using the random-effects model of meta-analysis. Heterogeneity was explored by meta-regression and subgroup analysis, performed using as covariate: type of coconut water, types of control treatment to which coconut water was contrasted, type of preservation, preservation time, and animal species. The overall results showed that coconut water elicited a positive effect on the total motility of sperm cells from cooled semen ($P < 0.05$), while for the fresh and cryopreserved semen no significant effect was observed. In addition, coconut water showed a positive effect on membrane integrity of sperm cells from fresh semen ($P < 0.05$). On the other hand, for cooled semen, a negative result was found ($P < 0.05$). Therefore, the use of coconut water as a seminal extender proved to be a viable alternative for seminal preservation in small ruminants.

Keywords: Coconut water. Extender. Sperm. Sperm quality.

RESUMO

REIS, Rogério Amorim dos, M.Sc., Universidade Federal de Viçosa, julho de 2021. **Água de coco como meio para preservação seminal em pequenos ruminantes: um estudo de meta-análise.** Orientador: Ciro Alexandre Alves Torres. Coorientadora: Bruna Waddington de Freitas.

Os diluentes à base de gema de ovo e leite têm sido amplamente utilizados na preservação seminal, entretanto, estes apresentam riscos sanitários pela transmissão de patógenos. Portanto, novos estudos têm surgido na busca por um diluente de origem vegetal, capaz de assegurar a qualidade espermática e ao mesmo tempo eliminar o risco de contaminação apresentado por aqueles de origem animal. Em função do exposto, uma revisão meta-analítica foi conduzida para determinar o efeito da água de coco como diluente seminal para a preservação de espermatozoides de pequenos ruminantes. Quatro bases de dados foram utilizadas para realizar as buscas bibliográficas, sendo estas PubMed, Science Direct, Scopus e Web of Science. Foram obtidas 88 comparações independentes dos 09 estudos selecionados, conforme os critérios de seleção deste estudo. A diferença média ponderada (DMP) entre os tratamentos com água de coco (diluentes com inclusão de água de coco) e tratamentos controle (diluentes sem adição de água de coco) foi avaliada por meio da utilização de um modelo de efeitos aleatórios. Para avaliar a heterogeneidade, foram realizadas análises de meta-regressão e subgrupo, utilizando como covariável: tipo de água de coco, tipo de tratamento controle ao qual a água de coco foi contrastada, tipo de preservação, tempo de preservação e espécie animal. A água de coco resultou em um incremento sobre a variável motilidade total para sêmen resfriado ($P < 0,05$), ao passo que, para o sêmen fresco e criopreservado, não foi observado qualquer efeito significativo. Para a variável integridade de membrana, observou-se um efeito positivo para sêmen fresco ($P < 0,05$). Para o sêmen resfriado, foi encontrado um resultado negativo ($P < 0,05$). O emprego da água de coco como diluente seminal apresentou-se como alternativa viável para a preservação seminal em pequenos ruminantes.

Palavras-chave: Água de coco. Diluente. Espermatozoide. Qualidade espermática.

SUMÁRIO

1 INTRODUCTION

The exposure of sperm cells to the low temperatures that are necessary for semen preservation process may hinder its viability and use, and also affects its integrity and morphology (Salamon and Maxwell, 2000; Castelo et al., 2008; Gangwar et al., 2008, 2015).

In seminal plasma, sperms have a limited survival time, thus the necessity of using diluents and cryoprotectants that can maintain sperm quality during the execution of preservation techniques and storage at low temperatures, providing an adequate environment for survival of these gametes and, consequently prolonging sperm viability (Daramola et al., 2016).

In seminal preservation, diluents being used can be of chemical, vegetable or animal origin, the latter two being the most frequently used (Marco-Jiménez et al., 2004). However, despite the positive results obtained from the use of egg yolk and milk as seminal extenders, they may present problems regarding risk of transmission of pathogens, leading to possible restrictions regarding the export of semen (Bousseau et al., 1998; Leite et al., 2011; Moreira, 2017).

Given the above, the search for a diluent that reduces the risk of contamination by animal pathogens has increased. With favorable results for its use, coconut water has been used as a seminal extender and has gained space in the midst of biotechnologies (Toniolli et al., 2010; Del Valle et al., 2013; Cardoso et al., 2005). In addition to having water, sugars, proteins, salts, and vitamins in its composition, coconut water is easy to prepare and has a low cost (Carvalho et al., 2006; Rondon et al., 2008). Moreover, improvements in sperm cells integrity and viability in small ruminants have already been reported with the use coconut water (Cavalcante et al., 2014; Daramola et al., 2016; Brito et al., 2019).

Therefore, it was hypothesized that coconut water used as seminal diluent contributes to the preservation of sperm parameters, ensuring seminal viability in small ruminants, making the ejaculate viable for use after refrigeration or cryopreservation. Hence, the aim of this study was to evaluate through a meta-analytical approach the effect of coconut water when being used as a seminal diluent on parameters correlated with sperm viability during seminal preservation in small ruminants.

2 THEORETICAL FRAMEWORK

2.1 Seminal preservation

The ejaculates of most domestic animals contain sperm cells in quantities greater than those necessary for fertilization to occur. Thus, dilution and preservation of the semen are ways to optimize its use, allowing for several inseminations with the same ejaculate (Bailey et al., 2000; Castelo et al., 2008).

Over the past few years, seminal preservation techniques have progressively evolved, with extenders and procedures for the preservation of seminal viability being described in several species (Küçük et al., 2014). On the other hand, a variation in the level of sensitivity in response to seminal preservation processes has been found due to the differences presented by sperm, which may occur between species and between males of the same species (Bailey et al., 2000; Curry, 2007).

Exposure of sperm to adverse conditions during seminal cooling or cryopreservation in the absence of diluents can impair the viability of such cells, inducing damage due to rapid temperature reduction as well as oxidation of essential cell constituents (Purdy, 2006; Curry, 2007; Sieme et al., 2016). In addition to protecting sperm and providing an increase in the volume of the ejaculate, the diluent must have characteristics such as buffering effect, as these gametes are quite susceptible to pH changes; ability to maintain osmotic pressure; act as an energy substrate, providing energy for the sperm; and antimicrobial activity, which is achieved with the addition of antibiotics, aiming to reduce the load of non-pathogenic bacteria and the transmission of pathogenic bacteria that can compromise seminal quality (Bailey et al., 2000; Castelo et al., al., 2008; Nunes and Salgueiro, 2011).

It is known that the sperm membrane has a significant amount of polyunsaturated fatty acids (PUFA), which are responsible for contributing to its fluidity and flexibility (Lenzi et al., 2002). However, this also makes it highly susceptible to lipid peroxidation, also called lipoperoxidation (LPO) (Asadpour et al., 2011). LPO is an autocatalytic and self-propagating reaction where lipid constituents of the plasma membrane are oxidized, compromising its integrity and functionality (Sanocka and Kurpisz, 2004; Agarwal et al., 2014). Furthermore, during the cryopreservation process, part of the sperm also suffers mechanical damage (Bucak et al., 2007; Atessahin et al., 2008).

The execution of sperm preservation processes should strive for the least possible damage to the manipulated gametes since injuries such as LPO, formation of free radicals – also known as reactive oxygen species (ROS) –, changes in DNA, low motility, and vigor

negatively affect the quality of these cells, which can lead to low fertilization rates (Salamon and Maxwell, 2000; Watson, 2000; O'Hara et al., 2010).

2.1.1 Cooling

The cooling process represents a way of storing semen in a liquid state with the establishment of reversible inhibition of cellular metabolism as a result of the reduced temperatures to which the ejaculate is submitted (Câmara and Guerra, 2011).

Cooled semen, as well as fresh semen, when compared to the cryopreserved one, has higher fertility. However, there is a directly proportional relationship between storage time and sperm quality, which is independent of the diluent, dilution rate, temperature or conditions of storage, which negatively influence the sperm as the incubation time increases. Furthermore, in species that have reproductive seasonality, its use is restricted due to the period of sexual activity of males (Salamon and Maxwell, 2000; Bezerra, 2010).

The metabolism of sperm cells at body temperature is high, however, at temperatures close to 5ºC, there is a reduction in the sperm catabolism necessary for the preservation of the ejaculate for long periods, since only about 10% of it is necessary for its survival (Bezerra, 2010). On the other hand, irreversible damage to gametes can occur due to the thermal shock caused by seminal cooling. In addition, it has been suggested that heat shock is probably related to the transition phase of lipids present in the cell membrane, which may, in addition to altering its function, interfere with protein channels, inducing an ionic imbalance that results in the appearance of membrane damage, altering its permeability and consequently causing reduction in sperm motility (Watson, 1996; Salamon and Maxwell, 2000; Câmara and Guerra, 2011; Madeddu et al., 2016).

During the transition phase, plasma membrane changes from fluid state to gel state due to a reorganization of the fatty acids present in it, resulting in a structure with greater permeability and more susceptible to rupture (Andrabi, 2007). During this period, cell dehydration also occurs (Sieme et al., 2016), which is an important process for reducing ice formation in the intracellular environment. The osmotic imbalance between the internal and external environments of these cells stimulates the efflux of water so that balance is restored; however, this process is influenced by the cooling curve (Nunes and Salgueiro, 2011; Sieme et al., 2016). When a very fast cooling curve is used, a greater concentration of water will be retained in the intracellular environment, enabling a larger formation of ice crystals and

membrane rupture, leading to the death of these cells (Andrabi, 2007). On the other hand, if the cooling curve is too slow, high cellular dehydration, reduced organelles, and exposure of cells to high concentrations of solute occur, affecting the composition of the plasma membrane and inducing irreversible damage to it (Gao and Critser, 2000; Yeste, 2016).

2.1.2 Seminal cryopreservation

Cryopreservation, a technique whereby cells, embryos, or tissues are conserved at temperatures below 0°C, preserving their composition, function, and viability indefinitely, has significant importance for reproductive biotechnologies such as conventional artificial insemination (AI) and in fixed time (FTAI); as well as for the production of embryos *in vivo* and *in vitro*, providing the diffusion of genetic material on a larger scale and its conservation for long periods (Pegg, 2002; Silva and Guerra, 2011; Li et al., 2018). In contrast, the sperm response to cryopreservation varies between individuals of the same species as well as of different species (Küçük et al., 2014).

When cryopreserved, sperm are subjected to stressors such as cold shock reactions and oxidative stress. As a result, a negative impact on cell function, membrane integrity, and motility occur as a result of the triggering of chemical reactions that cause changes in the lipid composition of the sperm membrane (Saraswat et al., 2012; Gangwar et al., 2015; Ondřej et al., 2015, 2019). Damage to sperm during semen manipulation occurs mostly as a result of oxidative stress caused by the production of ROS (Agarwal et al., 2014; Tariq et al., 2015; Kumar et al., 2019). Oxidative stress also induces the formation of ROS by dead sperm present in the ejaculate and by atmospheric oxygen (Bucak et al., 2009).

Sperm, under aerobic conditions, produce ROS as a result of their metabolism and the presence of oxygen, just like all living cells. In large amounts, ROS becomes harmful to these gametes, which are extremely susceptible to oxidation by such molecules due to their membrane composition (De Lamirande et al., 1997; Aitken et al., 2006). On the other hand, at physiological levels, they participate in important processes such as sperm capacitation and acrosome reaction, necessary events for egg fertilization (De Lamirande et al., 1997; Aitken and Baker, 2004; Du Plessis et al., 2015).

Enzymatic and non-enzymatic mechanisms against the production of ROS are present in sperm and seminal plasma. However, this system becomes deficient due to the "disposal" of a large part of the cytoplasm, where these enzymes are present during spermatogenesis (Aitken

et al., 2006; Partyka et al., 2012). This process, associated with the dilution the ejaculate in seminal diluents, causes dissolution of these antioxidant agents and the exposure of sperm to free radicals, causing an imbalance between the antioxidant system and the concentration of ROS (Agarwal et al., 2014; Fanaei et al., 2014; Tariq et al., 2015).

2.2 Cryoprotectants and seminal extenders

The absence of extenders for cooling or seminal cryopreservation exposes sperm to adverse conditions that can impair the viability of these cells, inducing damage due to the rapid temperature reduction as well as the oxidation of essential cell constituents (Purdy, 2006; Curry, 2007; Sieme et al., 2016).

The use of a diluent capable of ensuring that the plasma membrane is not compromised and the maintenance of osmolarity, isotonicity, and pH of the medium is extremely important to obtain good quality semen. These can be made from different compounds such as egg yolk, lactose, sodium citrate, skimmed milk, among others (Rondon et al., 2008; Oliveira, 2016). In addition to acting to stabilize the plasma membrane and as an energy substrate, extenders also participate in maintaining an adequate environment for sperm survival, preserving the viability of the cells (Purdy, 2006; Vidal et al., 2013).

In addition to diluents, it is necessary to add some cryoprotective agents so that damage to the sperm is reduced. These agents must have physicochemical characteristics such as low molecular weight, high solubility in aqueous media, and low toxicity to cells, in addition to maintaining a conducive environment to sperm survival as well as protecting them against injuries arising from this process (Gonzalez, 2004; Purdy, 2006).

Cryoprotectants can be divided into penetrating, which act inside the cell to minimize injuries caused during manipulation, whether they are of chemical or mechanical origin, and non-penetrating, acting outside the cell, increasing the osmolarity of the medium and causing an efflux of the water present in sperm, preventing the formation of ice crystals inside the cell during cryopreservation (Castelo et al., 2008; Kulaksiz et al., 2013). Glycerol, ethylene glycol and dimethyl sulfoxide are examples of penetrating cryoprotectants, formed by molecules that can cross the plasma membrane (Purdy, 2006). As non-penetrating cryoprotectants, the ones based on egg yolk and milk are the most frequently used. They have macromolecules, proteins and lipids in their composition (Salamon and Maxwell, 2000; Oliveira, 2016; Tarig et al., 2017).

2.2.1 Egg yolk

Since the discovery of the protective effect exerted by egg yolk on sperm preservation, this cryoprotectant has been widely used with considerable efficiency in seminal dilution, protecting sperm from heat stress and preserving their functions after storage (Phillips, 1939; Barak et al., 1992; Bergeron et al., 2004; Dong et al., 2011).

The main constituent pointed out as responsible for attributing the protective effect to the egg yolk diluent is the fraction of low-density lipoproteins (LDL – Low-Density Lipoproteins) (Marco-Jiménez et al., 2004). Some mechanisms have been suggested to explain the positive effect it plays, including the interaction of LDL with the plasma membrane by stabilizing it, and the formation of a layer on the surface of the sperm and the replacement of phospholipids lost during preservation (Graham and Foote, 1987; Moussa et al., 2002; Del Valle et al., 2013). Subsequently, a new mechanism of action for LDL was suggested through binding seminal plasma proteins, preventing their adhesion and action on sperm (Manjunath, 2012).

Seminal plasma proteins are represented by two main families: spermadesins and those that have the fibronectin type II domain (FBN II), which are the main proteins of the seminal plasma of bulls, commonly called BSPs (Bovine Seminal Plasma proteins) (Watson, 1981; Moussa et al., 2002; Bergeron et al., 2004; Dong et al., 2011). BSPs are capable of binding with the fraction of low- and high-density lipoproteins (LDL – Low-Density Lipoproteins and HDL – High-Density Lipoproteins, respectively) with choline groups of sperm membrane phospholipids and with heparin (Desnoyers and Manjunath, 1992; Manjunath et al., 1989; Manjunath and Thérien, 2002). After the discovery of BSPs, homologous proteins were identified in goats (GSP – Goat Seminal Plasma proteins) and sheeps (RSP – Ram Seminal Plasma proteins), which also interact with membrane lipid fractions. In mammals, it is suggested that the protective mechanism that occurs due to the presence of LDL is the same, since proteins that interact with lipid fractions – or that are homologous to them – seem ubiquitous (Villemure et al., 2003; Bergeron et al., 2005; Bergeron and Majunath, 2006).

During ejaculation, when in contact with sperm, seminal plasma proteins bind to membrane phospholipids, inducing an efflux of cholesterol and phospholipids from the plasma membrane, initiating a cascade of events that will lead to sperm capacitation (Thérien et al., 1998, 1999; Manjunath and Thérien, 2002; Bergeron and Majunath, 2006). The stimulation of the efflux of lipid content present in the plasma membrane by BSPs is time-concentration dependent (Bergeron et al., 2004; Manjunath, 2012).

In vivo, this destabilization is necessary for sperm capacitation to occur. However, for seminal preservation, this effect is harmful, since a greater susceptibility to heat stress is correlated with a lower presence of cholesterol in these cells (Darin-Bennett and White, 1977). On the other hand, the use of media with high concentrations of LDL, such as those based on egg yolk, has been shown to have a beneficial effect on the destabilization of the membrane, since BSPs interact with the lipoproteins from the diluent to the detriment of those present in the sperm membrane (Thérien et al., 1998, 1999; Manjunath and Thérien, 2002; Bergeron and Majunath, 2006; Prapaiwan et al., 2016).

2.2.2 Milk

The use of milk as a seminal extender, whether it is whole, skimmed, or reconstituted, has shown positive results, which have been attributed to the protein fraction of milk, with cow's milk being preferable to that of other species (Salamon and Maxwell, 2000).

The use of skimmed milk as a seminal diluent demonstrated a protective effect on sperm cells during the preservation of these gametes. However, it contains a very small fraction of lipids in its composition (≤ 0.1%), mainly represented by triglycerides, which indicates that the protective effect in this diluent is not performed by the lipid fraction (Foote et al., 2002; Leboeuf et al., 2003; Bergeron and Majunath, 2006; Bergeron et al., 2007).

The main protein present in milk is casein, which has been previously suggested as the agent responsible for the protective effect of seminal extenders based on this product (Bergeron and Majunath, 2006). Later, it was found that the protective mechanism exerted by milk-based extenders occurred by preventing the binding of BSPs to the sperm, similar to the mechanism exerted by egg yolk, preventing the efflux of lipid constituents stimulated by its interaction with seminal proteins (Bergeron et al., 2007). Unlike the mechanism of uptake of BSPs by LDL present in egg yolk, the protection exerted in milk occurs due to an interaction between the casein micelles or serum proteins and the seminal plasma proteins (Lusignan et al., 2011), enabling sperm preservation during storage at low temperatures (Bergeron et al., 2007; Manjunath, 2012).

2.2.3 Nucifera L. coconut

Coconut water contains water, sugars – such as sucrose, glucose and fructose –, proteins and mineral salts in its composition. Furthermore, the presence of ascorbic acid (vitamin C) and enzymes such as superoxide dismutase (SOD) give it an antioxidant capacity (Carvalho et al., 2006; Salim et al., 2018). These characteristics, associated with the search for a diluent without additives of animal origin, made coconut water a target for research, gaining space in areas such as biotechnology, where it is being used for seminal dilution and conservation (Carvalho et al., 2006; Rondon et al., 2008).

In addition to the low cost, the ease of preparation is another favorable point for using coconut water-based extenders. On the other hand, the difficulty of storage for long periods of time, the availability of coconut in regions devoid of the fruit, and the variability of the biochemical constitution of the fruits were obstacles to its use. In this sense, powdered coconut water (ACP; ACP Biotechnology, Fortaleza-CE, Brazil) was elaborated, aiming to overcome the difficulties of using coconut water. It was obtained from the dehydration of coconut water, being characterized by standardization and stabilization, and maintaining its biochemical characteristics, which provides greater longevity without compromising its quality. Furthermore, after dissolution, it has biochemical characteristics very similar to those found in fresh coconut water, and it can easily be stored and sent to regions where fresh coconuts are not available (Cardoso et al., 2005; Nunes and Salgueiro, 2011).

The use of coconut water-based extenders is a viable alternative in the seminal cryopreservation of several animals, such as goats (Daramola et al., 2016), dogs (Cardoso et al., 2003; Cardoso et al., 2005), angola (*Numida meleagris*) (Rondon et al., 2008), capuchin monkeys (De Araújo et al., 2009), fishes (Viveiros et al., 2010) and pigs (Toniolli et al., 2010). Furthermore, more recent works have shown favorable results for the use of coconut water in characteristics such as viability, acrosome and sperm DNA integrity (Cavalcante et al., 2014), motility, mitochondrial activity, and sperm membrane integrity (Daramola et al., 2014; Brito et al., 2019), indicating that coconut water has the potential to be used in seminal cryopreservation in small ruminants.

3 MATERIALS AND METHODS

3.1 Search strategy

This study was conducted in accordance with the Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Liberati et al., 2009). To perform the meta-analysis, an extensive bibliography search was conducted using the electronic databases Medline/PubMed, Science Direct, Scopus and Web of Science, completed in October 2020. For all databases, the terms ‘coconut water’ and ‘semen’ were adopted for the search strategy (supplementary table 1).

3.2 Inclusion and exclusion criteria

The following inclusion criteria were used to identify appropriate studies: 1) the authors performed seminal preservation using goats and/or sheep as animal models; and 2) included coconut water in the medium for seminal preservation. The exclusion criteria were: 1) the authors did not use coconut water; 2) used other species than goats and/or sheep; 3) did not work with semen; and 4) the study was a review article, letter to the editor, case study, comment, or editorial.

The articles selected by title and abstract were submitted to a new analysis, performed through a complete reading, to confirm their relevance regarding the selection and inclusion criteria of this study. To be included in the meta-analysis, studies should have a control group free of any inclusion of coconut water.

3.3 Data extraction

Firstly, Mendeley software (Mendeley Desktop; version 1.19.4) was used to remove duplicates. Then, two independent authors screened the retrieved studies according to the inclusion and exclusion criteria. Afterwards, the titles and abstracts of each study were investigated, and then the full-texts of studies deemed relevant were retrieved to assess suitability for the meta-analysis, with discrepancies resolved through a third reviewer. A Microsoft Excel form was then created based on the following information: first author, publication reference, control treatment with which coconut water was contrasted, type of coconut water, animal species, type of preservation and evaluation time. The mean values, variances (i.e., standard deviation - SD and standard error - SE) and number of replicates for the following response variables: percentage of living cells, total and progressive motility, and

membrane integrity were extracted for control group (treatments without the addition of coconut water) and coconut water group (treatments including any level of coconut water).

3.4 Data synthesis and analysis

3.4.1 Weighted mean difference and publication bias

A meta-analysis was conducted using the R Statistical Software (Metafor package, version 3.4) (Viechtbauer, 2010). The effects of coconut water as a seminal extender for small ruminants were evaluated using the random-effects model to examine the weighted mean difference (WMD) between coconut water treatments (diluents that included coconut water) and control treatments (diluents without any addition of coconut water). Treatment means were weighted by the inverse of the variance for the random-effects model, as proposed by Der-Simonian and Laird (1986).

Between-studies variability (i.e. heterogeneity of the treatment effect) was evaluated using both the chi-square ($\boldsymbol{Q}$) test of heterogeneity and $\boldsymbol{I^2}$ statistics, which measures the percentage of variation due to heterogeneity. Negative I^2 values were assigned as zero values. An I^2 value of less than 25% indicates low heterogeneity, whereas values between 25 and 50% denote moderate heterogeneity, and those above 50% denote high heterogeneity (Higgins et al., 2003).

Publication bias was evaluated using the funnel plot (Light and Pillemer, 1984) and asymmetry test (indicative of publication bias) which was carried according to the Egger regression asymmetry test, among the WMD and SE (Egger et al., 1997). Statistical significance was declared when $P \leq 0.05$. Outliers were removed when the studentized residuals were greater than -2.5 or less than 2.5.

3.4.2 Meta-regression and subgroup analysis

A meta-regression analysis was conducted to identify the effects of categorical covariates and to select them to then perform a subgroup analysis. A mixed-model was applied to adjust the data in the meta-regression analysis using WMD as a dependent variable.

The mixed-effects models were given by:

$\Theta i = \beta + \beta i\ xij + \ldots \beta ip\ xip + \mu i$

Where Θi = the true treatment effect on the ith explanatory variable; β = the overall true effect of the treatment; xij = the value of the jth covariate (j = 1, 2, ..., p) for the ith explanatory variable; βi = change in size from the true effect to the unity increase in the j-th covariate; and $\mu i \sim N (0\ \tau^2)$. Here τ^2 indicates the amount of heterogeneity not explained by the covariates (Viechtbauer, 2010).

The between-study variance (Tau-squared = τ^2), or measure of the between-study variance, is the one used in Der-Simonian and Laird's (1986) random-effects meta-analyses. However, it is less adequate when covariates are included (Thompson and Sharp, 1999). Restricted Maximum Likelihood estimation (RML) approach was used to estimate τ^2 because it is less likely to underestimate or produce biased estimates of variance (Thompson and Sharp, 1999; Viechtbauer, 2005).

Null hypothesis tests for covariate coefficients were obtained using Wald's multiparameter test (Harbord and Higgins, 2008). The adjusted R^2 was used for the model, which represents the proportion of between-study variance (heterogeneity) explained by the covariates (Harbord and Higgins, 2008; Viechtbauer, 2010). This was calculated by comparing the estimated between-study variance when covariates were included in the model (σ^2) with the corresponding values when they were excluded (σo^2); adjusted R^2 (%) = $(\sigma o^2 - \sigma^2) / \sigma o^2 \sigma o$.

The criteria for the meta-regression were: 1) $P \leq 0.05$ for the heterogeneity test, 2) $P \geq 0.05$ for the funnel test, and 3) no observation with residual values studentized out of the range of -2.5 to 2.5 (outliers). WMD was assessed using subgroup analysis when categorical covariates were significant in the meta-regression analysis ($P \leq 0.10$).

The covariates were divided as follows: type of control treatment compared to coconut water (TRIS, physiologic solution with 0.5 % glucose and egg yolk, egg yolk), type of coconut water (ACP-101, ACP-101c, ACP-102c, *Nucifera* Coconut, Green and Red Coconut Water – *Viridis* and *Rubescens*, respectively – young and mature, and Light Green Coconut Water), type of preservation (fresh, cooled or cryopreserved), animal species (goats or sheep) and evaluation time (≤ 1h, 12 to 24h, > 24 to 49h, > 73 to 97h, 121 to 145h, 169 to 193h and 720h).

4 RESULTS

Altogether, 579 articles were retrieved from the selected online databases. Among these, 105 were duplications. Three studies were manually included. Nine works were eventually

included in the final sample of this study according to the pre-established selection and inclusion criteria, as shown in the diagram (Fig. 1). Seven were peer-reviewed publications, and two were master's thesis. The included studies were published between 2007 and 2019 (supplementary table 2).

The dataset analyzed in this research consisted of a total of 88 independent comparisons obtained from the nine selected studies. Seventy-nine means were evaluated for the goat species and nine means for the sheep species, representing 89.8% and 10.2%, respectively, of the total evaluated means. Cooled semen had the largest number of comparisons, totaling 68 means (77.3%), while cryopreserved and fresh semen had 13 (14.8%) and seven (7.9%) means respectively.

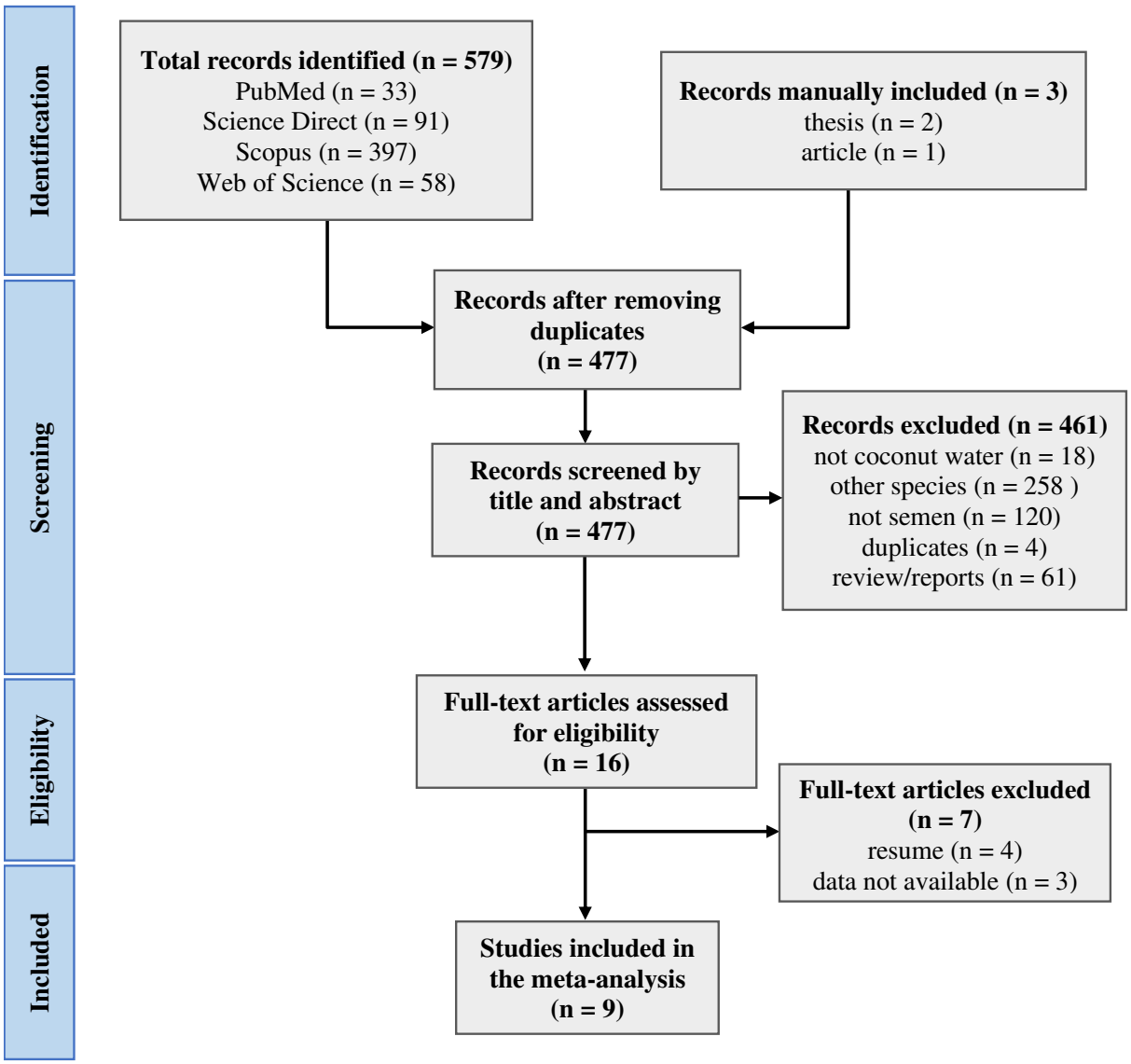

Fig. 1. Flowchart for selection of studies.

4.1. Asymmetry analysis of the funnel plot and meta-regression

As noted in supplementary table 3, publication bias, assessed by the funnel test asymmetry analysis, did not show evident statistical significance (P > 0.05). The heterogeneity present in the studies showed significance (P < 0.05) for all variables. The variables percentage of living cells, vigor, progressive motility, and membrane integrity showed high heterogeneity (> 50%) while the variable of total motility showed moderate heterogeneity (≥ 25% and ≤ 50%).

The meta-regression analysis was performed to enable the identification of covariates responsible for explaining the heterogeneity present between the studies (supplementary table 4).

4.2. Subgroup analysis

By analyzing the control treatment subgroup (Fig. 2) it was possible to notice that the percentage of living cells varied depending on the type of control treatment by which the coconut water was contrasted with. When comparing egg yolk-based extenders vs. coconut water extenders, no effect was observed (WMD = 0.42; P = 0.84), whereas a reduction in the number of live cells was observed for the TRIS vs. coconut water (WMD = -3.86; P < 0.0001).

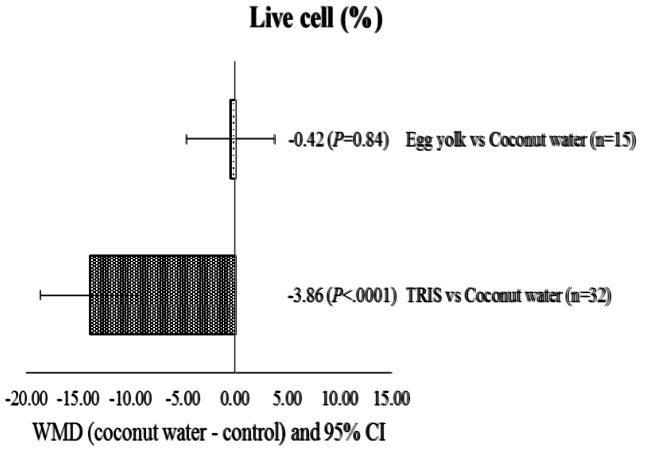

Fig. 2. Subgroup analysis (subgroup = control treatment) of the effect of coconut water-based extender for seminal preservation in small ruminants, WMD = weighted mean difference between treatment and control treatments.

The results of the variables analyzed as a function of the type of coconut water used are shown below (Fig. 3).

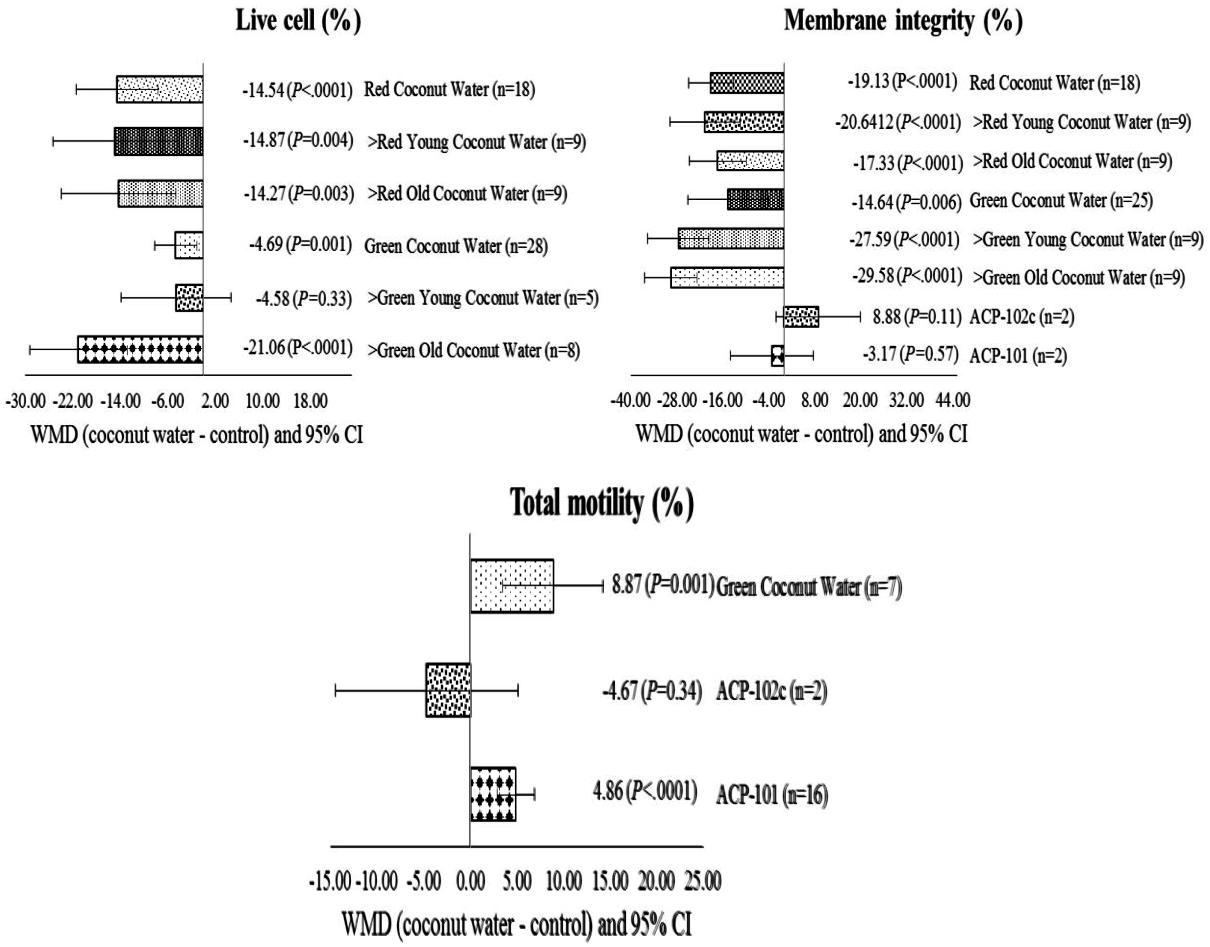

Fig. 3. Subgroup analysis (subgroup = type of coconut water) of the effect of coconut water-based extender for seminal preservation in small ruminants, WMD = weighted mean difference between coconut water and control treatments.

A reduction in the percentage of live cells regardless of the degree of maturity of the fruit occurred for the red variety, whether young (WMD = -14.87; P = 0.004) or mature (WMD = -14.27; P = 0.003). For the green variety, the use of coconut water from the young fruit had no effect for the same variable (WMD = -4.58; P = 0.33), while the use of the mature fruit resulted in a reduction in the number of live cells (WMD = -21.06; P < 0.0001). The use of coconut water caused a reduction in membrane integrity regardless of variety and degree of maturity (P < 0.05). Comparing the red and green varieties it appears, in general, that the red variety had less beneficial results (WMD = -19.13; P < 0.0001) compared to the green one (WMD = -14.64; P = 0.006). When ACP-101 and ACP-102c were used, which are, respectively, specific for goats and sheep, no effect was observed on this variable. Analyzing the total motility, it was noticed that the use of green coconut water and ACP-101 resulted in an increase in this variable. The use of green coconut water was responsible for the greatest increase on this

variable (DMP = 8.87; P = 0.001), followed by ACP-101 (DMP = 4.86; P < 0.0001). The use of ACP-102c had no effect on the same variable (DMP = -4.67; P = 0.34).

The response for percentage of living cells, progressive motility and membrane integrity varied as a function of the evaluation time (Fig. 4). The percentage of live cells had no significant effect for the evaluation periods of ≤ 1h (WMD = 0.19; P = 0.92), 12 to 24h (WMD = -0.04; P = 0.99) and > 24 at 49h (WMD = - 4.68; P = 0.06). On the other hand, this variable underwent a directly proportional reduction as the evaluation time was prolonged: > 73 to 97h (DMP = -7.47; P = 0.04), 121 to 145h (WMD = -12.68; P = 0.001) and 169 to 193h (WMD = -13.42; P = 0.001). There was no significant effect on progressive motility for storage periods of ≤ 1h (WMD = -1.74; P = 0.13) and 12 to 24h (WMD = - 1.17; P = 0.61), however, a reduction on this variable was observed for storage times starting at 24h (WMD = - 8.61; P = 0.04).

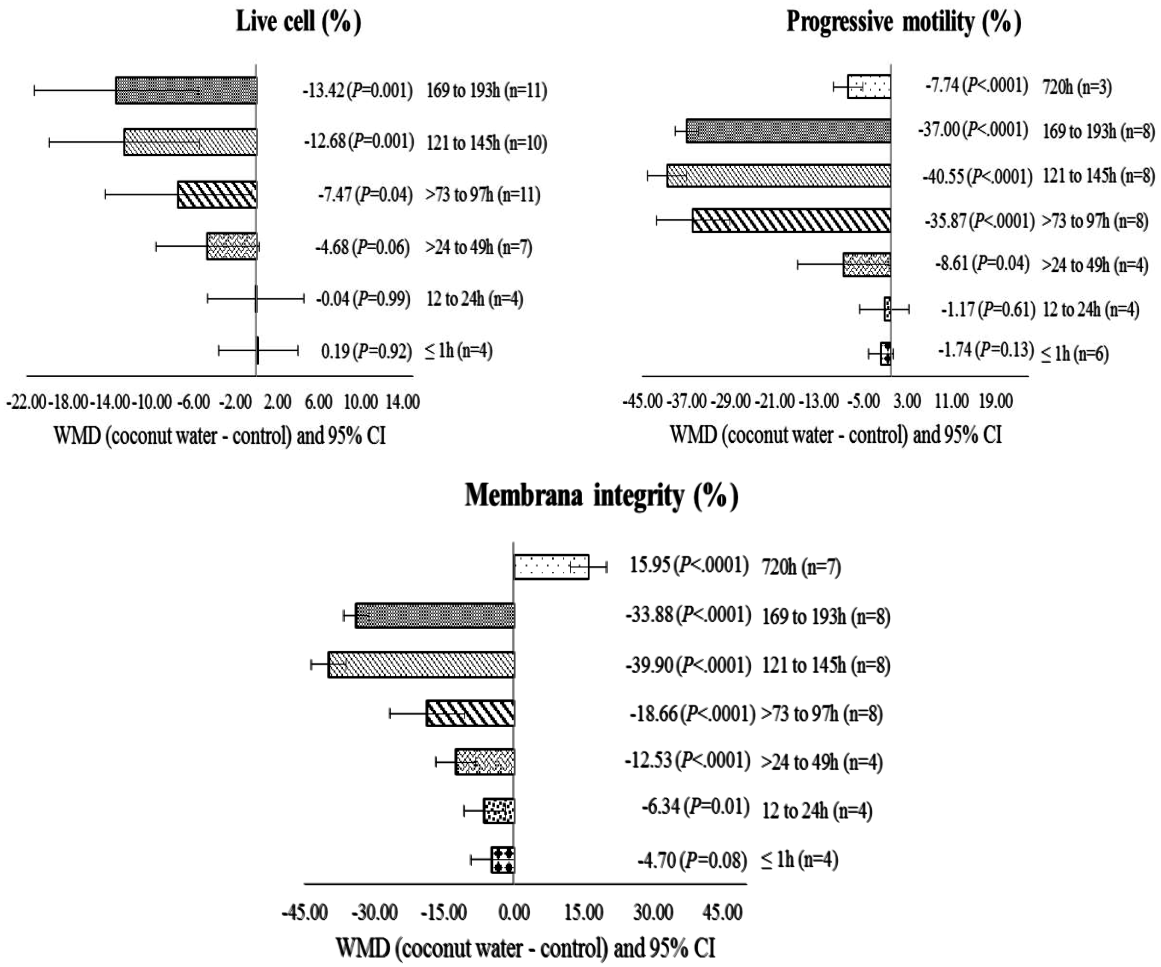

Fig. 4. Subgroup analysis (subgroup = preservation time - h) of the effect of coconut water-based extender for seminal preservation in small ruminants, WMD = weighted mean difference between coconut water and control treatments.

In relation to the membrane integrity (Fig. 4), this had no significant effect during the storage period ≤ 1h (DMP = - 4.70; P = 0.08). On the other hand, a significant reduction when the 12 to 24h (WMD = - 6.34; P = 0.01), > 24 to 49h (WMD = - 12.53; P < 0.0001), > 73h to 97h (WMD = - 18.66; P < 0.0001), 121 to 145h (WMD = - 39.90; P < 0.0001) and 169 to 193h (WMD = - 33.88; P < 0 .0001) evaluation periods were used was observed. The best result for the use of coconut water on membrane integrity was achieved in the 720h evaluation period (WMD =19.95; P < 0.0001), which refers to the semen evaluation at the mark of 30 days after cryopreservation.

The response of spermatozoa preserved in coconut water-based medium regarding total motility and membrane integrity was affected by the type of preservation (Fig. 5).

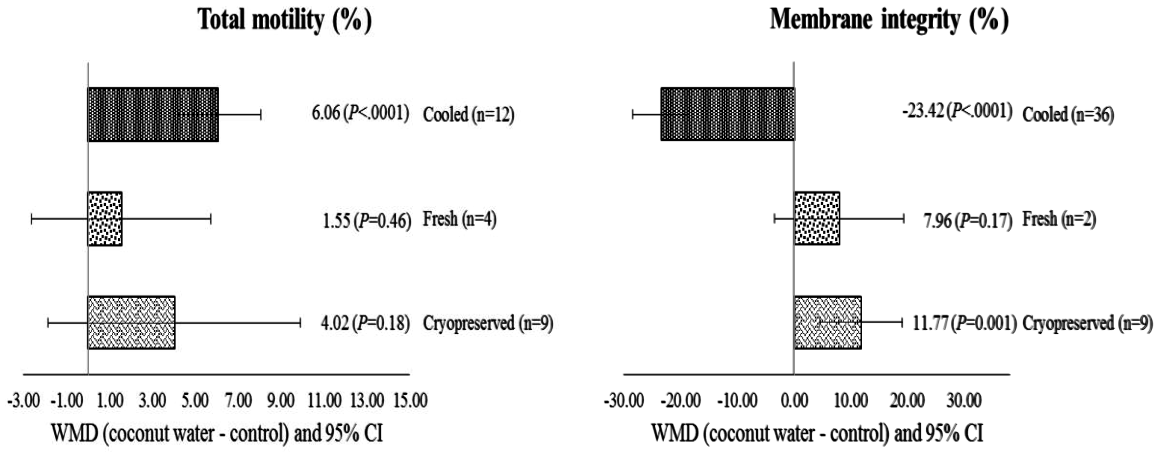

Fig. 5. Subgroup analysis (subgroup = type of preservation) of the effect of coconut water-based extender for seminal preservation in small ruminants, WMD = weighted mean difference between treatment and control treatments.

For total motility in cooled semen, a significant increase was obtained with the use of coconut water (WMD = 6.06; P < 0.0001), while for fresh (WMD = 1.55; P = 0 .46) and cryopreserved semen (WMD = 4.02; P = 0.18) no effect was observed. Membrane integrity was also influenced by the technique used. A considerable reduction on this variable for cooled semen was observed when using coconut water (WMD = - 23.42; P < 0.0001), while for fresh semen there was no change (WMD = 7.96; P = 0.17). Cryopreserved semen showed superior results for membrane integrity (DMP = 11.77; P = 0.001).

Results obtained from the analysis of the animal species subgroup (Fig. 6) show that, for goats, the variables analyzed were influenced by the presence of coconut water in the medium for seminal preservation.

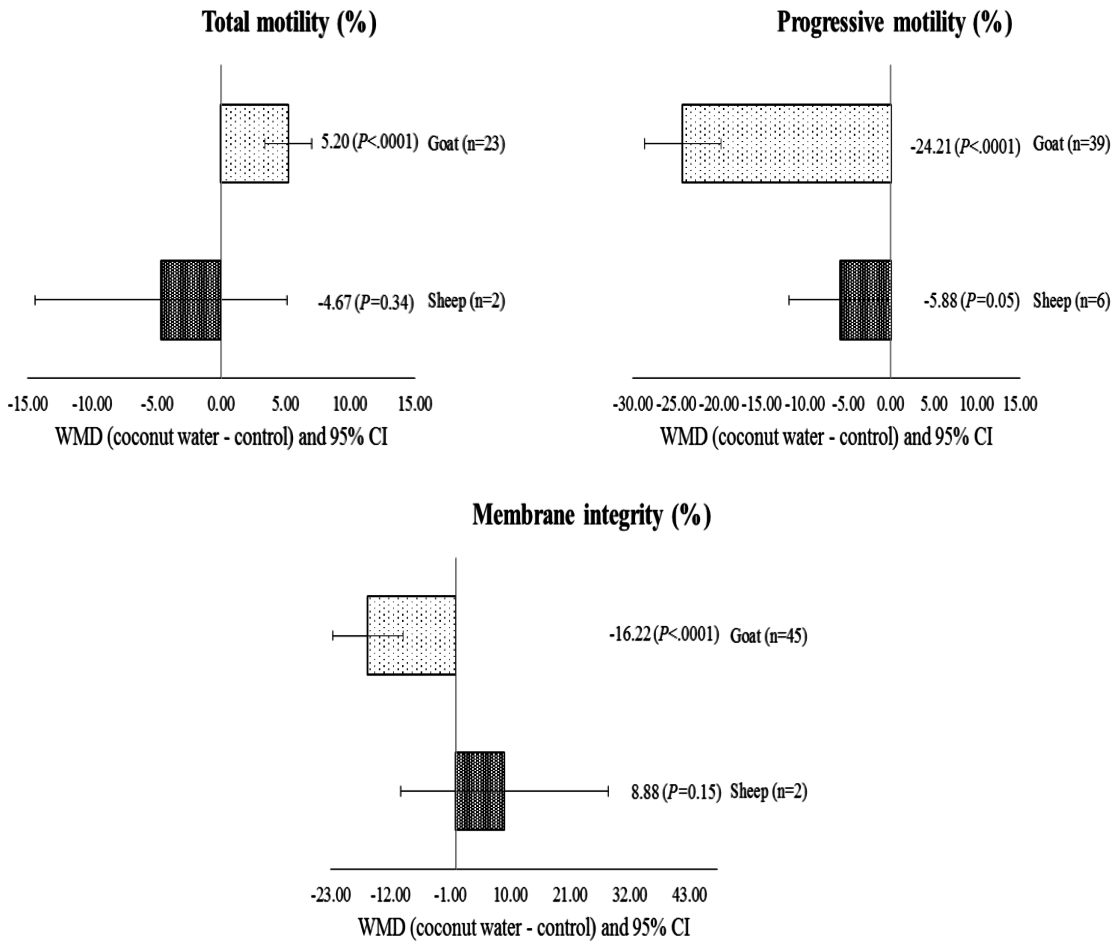

Fig. 6. Subgroup analysis (subgroup = species) of the effect of coconut water-based extender for seminal preservation in small ruminants, WMD = weighted mean difference between treatment and control treatments.

For the goat species, progressive motility (WMD = - 24.21; P < 0.0001) and membrane integrity (WMD = - 16.22; P < 0.0001) were reduced, while an increment was obtained for total motility (WMD = 5.20; P < 0.0001). For the sheep species, only progressive motility was influenced, showing a reduction in the presence of coconut water (WMD = -5.88; P = 0.05), while total motility (WMD = - 4.67; P = 0.34) and membrane integrity (WMD = 8.88; P = 0.15) did not show any significant effect.

5 DISCUSSION

The absence of effect observed on the number of live cells when comparing coconut water with egg yolk (Fig. 2) demonstrates a protective capacity of spermatozoa performed by coconut water during preservation. On the other hand, a different result was observed when compared to TRIS, with a reduction in the number of living cells due to the use of coconut

water, contrasting the hypothesis of this work. A similar result could also be observed, in general, on the percentage of living cells analyzed as a function of the type of coconut water (Fig. 3). This behavior has been reported in cats (Barbosa et al., 2020) and capuchin (*Cebus apella*) (Oliveira et al., 2011) using the same diluent, and being associated with the levels of sugars present in coconut water. Although they are important sources of energy for sperm metabolism (Daramola et al., 2016), the levels of sugars present in this type of diluent can be very high (Yong et al., 2009), which can lead to excessive cell dehydration as a result of the osmotic difference between the extra and intracellular media, making the survival of these gametes more difficult (Oliveira et al., 2011; Barbosa et al., 2020).

Results found for membrane integrity may have been influenced by the existing amino acid profile in coconut water (Fig. 3). Among these, proline, glycine and glutamine can be highlighted, which have been shown to act as cryoprotective agents for sperm cells (Kundu et al., 2001; Yong et al., 2009). In studies performed in cynomolgus monkeys (*Macaca fascicularis*), Li et al. (2003) tested different levels of these amino acids and identified optimal concentrations where their presence improved sperm motility and membrane integrity. On the other hand, the authors also observed that when concentrations of these agents were lower or higher than ideal, there was a significant reduction on the same parameters. Although amino acid concentrations have not been determined in this work, such factor could be directly linked to the reduction found for this variable since their abundance in coconut water has already been reported (Yong et al., 2009). In addition, coconut water contains vitamin C, which acts as an antioxidant agent (Agarwal and Sekhon, 2010; Memon et al., 2012) and has been reported to have a dose-dependent effect against oxidative stress on sperm cells in humans (Ahmad et al., 2017). However, results obtained in this research suggest that the concentration of this agent in the diluent was not sufficient to exert such protection.

No effect was found with the use of ACP-101 on the membrane integrity variable and with ACP-102c for membrane integrity and total motility ($P > 0.05$), respectively, which can be explained by the low sample number ($n = 2$). A positive effect on the sperm motility and membrane integrity variables has been reported with the use of ACP-102c (Brito et al., 2019).

The presence of amino acids in coconut water did not affect total motility (Fig. 3), in contrast to the findings described by Li et al. (2003). A positive effect of the presence of amino acids such as proline and glutamine on sperm motility was observed by Dorado et al. (2014) in donkeys (*Andalusian donkey*) after 72h of cooling. Although the mechanism of action for amino acids on sperm motility is not yet fully elucidated, an interaction of proline with plasma

membrane phospholipids has been identified, acting in its stabilization (Storey and Storey, 1990), while for glutamine a mechanism of cryoprotective action at the extracellular level has been suggested (Trimeche et al., 1996, 1999). In studies with liver cells, Marsh et al. (1990) concluded that the mode of action of amino acids may be related to the preservation of the mitochondrial function, which is of great importance for the generation of energy in all cell types (Piomboni et al., 2012). Thus, in addition to the action of the amino acids present in coconut water, it is possible that the metabolism of sugars present in the diluent – with consequent energy supply to the microtubules present in the spermatic flagellum – are responsible for the observed increase in total motility (Kewilaa et al., 2013; Nelson and Cox, 2014).

Results found in this research for sperm quality as a function of preservation time (Fig. 4) corroborate the results found in the literature by Rajashri et al. (2007). In the study using a breed of sheep from India, these authors observed a detrimental effect due to the prolongation of the semen cooling time on sperm quality. It is possible that the reduction in the percentage of living cells and progressive motility parameters found in this research were due to sperm hyperactivation. This is a process related to capacitation, where sperms have an altered motility pattern (Maxwell and Watson, 1996; Suarez and Ho, 2003) as a function of physiological factors, such as the levels of Ca^{2+} and ATP, through which the sperms acquire the ability to move effectively in the female reproductive system until reaching the oocyte (Ho et al., 2002). In contrast, hyperactivation can induce a drop in diluent energy levels as a result of increased ATP consumption by these cells, in addition to leading to exhaustion due to increased motility and metabolic activity (Gangwar et al., 2020). As a result, the viability of sperm under *in vitro* conditions is compromised, as the energy supply to these gametes is of great importance for the performance of several functions, including maintaining sperm motility and metabolism (Williams and Ford, 2001). Furthermore, it is known that the seminal cooling process has a negative effect on sperm quality (Pervage et al., 2009) as preservation time is increased, regardless of the type of diluent used, dilution rates or conditions of storage (Salamon and Maxwell, 2000; O'Hara et al., 2010).

Although the results found for the number of live cells for periods ≤ 1h, 12 to 24h and > 24 to 49h (Fig. 4) did not show statistical significance, they demonstrate that coconut water was able to preserve these gametes alive until the second day of storage, corroborating the results described by Salim et al. (2018) in works carried out with goats. The presence of essential compounds such as sugars, vitamins, minerals and amino acids in coconut water may

have contributed to this preservation (Yong et al., 2009; Daramola et al. 2016) since in addition to helping in cell dehydration these compounds act in the stabilization of the plasma membrane as cellular protectors (Hincha et al., 2006; Purdy, 2006; Naing et al., 2010), providing an adequate environment for the survival of these gametes.

No significant effect was observed on membrane integrity for the evaluation period ≤ 1h, with a significant reduction on this variable for the evaluation periods from 12 to 24h to 169 to 193h (Fig. 4).

ROS are one of the main causes of plasma membrane damage in sperm (Aitken and Krausz, 2001; Kumar et al., 2019). Although coconut water has in its composition antioxidant enzymes that act to prevent the formation of ROS, such as SOD, it can be suggested that the concentration of this agent in the diluent was not sufficient to prevent the involvement of the plasma membrane when the sperms were refrigerated (Fig. 4 and 5) (Nelson and Cox, 2014; Salim et al., 2018). The same behavior has already been reported by Salim et al. (2018) while working with cooled goat semen. These authors measured SOD levels at three different times during seminal preservation, correlating the results obtained with the membrane integrity of these gametes. No significant effect on this variable was observed until the third day of storage, being justified by the presence of a higher concentration of SOD in the diluent (D3 = 36.527 ± 2.20 ng/100µL). On the other hand, a reduction in the same variable was observed from the fourth to the eighth day of preservation, concomitant with the lower concentration of this enzyme in the diluent (D8 = 24.830 ± 8.93 ng/100µL). Furthermore, the release of an amino acid oxidase enzyme resulting from the presence of dead sperm in the semen acts on the degradation of aromatic amino acids present in the plasma membrane, producing peroxide (Shannon and Curson, 1972). In association, it should also be considered that in addition to the increase in metabolic activity and sperm degeneration (Gangwar et al., 2020) these gametes produce ROS under aerobic conditions as a function of their metabolism, contributing to the installation of oxidative stress by favoring pro-oxidant agents over those antioxidants (Lenzi et al., 2002; Piomboni et al., 2012; Gangwar et al., 2015; Tariq et al., 2015). Thus, as the number of dead sperms increases, oxidative stress is intensified, causing the oxidation of essential biomolecules such as proteins and lipids (Tariq et al., 2015; Rajashri et al., 2017). A contrary result was observed for membrane integrity in the 720h period (Fig. 5), however, this result refers to the 30-day cryopreservation period.

When using a coconut water-based extender, the cooled sperm, unlike fresh and cryopreserved sperm, presented a significant increase in total motility (Fig 5). During the

cooling process, there is a reversible reduction in the metabolic activity of these gametes and, concomitantly, of sperm catabolism, which contributes to the preservation of these cells (Vishwanath and Shannon, 2000; Bezerra, 2010). Divergent results were reported in llama (*Lama glama*) by Zampini et al. (2020) while using the techniques of cooling and seminal cryopreservation, presenting a significant reduction in sperm motility. These results confirm the variation in sensitivity to heat shock between species, which is dependent on the protein-lipid composition and the cholesterol/phospholipid ratio of the sperm plasma membrane (Curry, 2007; Nunes and Salgueiro, 2011). Species with higher amounts of saturated fatty acids and a higher cholesterol/phospholipid ratio are more tolerant to heat shock (Darin-Bennett and White, 1977). Furthermore, the efficiency of coconut water in preserving sperm motility in small ruminants has been previously reported, with results above 60% for this variable in cooled semen while using coconut water as a seminal diluent (Salim et al., 2018; Vlad et al., 2018).

Despite the lack of effect found for the total motility variable for fresh and cryopreserved semen, these results demonstrate that coconut water was able to preserve motility as much as the extenders to which it was contrasted (Fig. 5) with, representing a favorable point to its use as a seminal diluent, as proposed in this research.

A cryoprotective effect of coconut water on sperm membrane integrity was observed during the cryopreservation process (Fig. 5). Such increase may have occurred due to the establishment of a cryoprotective layer through the interaction of amino acids with the phospholipids present in the spermatic membrane, protecting these cells against the formation of ice crystals (Kundu et al., 2001). One of the main events that cause damage to the plasma membrane during cryopreservation is the formation of intracellular ice crystals (Nunes and Salgueiro, 2011; Sieme et al., 2016). However, it is known that the presence of sugars in the associated diluent to the cooling curve is responsible for sperm dehydration, reducing or even preventing the formation of ice crystals inside the sperm (Purdy, 2006; Naing et al., 2010). If the cooling curve is slow, water moves across the plasma membrane and participates in the formation of ice in the extracellular environment. On the other hand, if the cooling curve is slow, ice crystals form in the intracellular environment (Devireddy et al., 2000). In addition, sugars also interact with membrane phospholipids in the membrane reorganization process, resulting in a cell with greater capacity to survive cryopreservation (Aisen et al., 2002), which explains the observed increase in membrane integrity when sperms were cryopreserved.

The observed increase in total motility for the goat species (Fig. 6) may be associated with the mechanisms responsible for controlling the energy supplied to sperm during sperm

preservation. These mechanisms play a crucial role in maintaining the viability of these gametes, since the reactions that maintain the functional status of sperm demand a high energy consumption, practically in their majority (Rodriguez-Gil, 2006). Although glycolysis and the Krebs cycle are accepted as the mechanisms responsible for providing energy to sperm in mammals, the pentose-phosphate (PPP) pathway, an alternative pathway for the degradation of intermediate metabolites of glycolysis, should be considered as well (Williams and Ford, 2001; Piomboni et al., 2012; Nelson and Cox, 2014). In goats, Qiu et al. (2016) found that PPP was more efficient than glycolysis for maintaining motility in cooled semen. The same authors suggested that the occurrence of glycolysis with more intermediate metabolites, such as fructose-6-phosphate, is responsible for the improvement in this parameter. During the oxidation process of glucose-6-phosphate from glycolysis by PPP, fructose-6-phosphate is formed by the non-oxidative phase of this pathway and NADPH. At the sperm level, these metabolites can be of great importance, and it is suggested that the PPP-derived fructose-6-phosphate participates in glycolysis with consequent production of ATP, acting on motility, while NADPH, an electron donor, acts by reducing free radicals and consequently the attack on sperm by ROS (Nelson and Cox, 2014; Qiu et al., 2016).

No effect was observed for the variables of total motility and membrane integrity for the sheep species (Fig. 6), which may have occurred due to the small sample number (n = 2).

As mentioned earlier, the supply of energy for the maintenance of sperm motility is extremely important (Williams and Ford, 2001; Mukai and Okuno, 2004; Rodriguez-Gil, 2006). Working with chilled goat sperm in coconut water-based media, Salim et al. (2018) found results above 55% for the variable progressive motility up to the second day of storage, with a significant reduction for this same variable from the third day on. As well as Rajashri et al. (2017), Salim et al. (2018) attribute this reduction to the depletion of energy sources present in the extender, as well as to sperm exhaustion resulting from its metabolic activity, which may explain the results obtained in this research for progressive motility (Fig. 6). For the sheep species, it is possible that the presence of the seminal protein zinc-alpha-2-glycoprotein (ZAG-2) has contributed to the reduction in this parameter (Qu et al., 2007). This protein is characterized by a dual mechanism of action, stimulating an increase in sperm motility by binding to these cells – in fresh semen, soon after ejaculation, or in a short period of incubation. On the other hand, when the semen is preserved for longer periods, cooled or cryopreserved, this stimulus becomes harmful to the gametes (Soleilhavoup et al., 2014; Leahy et al., 2019).

A great similarity between the GSP-14 and -15 kDa proteins, present in the seminal plasma of goats, and the RSVP-14 protein, has already been reported, indicating that their mechanism of action occurs in a similar way (Barrios et al., 2005; Cardozo et al., 2008). Protein RSVP-14 and -20, present in seminal plasma of sheep, demonstrated a protective effect on membrane integrity during seminal preservation in this species, being linked to the presence of enzymes preventing oxidative stress such as glutathione peroxidase (GPx) and SOD (Marti et al., 2007). On the other hand, it has been suggested that proteins that contain the FBN II domain, such as GSP-14 and -15 kDa proteins, have a dual action mechanism – acting, at first, in the stabilization of the plasma membrane, through interaction with phospholipids, and later in its modification (Barrios et al., 2005; Cardozo et al., 2008). *In vivo*, it is known that the reorganization of the plasma membrane is necessary for capacitation and fertilization, however, this mechanism becomes harmful to sperm under *in vitro* conditions since the continuous exposure to these proteins causes a reduction in the levels of cholesterol present in the membrane, increasing the susceptibility of these cells to the low temperatures used for seminal preservation (Darin-Bennett and White, 1977; Bergeron et al., 2005; Manjunath, 2012), which may justify the result found.

6 CONCLUSION

Coconut water used as a seminal diluent in small ruminants results in the increment of total motility and sperm membrane integrity for cooled and cryopreserved semen, respectively. The absence of significant effect found for some of the analyzed variables demonstrates a sperm preservation capacity by coconut water like the extenders to which it was contrasted with, making it a viable alternative to seminal preservation in small ruminants.

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Supplementary table 1. Description of the study's searching process using selected descriptors and databases.

Search	Database: PubMed		
#	**Query**	**Data/Time**	**Retrieved items**
1	Search: coconut water	10/19/2020 – 08:43am	1100
2	Search: semen	10/19/2020 – 08:44am	41.753
1 AND 2	Search: (coconut water) AND (semen)	10/19/2020 – 08:46am	33
	Database: Science Direct		
#	**Query**	**Data/Time**	**Retrieved items**
1	(("coconut water") AND (sperm))	10/20/2020 – 07:08am	91
	Database: Scopus		
#	**Query**	**Data/Time**	**Retrieved items**
1	ALL (coconut AND water)	10/19/2020 – 08:50pm	42.565
2	ALL (semen)	10/19/2020 – 08:50pm	131.994
1 AND 2	(ALL (coconut AND water)) AND (ALL (semen))	10/19/2020 – 08:51pm	397
	Database: Web of Science		
#	**Query**	**Data/Time**	**Retrieved items**
1	TS= coconut water	10/20/2020 – 07:04am	3.707
2	TS= semen	10/20/2020 – 07:05am	42.584
1 AND 2	#1 AND #2	10/20/2020 – 07:06am	58

Supplementary table 2. Studies included in this meta-analysis according to the pre-established criteria in this study.

Title	Publication year
The Effect of Egg Yolk with Coconut Water Diluter and Storage Time on Nubian Goat semen[1]	2007
Morfometria da cabeça de espermatozoides caprinos diluídos e criopreservados em meio à base de água de coco em pó (ACP-101c)[2]	2011
Avaliação de espermatozoides caprinos congelados em meio à base de água de coco em pó (ACP-101®) ou TRIS[3]	
In vitro evaluation of goat cauda epididymal sperm, cooled in different extenders at 4°C[3]	2013
Criopreservação de sêmen caprino e ovino em diluente de origem vegeta à base de água de coco em pó sem adição de gema de ovo[2]	
Criopreservação do sêmen ovino em meio diluente à base de água de coco em pó (ACP-102c) [3]	2014
Effects of coconut (Cocos nucifera) water with or without egg-yolk on viability of cryopreserved buck spermatozoa[3]	2016
Quality of boer goat liquid semen on different coconut water diluent (Cocos nucifera) during cold storage[3]	2018
Influence of Coconut Powder Water-Based Conservation Medium (APC-102c) for Maintaining Mitochondrial Activity of Cryopreserved Ram Sperm[3]	2019

[1] Article manually included; [2] Dissertation manually included; [3] Article included through the search and selection process according to the PRISMA diagram.

Supplementary table 3. Effects of coconut water-based extender for seminal preservation in small ruminants. Control treatments are represented by diluents without any addition of coconut water. N represents the number of comparisons for control and coconut water treatments. The weighted mean differences between control and treatments are presented (WMD). I^2 represents the proportion of total variation of effect size estimates due to heterogeneity. P value to $\chi 2$ (Q) test of heterogeneity and Egger's regression asymmetry test (Funnel test) are presented.

Item[1]	Control[2] mean (SD)	N[3]	Coconut water		Heterogeneity[5]		Funnel test[6]
			[4]$WMD_{Random\ effect}$ (95% CI)	*P* value	*P* valor	I^2 (%)	*P* valor
Live cells, %	58.11 (12.67)	47	-7.44 (-10.33, -4.55)	<.0001	<.0001	97.78	0.275
Vigor, %	2.17 (0.31)	15	0.50 (0.35, 0.65)	<.0001	0.001	62.13	0.461
Total motility, %	48.99 (17.35)	25	4.46 (2.28, 6.65)	<.0001	0.004	48.18	0.123
Progressive motility, %	50.35 (14.75)	45	-21.61 (-25.62, -17.59)	<.0001	<.0001	96.60	0.153
Membrane integrity, %	61.01 (15.01)	47	-14.98 (-21.15, -8.80)	<.0001	<.0001	96.70	0.478

Supplementary table 4. Meta-regression of the covariate effect on weighted mean difference (WMD) between coconut water and control treatments on parameters correlated with sperm viability. Adjusted R^2 is the proportion of the between-study variance (heterogeneity) explained by covariate. N represents the number of comparisons between control and coconut water treatments.

Dependent variable (Y, WMD)[2]	Meta-regression parameters (*P*-value[1])					Adjusted R^2 (%)	N[4]
	T-Control	Preserv-type	Species	Evaluation time (h)	PT*ET		
Live cells, %	-21.1 (<.01)	--	--	22.4 (0.01)	--	50.36	47
Vigor, %	-0.4 (<.01)	-0.3 (0.14)	--	0.2 (0.49)	--	100	15
Total motility, %	6.3 (0.22)	-13.1 (0.01)	--	2.1 (0.48)	--	100	25
Progressive motility, %	--	1.3 (0.89)	--	-35.9 (0.01)	--	98.47	45
Membrane integrity, %	--	52.2 (<.01)	--	-5.5 (0.06)	--	97.60	47

[1] T-Control = control treatment (PSG + Egg yolk; TRIS and Egg yolk); CWtype = coconut water type; Preserv-type = Preservation type (cryopreserved; fresh and cooled); Species (sheep and goat); PT*ET = Preservation type * Evaluation time.