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Programa de Pós-Graduação em Veterinária

Dissertação

Utilização de extrato de própolis verde no resfriamento de sêmen equino

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Utilização de extrato de própolis verde no resfriamento de sêmen equino

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“Até que tenhas amado a um animal,
uma parte da tua alma estará adormecida”

Anatole France

Resumo

SANTOS, Fernanda Carlini Cunha. **Utilização de extrato de própolis verde no resfriamento de sêmen equino**. 2014. 30f. Dissertação (Mestrado) - Programa de Pós-Graduação em Medicina Veterinária. Universidade Federal de Pelotas, Pelotas.

O resfriamento seminal tem como intuito o prolongamento da viabilidade espermática e capacidade fecundante pela redução no metabolismo energético dos espermatozoides, proporcionando período hábil para transporte do sêmen do garanhão até a égua. O processo de resfriamento pode resultar em alterações ultraestruturais, bioquímicas e funcionais na célula espermática. O objetivo deste experimento foi avaliar o efeito do extrato de própolis verde adicionado ao diluente para resfriamento de sêmen equino a 5ºC. Para isso, foram coletados 4 ejaculados de 5 garanhões da raça Crioula e Quarto de Milha (n=20). Estes ejaculados foram submetidos aos seguintes tratamentos: controle (diluente a base de leite desnatado) e grupos com adição de extrato de própolis verde em diferentes concentrações (2,5µl/mL; 5µl/mL; 7,5µl/mL; 10µl/mL). As análises espermáticas incluíram motilidade, funcionalidade mitocondrial, integridade de membrana plasmática, DNA e acrossoma, sendo utilizados ejaculados contendo ≥70% de células íntegras ás 0h. As avaliações foram realizadas ás 0; 30; 60; 120; 180; 240; 300; 360 e 1440 minutos durante o resfriamento a 5ºC. Os parâmetros seminais diminuíram de acordo com o aumento do tempo em resfriamento em todos tratamentos (P<0,05). A motilidade espermática atingiu 0% em 360 minutos nos tratamentos com própolis. A adição de extrato de própolis verde ao diluente de resfriamento seminal manteve as características estruturais da célula espermática equina, no entanto prejudicou as caraterísticas funcionais.

Palavras-chave: Diluente. Espermatozoide. Garanhão. Refrigerado.

Abstract

Santos, Fernanda Carlini Cunha. **Utilização de extrato de própolis verde no resfriamento de sêmen equino**. 2014. 30f. Dissertação de Mestrado - Programa de Pós-Graduação em Medicina Veterinária. Universidade Federal de Pelotas, Pelotas.

Seminal cooling aims to prolong spermatic lifespan and fertilizing capacity due to reduction in energy metabolism of spermatozoa, providing enough period for transportation from the stallion to the mare. Cooling process can result in ultraestructural, biochemical and functional alterations to spermatozoa. The aim of this study was to evaluate the effect of green propolis hydroalcoholic extract (GPHE) added into equine semen extender and stored at 5ºC. For that, semen was collected from five stallions (Crioulo and Quarter horse) in four opportunities (n=20). These ejaculates were submitted to the following treatments: control group (skim milk glucose semen extender) and groups with the addition of green propolis extract in different concentrations (2,5µl/mL; 5µl/mL; 7,5µl/mL; 10µl/mL). Seminal analyses included motility, mitochondrial functionality, integrity of plasma membrane, DNA and acrossome, being used ejaculates with ≥70% of intact cells. Evaluation was performed at 0; 30; 60; 120; 180; 240; 300; 360 and 1440 minutes during cooled storage at 5ºC. Seminal parameters declined according to storage time in all treatments ($P<0.05$). Sperm motility reached 0% in 360 minutes in the groups with propolis. The addition of green propolis extract into the semen extender maintained structural characteristics of stallion sperm cell, however it was detrimental no functional characteristics.

Keywords: Cooling. Equine. Extender. Spermatozoa.

Lista de Tabelas

Artigo 1 Green propolis extract in stallion semen

Lista de Abreviaturas

ANOVA - Analysis of variance

ATP - Trifosfato de adenosina

FITC-PNA - Fluorescein isothiocyanate labeled peanut agglutinin

GPHE - green propolis hydroalcoholic extract

IA - Inseminação artificial

REPROPEL - Núcleo de Ensino e Pesquisa em Reprodução Animal

ROS - espécies reativas de oxigênio

Sumário

1 INTRODUÇÃO

A indústria equina exerce importante papel na economia brasileira. O Brasil possui o maior rebanho de equinos da América Latina e o 3º maior rebanho do mundo, perdendo apenas para China (1º) e México (2°). O rebanho efetivo é de aproximadamente 8,5 milhões de equinos e 1,2 milhões de muares e jumentos. A produção de equinos gera 3,2 milhões de empregos diretos e indiretos, movimentando 7,3 bilhões de reais/ano (MAPA, 2014). Esta crescente e constante valorização econômica estimula investimentos na criação de equinos.

Em relação à reprodução equina, a principal biotecnologia é a inseminação artificial (IA) (LOOMIS, 2006). Esta biotécnica consiste na coleta de sêmen de um garanhão e manipulação do ejaculado para posterior deposição no trato reprodutivo de uma égua. Em equinos, a IA pode ser realizada com sêmen fresco, resfriado e congelado, sendo que no Brasil destaca-se a realização de inseminações com sêmen resfriado pela facilidade de transporte do material ao invés de transportar garanhões e éguas.

O principal objetivo do resfriamento seminal é prolongamento da viabilidade espermática e capacidade fecundante pela redução no metabolismo energético dos espermatozoides, proporcionando período hábil para transporte do sêmen (GRAHAM, 2011). As taxas de prenhez obtidas por IA com sêmen resfriado a 5ºC por até 24h são semelhantes as obtidas com sêmen fresco (JASKO et al., 1992).

Durante o período de resfriamento, o sêmen é mantido em baixas temperaturas após diluição em diluente seminal específico para espécie. As principais funções dos diluentes de sêmen incluem aumento no volume total do ejaculado, proteção contra choque térmico, proteção contra alterações no pH, fornecimento de energia disponível ao espermatozoide, balanço eletrolítico, balanço osmótico e inibição do desenvolvimento bacteriano (VERSTEGEN et al., 2005).

O processo de criopreservação pode causar danos ultraestruturais, bioquímicos e funcionais na célula espermática (WATSON et al., 2000). A constituição da membrana plasmática com elevada taxa de ácidos graxos insaturados predispõe ao choque térmico e aumenta a susceptibilidade dos

espermatozoides frente danos oxidativos causados pelas espécies reativas de oxigênio (ROS). Todos os componentes celulares incluindo lipídios, proteínas, ácidos nucleicos e açúcares são potenciais alvos do estresse oxidativo (AGARWAL et al., 2008). Em baixa concentração, as ROS são consideradas fisiológicas, atuando como mediadoras da função espermática (GRIVEAU et al., 1997). Porém, em alta concentração as ROS podem prejudicar a motilidade, capacidade de fertilização (BAUMBER et al., 2000), afetar vias metabólicas relacionadas à produção de ATP (ARAMLI et al.,2013), diminuindo assim a disponibilidade de energia para o metabolismo basal da célula espermática. O processo de resfriamento resulta em injúrias às organelas da célula espermática, sendo que a adição de substâncias com ação antioxidante ao meio diluidor auxilia na proteção do espermatozoide.

Os antioxidantes são substâncias que atuam na regulação, remoção e supressão da formação de ROS (SIKKA, 2004; MANEESH & JAYALEKSHMI, 2006), evitando o início ou a propagação das reações em cadeia de oxidação (DEGÁSPARI & WASZCZYNSKYJ, 2004) na maioria dos tecidos e fluidos orgânicos (BARREIROS *et al*., 2006). A adição de substâncias antioxidantes em concentrações adequados ao diluente de resfriamento tem como intuito auxiliar o prolongamento da viabilidade espermática.

A própolis é uma substância resinosa de composição complexa, coletada pelas abelhas (*Apis melífera*) a partir de heterogêneos tipos de plantas, com amplo espectro de atividades biológicas, tais como antibiótica, antifúngica, antiviral, anti-inflamatória, antitumoral e antioxidante (CASTILHO et al., 2009). Esta substância é composta principalmente por 55% de resinas vegetais, 30% de cera de abelha, 10% de óleos essenciais e 5% de pólen (GHISALBERTI, 1979). A diferença entre os tipos de própolis esta relacionada à origem botânica e à espécie de abelha. A própolis verde é um composto encontrado somente no Brasil, principalmente na região de São Paulo e Minas, e está associada à planta *Baccharis dracunculifolia* (PARK et al., 2002). O arbusto alecrim-do-campo (*Baccharis dracunculifolia*) é considerado uma invasora em várias regiões do país, sendo a própolis produzida por abelhas a partir dos ápices vegetativos desta planta (BANKOVA et al., 1999; OLIVEIRA & BASTOS, 1998).

A própolis possui mais de 160 componentes químicos, dentre estes há flavanoides, ácidos aromáticos, terpenoides, aldeídos, álcoois, ácidos alifáticos e

ésteres, aminoácidos, esteróides, açúcares entre diversos outros (MARCUCCI, 1995). Em humanos, resultados evidenciam que a própolis tem capacidade de proteger a célula espermática frente à ação deletéria do estresse oxidativo (RUSSO, et al., 2006). Em caprinos, estudos indicam que a própolis não apresenta efeito benéfico na criopreservação seminal (CASTILHO et al., 2009). Em contrapartida, ÖĞRETMEN et al. (2014) avaliou o efeito deste composto na criopreservação de sêmen de carpas e verificou maiores taxas de motilidade espermática e capacidade de eclosão de ovos nos grupos tratados com própolis.

Na busca por novas alternativas de antioxidantes de origem natural, que não sejam tóxicos para os espermatozoides, a própolis verde pode ser uma alternativa na composição de diluentes para o resfriamento de sêmen equino com intuito de melhorar a qualidade espermática.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Determinar o efeito da adição de extrato de própolis verde no diluente de resfriamento de sêmen equino.

2.2 OBJETIVO ESPECÍFICO

Avaliar o efeito da adição de diferentes concentrações de extrato de própolis verde em diluente de resfriamento de sêmen equino sobre a preservação da motilidade, funcionalidade de mitocôndria, integridade de membrana, DNA e acrossoma.

3 ARTIGO

GREEN PROPOLIS EXTRACT IN STALLION SEMEN

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GREEN PROPOLIS EXTRACT IN STALLION SEMEN

3.1 Abstract

The aim of this study was to evaluate the effect of green propolis hydroalcoholic extract (GPHE) added into equine semen extender and stored at 5ºC. It was performed 4 seminal collection from 5 fertile stallions (Crioulo and Quarter horse), totalizing 20 ejaculates. These ejaculates were submitted to the following treatments: control group (skim milk glucose semen extender) and groups with the addition of green propolis extract in different concentrations (2,5µl/mL; 5µl/mL; 7,5µl/mL; 10µl/mL). Seminal analyses included motility, mitochondrial functionality, integrity of plasma membrane, DNA and acrosome at 0; 30; 60; 120; 180; 240; 300; 360 and 1440 minutes during cooled storage at 5ºC. Parameters declined according to storage time in all treatments. Motility reached 0% in 360 minutes in all groups with GPHE. Addition of green propolis extract into the semen extender maintained structural characteristics of stallion sperm cell, however it was detrimental to functional characteristics during cooled storage.

Key-words: cooling, equine, extender, spermatozoa.

3.2 Introduction

Cooled-transported stallion semen is commonly used to breed horses but fertility achieved from breeding with this material is frequently reduced, as compared to breeding mares with fresh semen (Brinkerhoff et al., 2010). Many factors affect fertility rate of cooled semen and this include semen processing and cooling methodology, type of extender and individual differences inherent to stallions (Aurich, 2008). It is know that semen quality declines over time after collection, extension and cooled storage**.**

Cooling process induces cellular injuries, which is associated with membrane lipids disruption, resulting in mitochondrial damage and loss of plasma membranes and acrosome integrity (Parks & Graham, 1992). These events are accompanied by loss of motility, viability and fertilizing capacity of spermatozoa, being this

phenomenon called cold shock (Watson, 2000). Addition of antioxidants compounds aims to reduce these physiological changes from occurring during cooling process. Free radical induced oxidative damage to spermatozoa is one such condition which has recently gained a considerable attention for its role in inducing poor sperm function and infertility (Russo et al., 2006). Antioxidant activity of several compounds are been researched, aiming to reduce this phenomenon and to prolong the lifespan of cooled stallion semen (Ball et al., 2001; Ball, 2008).

Propolis is a natural resinous hive product with a complex chemical composition, which is collected by honeybees (*Apis mellifera*) from buds of different trees and modified in the beehive by addition of salivated secretions and wax. This natural compound is composed mainly with 55% vegetable resins, 30% beeswax , 10% essential oils and 5% pollen (Ghisalberti, 1979). The difference between the types of propolis is related to the botanical origin and bee specie. Green propolis is a compound found only in Brazil, in the São Paulo and Minas state, and it is associated to the plant *Baccharis dracunculifolia* (Park et al., 2002). This compound presents plenty of biological and pharmacological properties, such as immunomodulatory, antitumor, antiinflammatory, antibacterial, antiviral, antifungal, antiparasite and antioxidant activities (Sforcin et al., 2000; Gekker et al., 2005; Orsi et al., 2006; Freitas et al., 2006).

The most common formulation is propolis ethanol extract and this compound poses immunological properties in animals and humans (Frankiewicz & Scheller, 1984). In humans, results suggest that propolis possess the capacity to protect sperm cells from deleterious effect of oxidative attack (Russo et al., 2006). In boar, studies indicate that propolis did not possess positive effect on seminal cryopreservation (Castilho et al., 2009). On the other hand, Öğretmen et al. (2014) evaluated the effect of this compound in cryopreservation of carp sperm and reported higher motility and hatching ability in the groups treated with propolis. Because of these properties and the different uses of propolis, there is a renewed interest in its anti-oxidant activities and *in vitro* methods are useful for preliminary investigation of this natural product potential use (Heinrich et al., 2008).

The present study aimed to evaluate the effect of green propolis hydroalcoholic extract (GPHE) in stallion sperm parameters during chilled storage at 5ºC.

3.3 Materials and Methods

It was performed 4 seminal collection from 5 fertile stallions (Crioulo and Quarter horse), aged 3 to 7 years, totalizing 20 ejaculates.

All stallions were allocated in the city of Pelotas, Rio Grande do Sul (RS), Brazil. The ejaculates were collected during reproductive season, after 2 days of sexual arrest. A mare in estrus was used for semen collection using an artificial vagina (Hannover model, Minitub, Tiefenbach, Germany) fitted with a nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA). Ejaculates with ≥70% progressive motility and morphologically normal sperm were used in the experiment (CBRA, 2013).

The green propolis was collected in São Paulo state, Brazil, by Apis Nativa Produtos Naturais Ltda (PRODAPYS) and stored at 5ºC. The extract was prepared in Federal University of Pelotas (UFPel), according to the technique described by Paulino et al. (2002) modified. A sample of propolis was frozen at -70ºC for later grinding. Extraction was performed in a solution with alcohol 96º GL, under stirring, at 37ºC for 24 hours. Next, the solvent was evaporated and the resulting dry matter was dissolved in phosphate buffer (pH 6.2) at a final concentration of 40 mg/mL (Fischer et al., 2010). Chemical composition was determined by high performance liquid chromatography (HPLC) with a device Hitachi-Merck (Germany) equipped with a pump (Model L-7100 Merck-Hitachi) and a detector drag (L-7455, Merck-Hitachi). Separation was performed on a Lichrochart 125-4 column (Merck, Darmstadt, Germany) as described by Marcucci et al. (2001). Components detection were monitored at 280 nm and standard compounds were co-chromatographed with the extract. Data analysis was performed on Merck-Hitachi D-7000 (Chromatography Data Station - DAD Manager). Green propolis extract composition is exposed in Table 1. The GPHE pH was 3.9 and was adjusted to 6.7, similar to the base extender.

Semen samples were diluted in skim glucose-milk base extender (Kenney, 1975) at concentration of $50x10^6$ spermatozoa/mL. Next, semen samples were submitted to the following treatments: control group (only skim milk glucose semen extender) and groups with the addition of green propolis extract in different concentrations (2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL). After submission to treatment, semen samples were stored at 5ºC in a commercial refrigerator (Minitub^{TM}).

Samples were evaluated immediately after warming-up, in a 37 ºC water bath for 5 minutes prior to analysis, following 0; 30; 60; 120; 180; 240; 300; 360 and 1440 minutes of storage at 5ºC. Parameters analyzed were: motility, mitochondrial functionality, integrity of plasma membrane, DNA and acrosome of stallion spermatozoa.

Table 1. Green propolis hydroalcoholic extract composition.

Compound	Concentration (mg/g)
P-coumaric acid	4.673
3-prenyl-4-hydroxycinnamic acid	1.951
Canferide	5.623
3,5-diprenyl-4-hydroxycinnamic acid	16.322
Cinnamic acid derivative	6.692
6-propenoic acid-2 ,2-dimethyl-8-prenyl-2H-1-benzopyran	0.163

Sperm concentration was determined using a Neubauer chamber in an optic microscope at 400x magnification. Morphology was assessed in a slide covered with a coverslip in a phase-contrast microscope at 1000x magnification. Motility was assessed by a single trained person, in a heated slide covered with a coverslip in phase-contrast microscopy at 200x magnification (BX 41 Olympus América, Inc., São Paulo, SP, Brazil). Mitochondrial functionality was examined by staining the cells with Rhodamine 123 (Sigma 83702), which is selectively accumulated by the mitochondria. Intense green accumulate in the intermediate piece was consider intact and mat green was considered damaged (Grasa et al., 2004). Plasma membrane integrity was evaluated using the fluorescent probes carboxyfluorescein diacetate (Sigma C5041) and propidium iodide (Sigma P4170), as described by Harrison and Vickers (1990). Green and red fluorescence were interpreted as an intact and damaged membrane, respectively. The DNA integrity was evaluated with acridine orange (Sigma A6014) test. Green and red fluorescence were interpreted as an intact and damaged DNA, respectively (Bencharif et al., 2008). Acrosome integrity was evaluated with propidium iodide and FITC-PNA (Sigma L7381) dyes as described by García-Macías et al. (2007). Sperm were classified as having an intact acrosome when this local was stained homogeneous fluorescent green, and a reacted acrosome when the fluorescent green was present in the sperm equatorial

region or when it was absent from the head. These parameters were analyzed in an epifluorescence microscope (Olympus BX 51, América INC, São Paulo, Brazil) at 1000x magnification and 100 spermatic cells were counted.

All variables were normally distributed according to the Shapiro-Wilk test. The combinations effects of Time x Treatment on the responses were tested by ANOVA, with comparisons of means performed with Tukey's test. The program Statistix 9® (Statistix, Statistix 9 for Windows, Analytical Software, Tallahassee, FL, USA, 2008) was used for statistical analyses. Significance was assigned to all values $P<0.05$.

This experiment used animals in the experimental model and had the approval of the Institutional Research Ethics Committee of UFPel by the number 1946.

3.4 Results

From 60 minutes, motility declined significantly in all treatments. Since 30 minutes, motility in treated groups were inferior to the control ($P<0.05$). After 360 minutes of cooled storage, motility reached 0% in the treatments with propolis (Table 2).

Table 2. Motility (mean±standard error mean) of stallion semen (n=20) submitted to different treatments with green propolis hydroalcoholic extract (GPHE) into extender and stored at 5ºC for 1440 minutes.

Minutes	Control	2.5µl/mL	5µl/mL	7.5µl/mL	10µl/mL
0	94.0 ± 0.4 $^{A\,a}$	94.0 ± 0.4 $^{A\,a}$	94.0 ± 0.4 $^{A\,a}$	94.0 ± 0.4 $^{A\,a}$	94.0 ± 0.4 $^{A\,a}$
30	92.0 ± 0.5 $^{A\,ab}$	75.0 ± 1.7 $^{B\,ab}$	75.2 ± 1.9 $^{B\,ab}$	73.0 ± 1.3 $^{B\,b}$	65.7 ± 3.7 $^{B\,ab}$
60	91.5 ± 0.5 $^{A\,abc}$	60.5 ± 3.6 $^{B\,bc}$	53.5 ± 4.4 $^{B\,bc}$	52.0 ± 3.6 $^{B\,bc}$	47.5 ± 3.9 $^{B\,bc}$
120	90.0 ± 0 $^{A\,bc}$	44.5 ± 3.5 $^{B\,bcd}$	44.2 ± 3.9 $^{B\,bcd}$	43.5 ± 4.5 $^{B\,bcd}$	39.5 ± 4.1 $^{B\,bcd}$
180	89.5 ± 0.3 $^{A\,bc}$	34.7 ± 4 $^{B\,bcd}$	32.2 ± 3.2 $^{B\,bcd}$	33.0 ± 3.2 $^{B\,bcd}$	28.5 ±3 $^{B\,bcd}$
240	88.0 ± 0.5 $^{A\,bc}$	28.2 ± 4.2 $^{B\,bcd}$	23.7 ± 2 ^{B,cd}	22.7 ± 1.7 $^{B\,bcd}$	19.7 ± 2.2 $^{B\,bcd}$
300	88.0 ± 0.5 $^{A\,cd}$	10.0 ± 1.4 $^{B\,cde}$	7.7 ± 1 $^{B\,cde}$	9 ± 1.2 $^{B\,cde}$	7.2 ± 1.4 $^{B\,cde}$
360	88.0 ± 0.5 $^{A\,cd}$	0.0 ± 0.0 $^{B\,de}$	0.0 ± 0.0 $^{B\,de}$	0.0 ± 0.0 $^{B\,de}$	0.0 ± 0.0 $^{B\,de}$
1440	54.0 ± 4.1 $^{A\,d}$	0.0 ± 0.0 $^{B\,e}$	0.0 ± 0.0 $^{B\,e}$	0.0 ± 0.0 $^{B\,e}$	0.0 ± 0.0 $^{B\,e}$

^{a,b,c,d,e} Different letter indicates statistical difference in columns (ANOVA, $P<0.05$)

^{A,B} Different letter indicates statistical difference in lines (ANOVA, $P<0.05$)

Control group (only skim milk glucose semen extender) and groups with the addition of GPHE in different concentrations 2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL

Regarding mitochondrial functionality, cooling process reduced this organelle quality with no statiscal difference between all treatments (Table 3).

Table 3. Mitochondrial functionality (mean±standard error mean) of stallion semen (n=20) submitted to different treatments with green propolis hydroalcoholic extract (GPHE) into extender and stored at 5ºC for 1440 minutes.

Minutes	Control	2.5µl/mL	5µl/mL	7.5µl/mL	10µl/mL
0	75.4 ± 3.8 ^{a}	75.4 ± 3.8 ^{a}	75.4 ± 3.8 ^{a}	75.4 ± 3.8 ^{a}	75.4 ± 3.8 ^{a}
30	60.3 ± 6.1 ^{ab}	54.1 ± 7.3 ^{ab}	54.7 ± 7.0 ^{ab}	55.8 ± 6.9 ^{ab}	52.1 ± 6.8 ^{ab}
60	57.5 ± 5.5 ^{ab}	51.4 ± 6.9 ^{abc}	43.6 ± 6.4 ^{abc}	50.5 ± 6.7 ^{ab}	46.8 ± 6.1 ^{ab}
120	43.6 ± 5.7 ^{bc}	42.6 ± 5.4 ^{bcd}	37.2 ± 5.5 ^{bc}	43.3 ± 5.3 ^{bc}	41.8 ± 6.1 ^{bc}
180	37.9 ± 6.2 ^{bc}	37.5 ± 5.4 ^{de}	39.9 ± 6.0 ^{cd}	35.1 ± 7.6 ^{bcd}	33.1 ± 6.9 ^{bcd}
240	36.3 ± 6.2 ^{bc}	32.4 ± 6.9 ^{cde}	34.4 ± 5.3 ^{cd}	39.0 ± 6.4 ^{cd}	34.4 ± 6.4 ^{cd}
300	44.3 ± 4.9 ^{bc}	36.3 ± 5.7 ^{bcd}	38.6 ± 7.1 ^{bc}	40.4 ± 7.4 ^{bc}	37.2 ± 7.3 ^{bcd}
360	41.5 ± 4.8 ^{bc}	29.7 ± 4.6 ^{cde}	34.5 ± 7.4 ^{bcd}	37.7 ± 7.2 ^{bcd}	33.8 ± 6.8 ^{bcd}
1440	21.4 ± 3.4 ^{c}	12.4 ± 3.0 ^{e}	17.3 ± 4.0 ^{d}	15.9 ± 3.8 ^{d}	18.5 ± 5.7 ^{d}

^{a,b,c,d,e} Different letter indicates statistical difference in columns (ANOVA, P<0.05)

Control group (only skim milk glucose semen extender) and groups with the addition of GPHE in different concentrations 2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL

From 300 minutes, plasma membrane integrity declined significantly in the control group and no period of time there was difference between all treatments (Table 4).

Table 4. Plasma membrane integrity (mean±standard error mean) of stallion semen (n=20) submitted to different treatments with green propolis hydroalcoholic extract (GPHE) into extender and stored at 5ºC for 1440 minutes.

Minutes	Control	2.5µl/mL	5µl/mL	7.5µl/mL	10µl/mL
0	82.8 ± 1.7 ^{a}	82.8 ± 1.7 ^{a}	82.8 ± 1.7 ^{a}	82.8 ± 1.7 ^{a}	82.8 ± 1.7 ^{a}
30	79.7 ± 2.8 ^{ab}	83.3 ± 2.4 ^{a}	80.3 ± 3.2 ^{a}	77.6 ± 3.7 ^{a}	79.6 ± 2.8 ^{a}
60	75.5 ± 2.9 ^{ab}	76.1 ± 4.7 ^{ab}	79.0 ± 3.7 ^{ab}	76.2 ± 3.9 ^{a}	76.9 ± 3.8 ^{ab}
120	68.8 ± 3.0 ^{abc}	69.4 ± 2.9 ^{abc}	71.3 ± 3.0 ^{abc}	69 ± 3.2 ^{ab}	67.9 ± 3.8 ^{abc}
180	58.9 ± 4.2 ^{bc}	60.5 ± 4.2 ^{cd}	66.6 ± 4.6 ^{bcd}	60.5 ± 4.6 ^{bc}	57.7 ± 4.3 ^{cd}
240	65.2 ± 3.6 ^{abc}	53.7 ± 6.3 ^{bcd}	50.4 ± 6.2 ^{cd}	46.3 ± 5.7 ^{bc}	54.7 ± 6.1 ^{bcd}
300	52.5 ± 5.5 ^{c}	39.7 ± 5.2 ^{d}	41.7 ± 5.5 ^{d}	43.5 ± 6.3 ^{c}	43.2 ± 7.0 ^{d}
360	51.1 ± 5.8 ^{c}	38.2 ± 5.6 ^{d}	41.6 ± 6.4 ^{d}	43.6 ± 6.8 ^{c}	38.9 ± 6.3 ^{d}
1440	52.2 ± 3.6 ^{c}	36.6 ± 4.5 ^{d}	43.2 ± 4.6 ^{d}	37.6 ± 4.3 ^{c}	37.6 ± 5.1 ^{d}

^{a,b,c,d,e} Different letter indicates statistical difference in columns (ANOVA, P<0.05)

Control group (only skim milk glucose semen extender) and groups with the addition of GPHE in different concentrations 2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL

DNA integrity declined during the evaluation period with no statiscal difference between treatments (Table 5).

Table 5. DNA integrity (mean±standard error mean) of stallion semen (n=20) submitted to different treatments with green propolis hydroalcoholic extract (GPHE) into extender and stored at 5ºC for 1440 minutes.

Minutes	Control	2.5µl/mL	5µl/mL	7.5µl/mL	10µl/mL
0	99.1 ± 0.1 ^{a}	99.1 ± 0.1 ^{a}	99.1 ± 0.1 ^{a}	99.1 ± 0.1 ^{a}	99.1 ± 0.1 ^{a}
30	82.4 ± 6 ^{ab}	83 ± 6.2 ^{abc}	80.7 ± 6.2 ^{bc}	82.4 ± 5.4 ^{abcd}	76.5 ± 7.0 ^{bc}
60	83 ± 4.3 ^{ab}	87.6 ± 3.2 ^{abc}	84.8 ± 4.0 ^{abc}	87.8 ± 6.3 ^{bcd}	83.5 ± 4.6 ^{abc}
120	80.9 ± 3.8 ^{b}	84.4 ± 3.5 ^{bc}	84.9 ± 4.2 ^{bc}	87.8 ± 3.6 ^{bcd}	87.7 ± 3.4 ^{bc}
180	89.9 ± 2.8 ^{ab}	87.7 ± 4.8 ^{ab}	92.9 ± 2.6 ^{ab}	87.1 ± 4.6 ^{abc}	91.7 ± 3.2 ^{ab}
240	87 ± 4.3 ^{ab}	92.1 ± 2.3 ^{ab}	84.6 ± 5.6 ^{ab}	89.8 ± 3.7 ^{ab}	85.1 ± 5.5 ^{ab}
300	86.9 ± 3.6 ^{b}	84.3 ± 5.0 ^{abc}	88.0 ± 4.4 ^{abc}	85.9 ± 4.2 ^{abcd}	86.7 ± 5.1 ^{abc}
360	82.3 ± 3.6 ^{b}	78.0 ± 5.0 ^{c}	84.4 ± 4.3 ^{bc}	81.8 ± 3.7 ^{cd}	80.9 ± 5.4 ^{bc}
1440	83.1 ± 3.4 ^{b}	77.2 ± 4.5 ^{c}	82.3 ± 3.9 ^{c}	80.7 ± 3.6 ^{d}	77.9 ± 4.6 ^{c}

^{a,b,c,d,e} Different letter indicates statistical difference in columns (ANOVA, P<0.05)

Control group (only skim milk glucose semen extender) and groups with the addition of GPHE in different concentrations 2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL

Acrosome integrity decreased according to increased storage time with no statistical difference between treatments (Table 6).

Table 6. Acrosome integrity (mean±standard error mean) of stallion semen (n=20) submitted to different treatments with green propolis hydroalcoholic extract (GPHE) into extender and stored at 5ºC for 1440 minutes.

Minutes	Control	2.5µl/mL	5µl/mL	7.5µl/mL	10µl/mL
0	73.0 ± 6.3 ^{a}	73.0 ± 6.3 ^{a}	73.0 ± 6.3 ^{a}	73.0 ± 6.3 ^{a}	73.0 ± 6.3 ^{a}
30	58.7 ± 7.2 ^{ab}	78.1 ± 4.0 ^{a}	62.9 ± 6.6 ^{a}	67.5 ± 6.6 ^{a}	60.4 ± 7.1 ^{ab}
60	54.6 ± 6.7 ^{ab}	59.1 ± 7.5 ^{ab}	54.0 ± 7.7 ^{a}	67.5 ± 5.0 ^{ab}	68.7 ± 4.2 ^{ab}
120	57.5 ± 7.6 ^{ab}	70.0 ± 5.9 ^{ab}	66.3 ± 7.0 ^{a}	63.7 ± 6.4 ^{ab}	61.7 ± 8.3 ^{ab}
180	57.5 ± 5.5 ^{ab}	70.1 ± 5.4 ^{ab}	54.4 ± 7.4 ^{a}	69.2 ± 6.2 ^{a}	73.3 ± 4.9 ^{ab}
240	55.7 ± 5.9 ^{ab}	49.3 ± 7.9 ^{ab}	60.7 ± 6.9 ^{a}	46.7 ± 7.9 ^{ab}	70.9 ± 8.4 ^{ab}
300	49.7 ± 7.2 ^{ab}	51.6 ± 8.7 ^{ab}	63.3 ± 7.8 ^{a}	46.1 ± 7.3 ^{ab}	60.3 ± 7.3 ^{ab}
360	43.1 ± 7.2 ^{b}	53.3 ± 7.9 ^{ab}	60.7 ± 7.0 ^{a}	39.8 ± 6.6 ^{b}	61.0 ± 5.8 ^{ab}
1440	58.7 ± 6.9 ^{ab}	33.8 ± 6.6 ^{b}	47.8 ± 7.0 ^{a}	39.7 ± 7.0 ^{ab}	59.5 ± 5.1 ^{ab}

^{a,b,c,d,e} Different letter indicates statistical difference in columns (ANOVA, P<0.05)

Control group (only skim milk glucose semen extender) and groups with the addition of GPHE in different concentrations 2.5µl/mL; 5µl/mL; 7.5µl/mL; 10µl/mL

3.5 Discussion

Cooled semen is worldwide used in equine breeding management and the addition of antioxidants is been researched aiming to provide better sperm quality during cooled storage at 5ºC. In the present experiment, addition of green propolis

extract at 2.5-10µl/mL did not improve sperm parameters during cooling. The concentrations used were established based on previous results obtained in our lab (unpublished data). Laboratorial evaluation was performed for 1440 minutes once equine semen can be maintained refrigerated at 5ºC for up to 24h. Supplementation with antioxidants has been reported to improve the viability and motility of cryopreserved spermatozoa of several mammalian species (Pomaraes et al., 1995; Maxwell & Stojanov, 1996; Foote, 2002; Funahashi & Sano, 2005).

In goats, Castilho et al. (2009) researched about propolis and ascorbic acid on semen cryopreservation and found that general means of sperm motility, vigor, eosin-nigrosin test and hyposmotic swelling test obtained after thawing were different, so that the extender with ascorbic acid and the control were similar and higher than the extenders containing propolis. Authors concluded that propolis was not effective in maintaining sperm integrity after thawing and consider this compound toxic to goat spermatozoa.

In contrast with the present results, a study by Russo et al. (2006) indicated that Chilean propolis hydroalcoholic extract has the capacity to protect human spermatozoa from the genotoxic action of benzo[a]pyrene *in vitro*, and to contrast strong oxidative attack, inhibiting lipid peroxidation and LDH release, which decreasing membrane fluidity is involved in the reduction of fertilization capacity. These authors reported the antioxidant action of propolis and its beneficial effect in human spermatozoa regarding free radical release and this action was mainly attributed to phenolic compounds.

Ögretmen et al. (2014) evaluated the effect of propolis on cryopreservation and fertilization ability of carps (*Cyprinus carpio*) and observed that extenders containing propolis exhibited higher percentage motility and motility duration than control group. Concentrations of 0.8 mg ml^{-1} and 1 mg ml^{-1} showed significant positive effects on post thaw motility and hatching ability. These authors concluded that propolis maintained spermatozoa integrity during fish semen cryopreservation process.

In our study, it was investigated the effect of propolis on motility, mitochondrial functionality, plasma membrane, DNA and acrosome integrity of stallion spermatozoa stored at 5ºC for 1440 minutes. In our conditions, no improvement in sperm parameters was obtained by the addition of 2.5-10µl/mL of propolis into the cooling extender.

All treatments with propolis addition presented motility equal to 0% after 360 minutes of cooled storage. Stallion sperm need to sustain motility to reach and fertilize the oocyte in the mare. The main energetic source for motility is ATP, produced by mitochondria in the mid piece by aerobic respiration (Travis et al., 1998). In low concentrations, ROS are considered physiological, acting as mediator of sperm functions (Griveau et al., 1997). However, in high concentrations ROS may affect negatively motility, fertilizing capacity (Baumber et al., 2000) and metabolic pathways related to ATP production (Aramli et al., 2013), thus it may induce cellular death due to lack of energy to basal metabolism of the cell. GPHE was able to maintain morphological characteristics of stallion spermatozoa, although different action of this compound was observed in functional characteristics.

3.6 Conclusion

Green propolis hydroalcoholic extract into stallion semen extender did not compromised mitochondria functionality, integrity of plasma membrane, DNA and acrosome of chilled-stored stallion spermatozoa at 5ºC, however it induced a decline in sperm motility.

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4 CONCLUSÃO GERAL

O extrato de própolis verde apresentou efeito negativo sobre a motilidade espermática de equinos durante o período de resfriamento. Em relação aos parâmetros funcionalidade de mitocôndria, integridade de membrana, DNA e acrossoma, a própolis não apresentou efeito prejudicial.

As concentrações de extrato de própolis verde avaliadas não apresentaram efeito benéfico na qualidade seminal durante o resfriamento, sendo sugerido a avaliação de menores concentrações deste componente em diluente seminal de equinos.

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