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Dissertação

Adição de quercetina e progesterona ao sêmen equino

Jorge Squeff Filho

Pelotas, 2018

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Adição de quercetina e progesterona ao sêmen equino

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“Uma mente que se abre a uma nova ideia jamais volta ao seu tamanho original”.
(Albert Einstein)

Resumo

SQUEFF, Jorge Filho. **Adição de quercetina e progesterona ao sêmen equino.** 2018. 65f. Dissertação (Mestrado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2018.

As variações e dificuldades em manter os parâmetros espermáticos da célula equina após manipulação (resfriamento e congelamento) nos leva a uma busca por substâncias que possam ser adicionadas aos meios de diluição de sêmen com o intuito de manter ou ainda melhorar a viabilidade celular. Com esse intuito, a presente dissertação testou, em dois experimentos, a adição de quercetina e progesterona ao sêmen equino. No experimento referente a adição da quercetina foram utilizados 10 cavalos da raça Crioula com idades entre 4 e 8 anos; a coleta de sêmen foi realizada com vagina artificial e 3 ejaculados de cada animal foi obtido (totalizando 30 ejaculados). As amostras foram submetidas a 5 diferentes tratamentos (0; 0,25mM; 0,5mM; 0,75mM e 1mM) de quercetina adicionadas previamente ao diluente de congelamento. As análises referentes aos parâmetros cinéticos da célula foram avaliadas de maneira subjetiva por uma pessoa treinada em microscópio contraste-fase e os parâmetros referentes a viabilidade da população espermática foram obtidos através de citometria de fluxo. A análise estatística realizada a partir dos dados obtidos não demonstrou diferença significativa entre os tratamentos e o controle ($P > 0,05$). O experimento referente a adição de progesterona foi realizado em duas etapas; a primeira etapa consistiu na realização de um teste de toxicidade do sêmen as diferentes concentrações do hormônio. A coleta do sêmen foi realizada com vagina artificial de um total de 10 cavalos da raça Crioula com idades entre 4 e 10 anos; posteriormente a isso as amostras foram submetidas a resfriamento até a temperatura de 5°C e adicionados os tratamentos contendo 7 diferentes concentrações de progesterona (0; 1; 2,5; 5; 10; 100 e 1000 ng/mL) em 3 tempos de exposição (0,5h; 3h e 24h). A segunda etapa consistiu na criopreservação do sêmen obtidos através de vagina artificial de 15 cavalos da raça Crioula com idades entre 4 e 10 anos. Os ejaculados foram submetidos aos tratamentos com 5 concentrações diferentes de progesterona (0; 1; 5; 10 e 100 ng/mL) adicionados ao meio diluente de criopreservação previamente ao congelamento. As análises em ambas as fases do experimento, dos parâmetros cinéticos espermáticos foram feitas pelo Sistema Computadorizado de Análise de Sêmen (CASA), bem como em ambas as avaliações feitas por citometria de fluxo referentes a viabilidade da população espermática.A análise estatística realizada posteriormente ao teste de toxicidade, ou seja, com o sêmen resfriado demonstrou melhora estatística ($P < 0,05$) nas concentrações de 5 e 1000 ng/mL no período de 3h de exposição em parâmetros relacionados com velocidade e distância percorrida do espermatozoide (DAP, DCL, DSL, VAP, VCL, VSL). Houve também aumento significativo na reação de acrossoma após 24 horas de exposição na concentração

de 5 ng/mL e diminuição significativa (P < 0,05) da peroxidação lipídica (LPO) nas concentrações de 10, 100 e 1000 ng/mL também no período de 24 horas. Os dados obtidos das amostras pós-descongelamento demonstraram uma diminuição (P < 0,05) da produção de espécies reativas de oxigênio (ROS), entretanto os outros parâmetros avaliados não diferiram estatisticamente do controle. A partir de todos os dados obtidos nos dois experimentos é possível afirmar que a adição de quercetina e progesterona ao meio de diluição, previamente a criopreservação não é interessante, uma vez que não impõe melhoras significativas na qualidade espermáticas; entretanto a adição de progesterona ao sêmen fresco resfriado demonstrou ações benéficaspara imediata utilização do sêmen, como hiperativação, capacitação e ação anti-oxidante.

Palavras-chave: congelamento; esteroide; flavonoide; garanhão; hormônio; toxicidade

Abstract

SQUEFF, Jorge Filho. **Quercetin and progesterone addition to the equine semen.** 2018. 65f. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2018.

The variations and difficulties on maintaining the equine sperm parameters after manipulation (cooling and cryopreservation) take the scientists in a journey researching for substances that are able to be added to the dilution semen medium in order to keep or improve sperm viability. That being said, the present study has tested, in two different experiments, the addition of quercetin and progesterone to the equine semen. In the quercetin experiment, 10 Crioulo horses with ages between 4 and 10 years old were used, semen collection were performed with artificial vagina and 3 ejaculates of each animal were obtained (totalizing 30 ejaculates). The samples were submitted to 5 different treatments (0; 0,25mM; 0,5mM; 0,75mM and 1mM) of quercetin added in the freezing medium before the cryopreservation process. The analyzes concerning kinect sperm parameters were assessed in a subjective manner by a trained person in phase-contrast microscope and the parameters related to sperm population viability were examined by flow cytometry. Statistical analysis made from data obtained did not showed significant difference between treatments and the control ($P > 0,05$). The progesterone addition experiment was performed in two phases; the first phase consisted in the realization of a sperm toxicity test to the different hormonal concentrations. Semen collection was also realized with artificial vagina in a total of 10 Crioulo horses in ages between 4 to 10 years; posteriorly semen samples were submitted to cooling process until reach 5°C and adding the treatments containing 7 different progesterone concentrations (0; 1; 2,5; 5; 10; 100 e 1000 ng/mL) in 3 different times of exposition (0,5h; 3h e 24h). The second phase consisted in the sperm cryopreservation from 15 Crioulo horses collected with artificial vagina and with ages between 4 to 10 years. The ejaculates undergone to 5 different concentrations of progesterone (0; 1; 5; 10 e 100 ng/mL) added to freezingmedium previously to the cryopreservation process. In both analysis concerning progesterone experiment, kinect parameters were obtained by the Computer Assistant Sperm Analysis (CASA), as well as the flow cytometer tests regarding viable sperm population were utilized. The statistical data showed that after the toxicity test, with cooled semen, a significant improvement ($P < 0,05$) with the concentrations of 5 and 1000 ng/mL for 3-hour of exposition in velocity and average distance sperm parameters (DAP, DCL, DSL, VAP VCL, VSL). There was a significant raising on acrosome reaction at the 24-hour exposition time with the 5 ng/mL concentration treatment and also a significant decrease ($P < 0,05$) of lipid peroxidation (LPO) in the concentrations of 10, 100 and 1000 ng/mL in the 24-hour time of exposition. Information collected from thawing samples demonstrated an impairment ($P < 0,05$) in the reactive oxygen species, however the other parameters

assessed did not differ from the control. From these data obtained in both experiments it is possible for us to conclude that the addition of quercetin to the freezing medium before the cryopreservation it is not interesting, once it does not presents significant improvements in sperm quality. On the other hand, the addition of progesterone to the fresh cooled semen demonstrated beneficial actions for the immediate use of semen, such as hyperactivation, capacitation and anti-oxidant effects.

Keywords: flavonoid; freezing; hormone; stallion; steroid; toxicity

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Sumário

1 Introdução

A equinocultura desempenha um importante papel na economia brasileira. A indústria do cavalo vem crescendo ao longo dos últimos 10 anos, em 2006 foram R$7,5 bilhões de faturamento bruto anual já em 2015 esse valor chegou aos R$16 bilhões de reais (CANAL RURAL, 2016). O rebanho brasileiro possui 5,5 milhões de equinos, sendo o quarto maior rebanho mundial ficando atrás dos Estados Unidos, China e México (IGBE, 2013). Em vista ao crescimento do setor é notório necessidade de investimentos na área de criação de equinos tanto para o melhoramento genético de cada raça, bem como investimento em pesquisas realizadas na área para melhorar a vida dos profissionais no campo.

Em relação a reprodução é de extrema importância ressaltar que diferentemente de outras espécies como bovinos e ovinos, que são selecionados para reprodução através de exames andrológicos (motilidade, morfologia, entre outros) os equinos são escolhidos, basicamente, pela aptidão à carreira atlética (provas equestres) ou morfologia. Essa afirmação reforça o que afirmam SULLIVAN et al (1975) e VOSS (1993), os equinos quando comparados as outras espécies domésticas são a espécie possuidora dos menores índices de fertilidade; sendo assim necessário aplicação de técnicas reprodutivas com o intuito de melhorar tais parâmetros.

O espermatozoide pode ser confundido como uma célula relativamente simples, uma vez que é composto basicamente por acrossoma, cabeça, peça intermediária e flagelo perdendo a maioria das organelas e do citoplasma durante o processo de maturação; entretanto realizar o processo da fecundação requer uma série de mudanças celulares e moleculares altamente específicas, fazendo com que o gameta masculino seja uma célula extremamente sofisticada e adaptável (VARNER et al., 2015).

Em relação as biotécnicas reprodutivas, em equinos, a inseminação artificial é a principal técnica realizada pelos profissionais da área (LOOMIS, 2006). Esta biotécnica pode ser realizada com sêmen fresco, resfriado ou ainda previamente congelado e descongelado no momento da aplicação no trato reprodutivo da fêmea;

destacando – se a vasta utilização do sêmen resfriado devido a sua facilidade de transporte. Em vista disso, os meios de diluição, manutenção e congelamento adicionados ao sêmen são de extrema importância, uma vez que essas soluções serão as responsáveis por proteger a célula espermática de eventos danosos como choque térmico, desidratação celular, criação de cristais intra e extracelulares.

Os processos de resfriamento e congelamento são complexos, necessitando de várias etapas para a correta realização; essa manipulação da célula espermática pode reduzir viabilidade e fertilidade devido a inúmeros fatores, mas entre os principais está a peroxidação lipídica da membrana plasmática causada pela excessiva produção e alta susceptibilidade do espermatozoide as espécies reativas de oxigênio (EROs). (GARCIA et al., 2011).

As grandes quantidades de ácidos graxos poli-insaturados presentes na membra plasmática da célula espermática equina são facilmente oxidados, tornando essa célula particularmente sensível a ação das espécies reativas de oxigênio. Além disso, o espermatozoide quase não possui citoplasma, consequentemente não possuindo quantidades intracelulares de agentes antioxidantes (FERREIRA et al., 1997).

Em adição a isso, uma das etapas mais importantes durante a manipulação do sêmen é a centrifugação – processo realizado para concentrar o número de espermatozoides – entretanto esse procedimento é responsável também por remover o plasma seminal, o que pode acarretar em um aumento na susceptibilidade doespermatozoide, uma vez que retira também as substâncias antioxidantes presentes no plasma (WAHEED et al., 2013).

Inúmeras substâncias têm sido adicionadas ao sêmen com o intuito de melhorar taxas de motilidade, viabilidade e diminuir a mortalidade celular durante a manipulação e criopreservação espermática. Várias substâncias relatadas na literatura apresentam efeito antioxidante e a quercetina é uma delas, um flavonoide natural amplamente encontrado em frutas, sementes, nozes e vegetais conhecida por possuir efeitos antioxidantes, anti-inflamatórios e antimicrobianos (GUARDIA et al., 2001). A sua ação antioxidante pode acontecer de três maneiras: pela diminuição na formação e sequestro dos radicais livres bem como, agindo como um quelante de íons metálicos (BEHLING et al., 2004).

Como um polifenol flavonoide sua fórmula molecular possui cinco grupamentos OH em sua estrutura (ALRAWAID E ABDULLAH, 2014); assim sendo,

o grande número de OH em sua estrutura, possui eficiente capacidade antioxidante, uma vez que possibilita a doação de até cinco elétrons e H+ às EROs, estabilizando-as (SILVA E GUERRA, 2012). Entretanto CAO et al., 1997; revela que os flavonoides podem possuir uma atividade pró-oxidante que está diretamente ligada ao número total de grupos hidroxilas presentes na molécula.

Outra substância que também foi adicionada ao sêmen e analisada os seus efeitos, no presente estudo, é a progesterona. Existem vários tipos de progestágenos, alguns exemplos são 5α-dihidroprogesterona, 17α-hidroxiprogesterona, 20α-tetrahidroprogesterona; entretanto a substância escolhida para ser utilizada na realização do experimento foi a progesterona (P4).

JINGCHUN LI et al., 2016 descreve esse hormônio como um forte estimulador do espermatozoide em mamíferos; outros estudos ainda relatam sua ação quimiotática podendo influenciar na migração espermática. CONTRERAS e LLANOS (2001) também demonstrou que a progesterona é capaz de estimular a capacitação espermática, hiperativação e iniciar a reação acrossomal em experimento conduzido com sêmen humano.

Em busca de novos conhecimentos e alternativas para tornar cada vez melhor e mais eficiente a manipulação espermática, ambas as substâncias citadas no texto foram adicionadas aos meios de manutenção e congelamento, em dois diferentes experimentos com o intuito de avaliar os efeitos e possíveis melhorias ao sêmen equino.

2 Artigos

2.1 Artigo 1

Quercetin in equine frozen semen

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QUERCETIN IN EQUINE FROZEN SEMEN

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Abstract

BACKGROUND: Supplementation of sperm diluents to diminish the damage caused by freezing process is broadly used in equine semen cryopreservation. **OBJECTIVE:** The present study aim is to measure the most appropriated quercetin concentration to be added into the dilution and frozen medium able to promote the maintenance of kinetic and equine sperm viability during the freezing process. **MATERIALS AND METHODS:** The present study added four different concentrations of quercetin into sperm freezing diluent before freezing-thawing process, spermatozoa population was analyzed by flow cytometry and a statistic study was conducted to detect significant differences between the control and treatments. **RESULTS:**Statistical analysis did not show any significant improvement or worsening on seminal parameters. **CONCLUSION:** In conclusion, within the utilized concentration, quercetin supplementation in equine freezing extender did not affected progressive motility, mitochondrial functionality, acrosome reaction, membrane integrity or DNA fragmentation index in post-thawed equine semen.

Keywords: Antioxidant, ATP, spermatozoa, ROS, flavonoid.

INTRODUCTION

Artificial insemination with frozen semen continues to increase in importance and popularity between owners and equine practitioners; however, conception rates with thawed frozen semen are lower compare to fresh or cooled semen (32). Sperm from a large number of the stallion population cannot be use for freezing because of unsatisfactory post-thaw spermatozoa quality and fertility rates (2).

Post-thaw motility and viability are reduced due to membrane damage caused by osmotic and oxidative stress during the process of freezing and thawing (33); Furthermore, a large number of variations in semen quality after the freezing process, thawing process and varying pregnancy rates after insemination occur between different stallions and ejaculates (23). According to DI SANTO (12), some damaging events such as cellular dehydration, thermal shock with intracellular and extracellular ice formation, and osmotic stress have been claimed to be the main insults occurring during cryopreservation.

As well as all cells of the body, spermatozoa demands oxygen to promote the oxidative metabolism of biological molecules. Such metabolism results in the generation of highly reactive oxygen species (ROS). The formation of ROS is a normal consequence of oxidative metabolism playing an essential and physiological role in the induction of vital sperm functions. Nevertheless, sperm centrifugation employed before freezing, to remove seminal plasma and concentrate spermatozoa, removes antioxidants present in semen, exposing spermatozoa to excessive ROS damage (5).

The damage chilling and freezing process cause to sperm cells that leads to cell death and a loss of fertility are relate to loss of membrane integrity, motility, poor mitochondrial functionality, DNA fragmentation, early acrosome reaction, among others.

One of the most toxic forms of ROS accepted is the hydrogen peroxide (H_2O_2), the action in mammalian semen is due to its ability to diffuse along both intracellular compartments and penetrate biological membranes (1). Due to a high content of polyunsaturated fatty acids, the sperm plasma membrane, in particular, is more susceptible to peroxidative damage.

Endogenous antioxidants protective capacity may be insufficient to prevent peroxidative damage during storage (4). For that reason, various antioxidants have been investigated in a large number of species as supplementation for semen diluents and the results show that antioxidants prevents the harmful effects of ROS on DNA integrity, motility, viability and acrosome integrity.

In the literature, a large number of substances with anti-oxidant action are describe, and quercetin is one of them; a natural non-enzymatic anti-oxidant belonging to the flavonoid group, widely found in fruits, vegetables, seeds, nuts, white wine (22). Quercetin is also well known for its many biological activities including antioxidant, anti-inflammatory (16), and antimicrobial properties. In the diary human diet quercetin consumption vary between 5 to 500 mg, making the 3, 5, 7, 3’-4’- pentahidroxi flavon the main flavonoid consumed by humans.

This compound can be found in red wine, black tea, beer, fruits (apple, grape, and strawberry), vegetables (onion, cabbage, broccoli), seeds and nuts; this great diversity makes quercetin a reliable choice to be tested as an anti-oxidant additive in thaw semen. BEHLING et al., (6) showed that the flavonoids have an effective anti-oxidant action due to its free radicals scavenge properties and for chelation of metallic ions, protecting the tissues from ROS and from the lipid peroxidation.

The anti-oxidant effect is focused on the hydroxyl radical (•OH) and to superoxide anion (•O2-) wich are highly reactive species involved in lipid peroxidation. Substitutions, the presence and location of the hydroxyl group (•OH), and the catechol-type B-ring make quercetin an effective antioxidant with higher ROS scavenger activity than vitamin E or C (29).

There are three different ways that quercetin is able to inhibit the free radical formation process, at its beginning (interaction with superoxide ions), at hydroxyl radical’s formation (metal chelation) and at the lipid peroxidation (reaction with lipid peroxi radicals) (6). Calcium influx regulation to sperm is another mechanism proposed which maintains internal adenosine triphosphate (ATP) concentration, avoiding acrosome reaction and premature capacitation (21) during sperm storage, improving sperm

longevity by saving spermatozoa limited energy content to be released into female genital tract.

We hypothesized the anti-oxidant effect of quercetin is beneficial to stallion spermatozoa when added in dilution and frozen medium, improving post-thaw equine semen parameters. That being said, the aim of this study was to measure the most appropriated quercetin concentration to be added into the dilution and frozen medium able to promote the maintenance of kinetic and sperm viability during the freezing process. The effect of four different quercetin concentrations (chosen based in data content in the literature) on membrane integrity, DNA fragmentation index, mitochondrial functionality and acrosome reaction in post-thaw equine semen were measured to confirm the sperm viability.

MATERIALS AND METHODS

Animal and semen collection

The experiment used animals in the experimental model had the approval of the Institutional Research Ethics Committee of Federal University of Pelotas (UFPel) by the number 1946.

For this experiment, 10 Crioulo horses aged 4 to 8 years were used during breeding season. Three ejaculates from each stallion (n=30) were collected using a teaser mare and Botucatu artificial vagina (Botupharma Ltda, Botucatu, São Paulo, Brazil).

Sperm analysis

Ejaculates were diluted in KENNEY (18) base extender at concentration of 50x106 spermatozoa/mL. Sperm 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 America, Inc., São Paulo, SP, Brazil).

Morphology was assessed in a slide covered with a coverslip in a phase-contrast microscope at 1000x magnification. Ejaculates with $\geqslant$ 70% motility and morphologically normal sperm were used.

Freezing process

Semen samples were centrifuged at 600g for 20 minutes. The pellet was resuspend in the freezing extender by POJ PRASATH et al. (26) modified (86mM Glicose; 3,2mM Tri-sodium Citrate; 2,5mM Di-sodium EDTA; 4mM Sodium Hydrogencarbonate; 0,25g Streptomycin; 50mL Lactose; 20mL egg yolk; 4mL dimetilformamide) at a final concentration of 100 million sperm/mL.

After, the treatments were submitted to: control (only freezing extender); addition of 0.25mM; 0.5mM; 0.75mM and 1mM of quercetin. The semen samples were packed into 0.5-mL straws, maintained at 5ºC for 20 minutes in a refrigerator (Minitub® 518C) and subsequently frozen in liquid nitrogen vapor, 6cm above the surface of liquid nitrogen, for 20 minutes. The straws were immersed in liquid nitrogen and stored at -196ºC until analysis. The thawing process was perform immerging the straws in water bath at 37ºC for 30 seconds and samples were immediately evaluated.

Parameters analyzed included progressive motility by phase-contrast microscopy and by flow cytometry were measured membrane integrity, mitochondrial functionality, DNA fragmentation index and acrosome reaction.

Analysis by flow cytometry

Attune Acoustic Focusing Cytometer® (Applied Biosystems) was used to perform flow cytometry. All cell analysis was stained with Hoechst (H33342), the population was detecting by photodetector VL1 (filter 450/40) and the results were assessed by Attune Cytometric Software v2.1. The carboxyfluorescein diacetate (membrane integrity); rhodamine 123 (mitochondria functionality) were detected by a BL1 photodetector (filter 530/30).

The red fluorescence used to measure membrane integrity, released by propidium iodide, was evaluated with the photodetector BL3 (640LP filter). Twenty thousand events were analyzed per sample at a flow rate of 200 cells/s. The debris (non-spermatozoa particles) were eliminate based on scatter plots (25).

Sample preparation

For all evaluations were added an aliquot of 10 µL of treated semen plus the respective fluorescent probe. The samples were incubated during 10 min in room temperature, after that 20 µL of Hoerchst 33342 solution (10mg/mL) was add to determine spermatozoa population and specific fluorescent dyes.

Posteriorly, 1 mL of Attune® Focusing Fluid (Life Sciences Solutions) were added to the sample and then brought to be analyze.

Membrane integrity

Carboxyfluorescein diacetate fluorescent (DCF) probes and propidium iodide (PI) were used to assess membrane integrity. DCF penetrates spermatozoa and is convert into a non-permeable fluorescent compound that retain in the cytoplasm, on the other hand IP only stain the nucleus of cells with injured membrane (3). Thus, were considered to have an intact plasma membrane, only gametes marked with DCF (13).

Mitochondrial functionality

Performed with rhodamine 123 fluorescent probe that stain active mitochondria. The more active is the mitochondria the higher is the green fluorescence (27). The spermatozoa were classified as high functional mitochondria (intense green fluorescence) or poor functional mitochondria (slight green fluorescence) (15).

DNA fragmentation index

DFI was evaluate using three probes combined. In a sample containing 10 µL of semen, the first probe add was 5 µL of TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2), the second was 10 µL of Triton (Triton X – 100, 0.1%) and finally, acridine orange was add only immediately before reading the sample (3).

Acrosome reaction

The acrosome integrity was estimated staining by the cells with propidium iodide (PI) and FITC-PNA. In this analysis, if the number of cells with acrosome reaction is high, worse is the results since - for the spermatozoon - an intact acrosome is essential to penetrate the oocyte pellucid zone.

Statistics

All variables were distributed according to the Shapiro-Wilk test. Data were evaluated by descriptive statistics, ANOVA analysis was performing by non-parametric measures with Kruskal-Wallis One-Way AOV, and posteriorly Dunn's All-Pairwise Comparison test was made. The program Statistix 10® (Statistix. Statistix 10 for Windows. Analytical Software, Tallahassee, FL, USA, 2014) was used for statistical analyses. Significance was assign to all values $p < 0.05$.

RESULTS

Data demonstrated in table 1 showed no significant differences ($p<0.05$) among quercetin concentrations and control.

Treatment concentrations correlated (Pearson) with progressive motility R=0,25 (p=0,01) and with acrosome reaction R=0,29 (p=0,003). Besides that, mitochondrial functionality correlated itself positively with acrosome reaction R=0,21 (p=0,03), DFI R=0,34 (p=0,0006) and negatively with cell integrity R= - 0,46 (p=0,0001) and that had direct correlation with membrane functionality R=0,99 (p=0,00001). In addition, the acrosome reaction had a direct correlation with DFI R=0,20 (p=0,04).

Table 1: Effects of different concentrations of quercetin (0 mM; 0.25 mM; 0.5 mM; 0.75 mM and 1mM) on Progressive motility, mitochondrial functionality, acrosome reaction, DNA fragmentation index (DFI), cell integrity and membrane functionality of the post-thaw equine semen.

	Quercetin Concentrations				
Evaluations	**0 mM**	**0,25 mM**	**0,5 mM**	**0,75 mM**	**1 mM**
Motility	17.3 ± 3.4[a]	9.6 ± 2.0[a]	10.9 ± 2.3[a]	4.9 ± 1.1[a]	7.9 ± 1.7[a]
Mitochondrial	24.1 ± 4.7[a]	22.7 ± 4.3[a]	15.7 ± 3.3[a]	25.9 ± 5.5[a]	23.9 ± 5.1[a]
Acrosome	24.4 ±4.8[a]	28.9 ±5.5[a]	11.4 ± 2.4[a]	27.5 ±5.9[a]	27.8 ± 5.7[a]
DFI	0.10 ± 0.2[a]	0.09 ±0.01[a]	0.10 ±0.02[a]	0.09 ±0.02[a]	0.09 ±0.01[a]
Cell Integrity	14.8 ± 3.0[a]	17.1 ± 3.7[a]	25.6 ± 5.7[a]	13.9 ± 3.0[a]	8.5 ± 1.9[a]
Membrane	14.6 ± 3.0[a]	16.6 ± 3.5[a]	25.4 ± 5.7[a]	13.8 ± 3.0[a]	8.6 ± 1.9[a]

Data presented in table 1 are correspondent to values of arithmetic mean followed by standard deviation. Values with $P < 0.05$ were considered statistically significant. Motility = Progressive Motility; Mitochondrial = Mitochondrial Functionality; Acrosome = Acrosome Reaction; DFI = DNA Fragmentation Index; Membrane = Membrane Integrity.

DISCUSSION

Since it is notorious that semen processing and manipulation, such as centrifugation and cryopreservation, may generate reactive oxygen species (ROS), many studies have examined the effect of *in vitro* antioxidant supplementation in protecting sperm from oxidative damage against LPO (10) or DNA (35). The present study used quercetin – a flavonoid family compound well known for its many biological activities – added to the semen sample before cryopreservation in four different concentrations to evaluate if the supplementation is beneficial to equine post-thaw semen parameters.

Quercetin is probably the most studied flavonoid compound in the male animal reproductive system, and it has been demonstrated to act against induced oxidative stress *in vivo* (31, 9) and *in vitro* (7). ROS and LPO (Lipid Peroxidation) analysis were not made in this experiment because the principal interest was to observe how this anti-oxidant action affects the other seminal parameters.

The evaluations employed in this experiment to measure the efficiency of quercetin in ameliorate thawed semen were motility, mitochondrial functionality, acrosome reaction, DNA fragmentation index, cell integrity and membrane integrity.

The study demonstrated no statistical differences ($p < 0.05$) between the concentrations analyzed. Overall, the considered antioxidant did not affect progressive motility or any other parameters analyzed whatsoever.

SEIFI-JAMADI et al. (28), in his study, also did not observed improvement in frozen-thawed equine sperm quality, except for motility and progressive motility. Beyond that, in higher concentrations (0,2 mM and 0,3 mM), the same author, reported a negative effect for progressive motility, viability and acrosome integrity. In another experiment, human semen was supplemented with quercetin and revealed a decreased in sperm motility and viability due to inhibition of Ca2+

- ATPase activity, a key enzyme related with sperm motility and male fertility (19).

According to DECKER (11) pH variations of the biological tissues is able to influence on the anti-oxidant flavonoid effect. More than that, the phenols, among them the flavonoids, because of its metal chelation action, may act as pro-oxidant substances as well. This pro-oxidant activity, presented by flavonoids is directly proportional to the number of hydroxyl groups found in the molecule (8); that being said, the information makes us conclude that the same structural attribute suggested to improve the anti-oxidant ability is able to exacerbate the oxidative stress, functional and structural cellular damage.

Divergent results in experiments with quercetin could be attributed to many factors such as different species, dosages, extenders, method of administration, which all of these coefficients may affect the anti-oxidant action. GIBB et al. (14) tested the anti-oxidant effect in post-thaw equine semen incubated at 37°C, during 3h, with a treatment of 0.15 mM of quercetin, however did not obtain significant improvement; yet the total motility percentage of post-thaw semen with this treatment were significantly higher ($P<0.05$) than the others. Besides that, intact acrosome percentage with quercetin supplementation were significantly higher ($P<0.05$) than those in the presence of catalase and cysteine.

Despite the numbers demonstrated no statistical difference between the treatments (table 1) in the present experiment, we believe the anti-oxidant effect of quercetin in equine semen cryopreservation may be dose dependent. SEIFI-JAMADI et al., (28), also related that in his study, lower viability and membrane integrity at higher concentrations of quercetin might be explained by quercetin pro-oxidant abilities.

Data presented in this experiment showed a positive correlation between mitochondrial functionality, acrosome reaction and DNA fragmentation index, which means, basically, that higher mitochondria produce ATP, higher occur cellular damage due to ROS production. Due to its chemical structure, OLIVEIRA et al., (24) manifested that quercetin reacts directly with mitochondrial membranes and components of the mitochondrial electron transport chain (METC), affecting the generation of ATP and the dynamics of the mitochondrial permeability transition pore (MPTP).

In LANG AND RACKER, (20) experiment, they showed that quercetin may act inhibiting mitochondrial ATP synthase (both soluble and particulate), which leads to a decreasing rates of ATP – dependent nicotinamide adenine dinucleotide (NAD+) reduction by submitochondrial particles (SMP, i.e., mitochondrial membranes whose components of the METC face the medium) isolated from beef heart.

The previously information induce us to conclude that quercetin have an anti-oxidant action not only because its ROS scavenger's effects, but as well as inhibiting the cellular metabolism acting directly on mitochondrial ATP production; showing that a poor mitochondrial functionality during the cryopreservation process could be interesting to lower the spermatozoa damage. A suggestion to verify this hypothesis is analyzing thawed semen parameters with the presence of quercetin and posteriorly wash the quercetin from the sample and reanalyze to evaluate if the mitochondrial functionality is getting back to normal criteria.

Another important parameter that determine sperm viability is DNA fragmentation index. In the present study DNA values had an inverse correlation with cellular integrity, which means that a low DNA fragmentation can be obtained through membrane protection. There are a few locals in the DNA molecule where quercetin could bind to such as the minor groove, major groove, between the two base pairs or outside of the helix by electrostatics (17). ZHANG et al. (34) also described that polyphenols can repair DNA damage caused by H2O2; showing another quercetin way of action, although we cannot conclude for sure which one (repair DNA or preventing from ROS damage) affected the DNA fragmentation in the present experiment.

The authors believe that the main DNA protection provided by quercetin occur outside the sperm and does not come in contact to genetic material. This hypothesis is taken into account because SUN et al., (30) showed in *in vitro* assays a low mutagen activity action depending on the dose at which quercetin is utilized.

In conclusion, within the utilized concentration, quercetin supplementation in

equine freezing extender did not affected improving or worsening the seminal parameters analyzed such as progressive motility, mitochondrial functionality, acrosome reaction, membrane integrity or DNA fragmentation index in post-thawed equine semen. In addition to it, more experiments should be done to elucidate its main action mechanism of quercetin to counteract ROS effects on the spermatozoa.

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2.2 Artigo 2

Effects of progesterone (P4) in equine semen

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EFFECTS OF PROGESTERONE IN EQUINE SEMEN (P4)

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ABSTRACT
The experiment was divided in toxicity containing seven different progesterone concentrations (0; 1; 2,5; 5; 10 and 1000 ng/mL) exposed to three different periods (0,5; 3 and 24 hours) and cryopreservation with the addition of five concentrations (0, 1, 5, 10 and 100 ng/mL). In both phases, Computer Assisted Sperm Analysis assayed the kinect parameters and flow cytometry were used to analyze sperm population. Results showed 5 and 1000 ng/mL treatments in the 3-hour exposition a significant statistically ($P < 0,05$) increase in distance and velocity parameters. Acrosome reaction increases statistically ($P < 0,05$) over time, exhibiting higher acrosome reaction at 24-hours exposition with the 5 ng/mL. Concentrations of 10; 100 and 1000 ng/mL at 24-hour exposition showed statistically ($P < 0,05$) lower values of Lipid Peroxidation when compared to the control group (0 ng/mL); ROS Total after thawing at the concentration of 5 ng/mL presented significant ($P < 0,05$) reduction compared to the 0 ng mL concentration group. The authors conclude is not recommended that the addition of progesterone to cryopreservation process; however, it is beneficial to the utilization on fresh, cooled and thawed semen at the moment of the artificial insemination, once promotes sperm acrosome reaction, hyperactivation and anti-oxidant effect.

Keywords: Stallion; Hormone; Capacitation; Steroid; Toxicity; Cryopreservation.

1. Introduction

Artificial insemination is the most commonly reproductive technique used in equine industry worldwide, this method can be perform with fresh, cooled or frozen semen. Therefore, the semen quality is a crucial part of the breeding horse programs [21]

One thing must be notice, and that is the method utilized to choose which stallion will be put to breed. Bulls, rams and other species with better post-thawing and fertilization rates are chosen by it's reproductive capacity measure by andrology tests; on the other hand, horses are chosen to reproduce basically by it's athletic life or morphology. That being said, strong

variations between different stallions in semen quality during and after the cryopreservation process and the pregnancy rate after artificial insemination remains as a critical point to the equine practitioners.

Sperm cryopreservation is a complex process that requires many steps to be performed correctly (semen collection, first dilution and centrifugation, second dilution on freezing extender, cooling, freezing and thawing). This technique continues to increase in importance and popularity between breeding associations and horse owners, due to its many advantages such as reducing sexual transmitting diseases, eliminating geographic barriers and maintenance, for a theoretical unlimited period of time, the genetic material of superior stallions.

The equine spermatozoa is susceptible to many injuries that can occur during the freezing process and - in particular - to oxidative stress. The formation of reactive oxygen species (ROS) is a physiological consequence of the oxidative metabolism; however, the high amount of PUFAs (Polyunsaturated Fatty Acids) within the membrane of the equine sperm cell makes it even more responsive to damage. According to [1], the oxidative stress is one of the major causes associated with reduced sperm motility, low plasma membrane integrity, mitochondrial function alteration [7], DNA alterations and acrosomal membrane injuries.

That being said, it becomes logical that an adequate freezing extender is a crucial part to the success of the freezing process. The main goals to semen extenders are secure the sperm cell from thermal shock, avoid osmotic stress and any damage than can be caused to the membrane fluidity, permeability and provide energy and substrate to the spermatozoa metabolism [13].

According to [3] loss of membrane fluidity and abnormal acrosome reaction can be caused by lipid peroxidation (LPO), following in decrease of sperm fertilization capacity. According to [8], mitochondrial dysfunction is one of the most causes of ROS formation; these insults embrace poor mitochondrial respiration rates, mitochondrial ROS production growth and oxidative harm to DNA, RNA, proteins and lipids [24].

After the freezing process and all the challenges and risks this event put the sperm cell to, it must be able to maintain its fertility capacity. Inside the female reproductive tract the spermatozoa undergo thru events like sperm capacitation, hyperactivation, acrosome reaction and fecundation. Sperm capacitation is a complicated process that includes biochemical alterations in membrane lipids and proteins, enhance in cyclic AMP level, ionic influxes and protein phosphorylation [28].

The progesterone is a strong stimulator of the mammalian spermatozoa; this hormone is release by the cumulus cells surrounding the oocyte and attracts the sperm towards the female gamete [9]. Experiment conducted in humans by [17] have showed that hormones such as progesterone and ANP (atrial natriuretic peptide) are able to influence the sperm migration.

Studies have shown that progesterone is able to enhance sperm functions that are crucial to fertilization process such as sperm capacitation, zona pellucida binding, hyperactivation and acrosome reaction [23]. Thru the pathway inside the female reproductive tract until the fecundation site, the spermatozoon population have been exposed to high levels of progesterone. Therefore, this steroid may play an important role in the selection of the spermatozoon capable of a successful fecundation among the entire population [6].

Furthermore, [14] suggested, in an experiment made with human semen, the existence of a non-genomic progesterone functional receptor that could be dependent to the integrity of plasma membrane and normal morphology.

Holding all that information, we hypothesized that the addition of progesterone to stallion semen could be beneficial to enhance the cell viability and fertility after cryopreservation. The experiment were divided in two phases; the first phase was realized toxicity process and the second phase was the cryopreservation process. In both phases,CASA Software assessed the kinect evaluations and sperm population parameters were analyzed by flow cytometry. The aim of this study was to determine the effect and the correct concentration of progesterone addition into the dilution and frozen medium capable of maintain and enhance sperm motility and viability during the toxicity and freezing manipulation.

2. Material and Methods

2.1. Animalsand semen collection

In this experiment, 25 Crioulo horses were used (10 to toxicity experiment and 15 to freezing experiment) ages between 4 to 10 years. The semen collection occurred between the months of May to August to the toxicity experiment and during the breeding season over the months of September to December to freezing study. One ejaculate from each horse were collected, totalizing 25 ejaculates. The semen were obtain using a teasing mare and Botucatu artificial vagina (Botupharma Ltda, Botucatu, São Paulo, Brazil).

2.2. Sperm analysis

After the semen collection, the sample was diluted 1:1 in [10] Skim-Milk Glucose extender (Instant Nonfat Dry Milk 2,4g; Glucose 4,9g; 2 mL Sodium Bicarbonate stock sol. = 7,5g/100mL; 94 mL Distilled Water); immediately posterior the semen collection, a first sperm motility test were performed in heated slide covered with a coverslip in phase-contrast microscopy at 200x magnification (BX 41 Olympus America, Inc., São Paulo, SP, Brazil) assessed by a single trained person.

After the sample transportation to the lab, the sperm motility were, once again, evaluated this time by the AndroVision®: CASA Software with PC and monitor (Minitube); only ejaculates with ≥ 70% motility sperm were used.

2.3. Toxicity process

This process consists in test the toxicity level of Progesterone (Sigma – Aldrich® P0130) to sperm of seven different P4 concentrations in three different times of exposition (30 minutes, 3 hours and 24 hours). In this experiment, the samples were divided in seven treatments with specific concentrations; control treatment (no hormone, only semen extender), treatment 1 (1 ng/mL), treatment 2 (2,5 ng/mL), treatment 3 (5 ng/mL), treatment 4 (10 ng/mL), treatment 5 (100 ng/mL) and treatment 6 (1000 ng/mL).

Once the sperm analysis is done and approved (<70% total motility), the samples were centrifuged at 600g for 10 minutes. The pellet was ressuspended in maintenance extender (Skim-Milk Glucose) to the concentration of 200.10^6 sptz/mL; after that 250 µL of semen were pipetted into seven different eppendorfs and taken to be cooled in the Minitube ® refrigerator at 5°C during 1 hour. After cooling the semen, 250 µL of each treatment containing double of the progesterone desirable concentration – also at 5°C – were added to the eppendorfs with the cooled semen making a 1:1 dilution for both semen and treatment concentration. At the final dilution, the semen concentration reached 100.10^6 sptz/mL and the treatment reached its desirable concentrations, previously explained.

After each exposition time (30 min, 3 hours and 24 hours), the semen were immediately analyzed; the parameters included total and progressive motility, DAP (distance average path, µm), DCL (distance curved line, µm), DSL (distance straight line, µm), VAP (Average Path Velocity, µm/s), VCL (Curvilinear Velocity), VSL (Straight Line Velocity), STR (Straightness, VSL/VAP, %), LIN (Linearity), WOB (wobble, VAP/VCL, %), ALH

(Amplitude of Lateral Head), BCF (Beat Cross Frequency) assayed by the Computer Assisted Sperm Analysis (AndroVision®). Flow cytometer Attune Acoustic Focusing Cytometer® were used to measure reactive oxygen species (ROS), lipid peroxidation (LPO), membrane integrity, membrane fluidity, mitochondrial functionality, acrosome reaction, only DNA fragmentation index were assay after the 24h exposition time.

2.4. Freezing process

The freezing process consists in analyze the effects of the addition of five different concentrations of Progesterone (Sigma – Aldrich® P0130) to the semen frozen medium. In this experiment, the samples were divided in 5 treatments with specific concentrations; control treatment (no hormone, only frozen semen extender), treatment 1 (1 ng/mL), treatment 2 (5 ng/mL), treatment 3 (10 ng/mL), treatment 4 (100 ng/mL).

Once the sperm analysis is done and approved (<70% total motility), the samples were centrifuged at 600g for 10 minutes. The pellet was ressuspend in fraction A (NaCitrate.2H2O 1,856g; Glucose 1g;water and egg yolk 20%) of the Egg Yolk Citrate freezing extender to the concentration of 250.10^6 sptz/mL. After that, semen were pipetted into five different eppendorfs and taken to be cooled in a cold chamber at 5°C during 1 hour and 30 min (cooling curve of -0,5°C / min) plus 30 minutes at 5°C to stabilize the sperm cell.

After cooling the semen, each treatment (without glycerol) were added, also at 5°C to prevent thermal shock; than the treatments were let to stabilize during more 30 minutes; the fraction B freezing extender (Egg Yolk Citrate) containing of glycerol were added to each treatment to the final concentration 5% of glycerol and let do stabilize again for 1 hour.

Posteriorly to stabilization, semen samples were packed into 0,5 mL strawsand subsequently disposed at 6cm above the surface of liquid nitrogen, for 20 minutes. The straws were immersed in liquid nitrogen and stored at -196ºC until analysis. The thawing process was perform immerging the straws in water bath at 37ºC for 30 seconds and samples were immediately evaluated. Parameters analyzed were the same assessed in the toxicity process (CASA and Flow Cytometry).

2.5. Analysis by flow cytometry

Attune Acoustic Focusing Cytometer® (Applied Biosystems) were used to perform the assessment by flow cytometry. The results were analyzed by Attune Cytometric Software v

2.1; cells, in all analysis, were stained with Hoechst (H33342) and the population was detect by photodetector VL1 (filter 450/40).

The red fluorescence issued from propidium iodide (membrane integrity) was evaluated by the photodetector BL3 (640LP filter). The carboxyfluorescein diacetate (plasma membrane integrity, reactive oxygen species); rhodamine 123 (mitochondria functionality) were read with a BL1 photodetector (filter 530/30). Twenty thousand events were analyzed per sample at a flow rate of 500 cells/s. The non-spermatozoa debris were eliminate based on scatter plots [18].

2.5.1. Sample preparation

For all the evaluation process an aliquot of 5 µL of treated semen were added plus the respective fluorescent probe. After that, the samples were incubated during 10 min in dry bath at 37°C; 20 µL of Hoerchst 33342 solution (10mg/mL) was add and let to remain in contact with the sperm for 1 min in all samples after the incubation time to determine spermatozoa population and specific fluorescent dyes. That preparation process were used in both toxicity and freezing experiments, with only a few differences to ROS and LPO evaluations.

During the toxicity analyzes ROS and LPO were assayed twice; ROS were analyzed once with 10 min in dry bath and a second evaluation occurred after 1 hours of incubation; LPO were analyzed also with after 10 min in the dry bath, but the second evaluation were made after 2 hours of incubation. In the thawing process, ROS and LPO samples were incubated during 15 min, five minutes longer than the other samples.

Posteriorly, 1,5 mL of Attune® Focusing Fluid (Life Sciences Solutions) were added to the sample and then brought to be analyze.

2.5.2. Reactive Oxygen Species (ROS)

Reactive oxygen species are produce only by living cells; the concentration of this free radical were detected and measure using carboxyfluorescein diacetate fluorescent (DCF) and propidium iodide (PI) probes. DCF emits a green fluorescence when oxidized and PI emits a red fluorescence. The calculation of ROS values were reached by the median intensity of green fluorescence used only to measure live sperm (negative PI) [20].

2.5.3. Lipid Peroxidation (LPO)

LPO basically consists, in the amount of sperm cells that were affected by the ROS production. In this analysis, sperm was assayed adding 1 µL of Bodipy into 5 µL of sperm sample. Lipid peroxidation rate was reached by the median of the green fluorescence intensity (peroxidized lipid)/median of the green fluorescence intensity plus median of the red fluorescence intensity (non-peroxidized lipid) x 100 [20].

2.5.4. Membrane integrity

The membrane was assess with carboxyfluorescein diacetate fluorescent (DCF) probes and propidium iodide (PI). DCF penetrates spermatozoa and converted in a non-permeable fluorescent compound that retain in the cytoplasm, whereas the IP only penetrates the nucleus of cells with injured membrane [2]. Thus, only gametes marked with DCF were considered to have an intact plasma membrane [19].

2.5.5. Membrane Fluidity

Membrane fluidity was determined using merocyanine 540 (M540) – a hydrophobic fluorescent dye – and propidium iodide (PI). In this evaluation, spermatozoa were divided into high fluidity and low fluidity; that was determined by the fluorescent intensity, the higher is the fluorescence higher is the fluidity [2]

2.5.6. Mitochondrial functionality

Performed with rhodamine 123 fluorescent probe that stain active mitochondria. The more active is the mitochondria the higher is the green fluorescence (SILVA et al., 2016). The spermatozoa were classified as high functional mitochondria (intense green fluorescence) or poor functional mitochondria (slight green fluorescence) [12].

2.5.7. Acrosome Reaction

The acrosome integrity was estimated staining by the cells with propidium iodide (PI) and FITC-PNA. In this analysis, if the number of cells with acrosome reaction is high, worse

is the results since - for the spermatozoon - an intact acrosome is essential to penetrate the oocyte pellucid zone.

2.5.8. DNA fragmentation index

DFI was evaluate using three probes combined. In a sample containing 10 µL of semen, the first probe add was 5 µL of TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2), the second was 10 µL of Triton (Triton X – 100, 0.1%) and finally, acridine orange was add only immediately before reading the sample [2]

3. Statistics

All variables were distributed according to the Shapiro-Wilk test. Data were evaluated by descriptive statistics, ANOVA analysis and posteriorly all means were assayed by the LSD Test. Cytometric data were also evaluated its correlations by the Pearson Test. The program Statistix 10® (Statistix. Statistix 10 for Windows. Analytical Software, Tallahassee, FL, USA, 2014) was used for statistical analyses. Significance was assign to all values $p < 0.05$.

4. Results

When assessed total and progressive motility, during the toxicity phase, until the concentration of 10 ng/mL for 3-hour exposition time, progesterone demonstrated no toxic effect to the sperm cell; however none of the concentrations tested avoided the decrease of this two variables in the 24-hour exposition period. Throughout the periods of time analyzed (0,5h; 3h and 24h) the control group did not showed any significant difference in the CASA software variables such as DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, ALH and BCF.

On the other hand, 5 and 1000 ng/mL concentration treatments in the 3-hour exposition time occurred a significant statistically ($P < 0,05$) increase in DAP, DCL, DSL, VAP, VCL, VSL parameters (table 3; table 4; table 5; table 6; table 7; table 8, respectively).

After freezing and thawing process, the CASA software also analyzed kinect cell parameters. In this assay, the 5 ng/mL concentration was lower ($P < 0,05$) than the control group in total motility, progressive motility, DCL and VSL; the other parameters obtained showed no significant difference among the treatments ($p > 0,05$) (table 14 and table 15).

The sperm cell population were also analyzed by flow cytometry; during the toxicity phase, progesterone in the concentration of 1000 ng/mL promoted acrosome reaction since the first contact (0,5h) demonstrating higher levels statistically significant ($P < 0,05$) than the other treatments with and without the hormone addition. In addition to that the 5 ng/mL progesterone treatment revealed that acrosome reaction increases statistically ($P < 0,05$) over time, exhibiting higher acrosome reaction at the 24-hours exposition time (Table 14).

Our data presented statistically lower ($P < 0,05$) values at membrane fluidity with the 5 ng/mL concentration compared with the treatment without the addition of the steroid hormone; besides that as well as the acrosome reaction, the membrane fluidity values raised with time. The membrane integrity assay produced data bringing forward that the 5 ng/mL concentration had statistically superiority than the control group ($P < 0,05$) and the same treatment also promoted membrane protection over the times stipulated.

Progesterone treatment effects proved at the concentrations of 10; 100 and 1000 ng/mL at 24-hour exposition time statistically ($P < 0,05$) lower values of Lipid Peroxidation (LPO) when compared to the control group (0 ng/mL). Mitochondrial functionality and Reactive Oxygen Species (ROS) reviews manifested no important alterations in relation to time and utilization of progesterone ($p > 0,05$) on the stallion sperm cell.

CASA data obtained after thawing demonstrated a significant reduction in total and progressive motility (Table 16). Flow cytometry analysis made after thawing the semen demonstrated statistically significant values only in ROS Total and ROS Viable variables. ROS Total corresponds median value of all sperm cells capable of produce reactive oxygen species and ROS viable corresponds only to viable cells (priopidium iodate negative). Our data showed that in ROS Total the concentration after thawing of 5 ng/mL presented significant ($P < 0,05$) reduction compared to the 0 ng mL concentration group; instead in both variables the concentrations 10 and 100 ng/mL had significant ($P < 0,05$) lower production of ROS than the control group (Table 17).

With the addition of progesterone the authors realized that membrane integrity had a positive correlation with viability ($r = 0,45$), in other words it means that both variables increase or decrease proportionately, which can be noticed in figure 1. The viability reduction is related to lipid peroxidation increment ($r = - 0,10$); acrosome reaction gain ($r = -0,16$); as well as the raise of membrane fluidity ($r = -0,42$). Acrosome reaction is directly correlated to Reactive Oxygen Species ($r = 0,14$). DFI (DNA Fragmentation Index) were assayed only at the 24-hour exposition time, however no significant differences ($P < 0,05$) between the treatments were demonstrated.

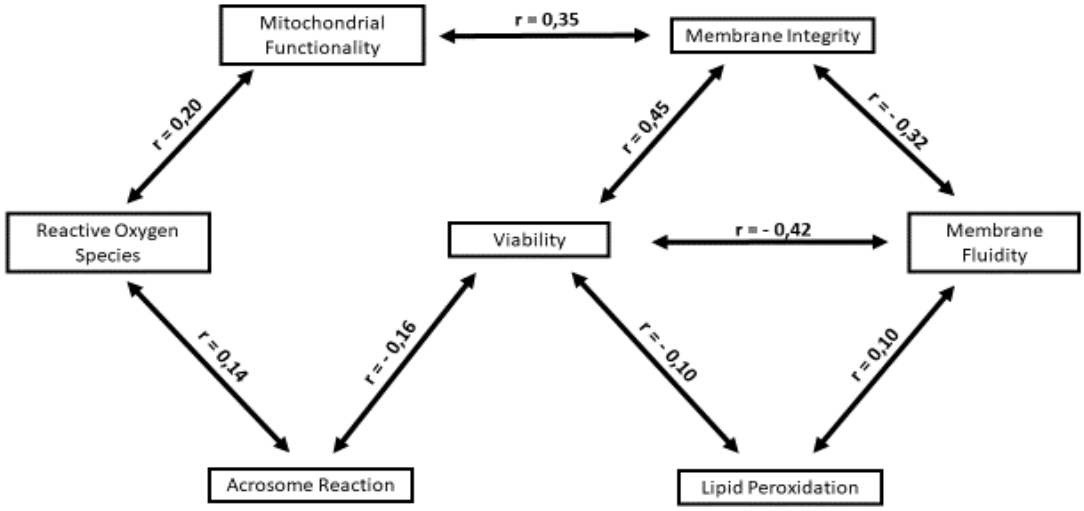

Figure 1: Image demonstrating significant (P < 0,05) correlations (Pearson) between Viability, Mitochondrial Functionality, Reactive Oxygen Species, Membrane Integrity, Acrosome Reaction, Lipid Peroxidation and Membrane Fluidity.

5. Discussion

The present experiment utilized progesterone, a steroid hormone produced by the follicular fluid; corpus luteum, presented in the ovary after the ovulation, and by the placenta during the pregnacy; this compound has the function of maintain the gestation during its whole period until birth. This steroid was added to the sperm in two different phases during the present experiment (toxicity and cryopreservation) to evaluate the effects on sperm kinect and viability parameters. According to [21] during cooling process, cold shock may damage different structural levels of the spermatozoon, which includes plasma, acrosomal and mitochondrial membranes, altering cellular functional integrity, and reducing sperm fertilizing ability.

Originally, progesterone was thought to act entering the cell thru a cytoplasmic receptor, bind to the cell DNA and act like a transcription factor [11]; however mature spermatozoa have stopped transcription and DNA replication, so the possibility of the hypothesis is very low [26].

[4] have demonstrated, thru the addition of a progesterone fluorescent marker, the presence of progesterone receptor in the stallion spermatozoa plasma membrane; three main

staining patterns were observed: 1) complete acrosomal cap and sperm head; 2) not stained at all and 3) cells with uneven stain degrees.

Our data have exhibited an increase in the parameters related with the sperm kinect movement and velocity starting from the three-hour progesterone exposition, which may be related to the calcium influx stimulated by the steroid hormone (capacitation and hyperactivation). [15]had presented data inferring that P4 and human follicular fluid are able to induce extracellular calcium influx and stimulates the acrosome reaction in capacitated human spermatozoa. According to [24] capacitation and hyperactivation are both calcium – dependent events.

At the 3-hour period of exposition to progesterone the concentration of 1000 ng/mL have enhanced sperm DAP, DCL, DSL, VAP, VCL, VSL and ALH; on the other hand, during the first half hour of exposition those variables at the 1000 ng/mL concentration are statistically lower ($P < 0,05$) than the group with 0 ng/mL of progesterone.

To explain these low values on the kinect sperm parameters at the 0,5-hour progesterone exposition, the authors believe that in the first 30 minutes, due to the high P4 concentration spermatozoa is exposed to (1000 ng/mL), occurs a very intense hyperactivation of the cells. [27]exhibited similar data in an experiment made with human semen which progesterone not only induced acrosome reaction but promoted hyperactivation of the sperm motility as well.

It is possible that this intense hyperactivation phenomenon hinders the correct sliding of the microtubules presented at the spermatozoon tail and consequently decreasing the velocity parameters of the sperm cell; after 3 hours it is likely that there has been a stabilization of this concentration and the sperm cell is able to retake its slipping movement.

Besides that, P4 promoted significantly increase ($P < 0,05$) in acrosome reaction at 30 minutes of exposition in the 1000 ng/mL concentration.In addition to that [5] did not visualized an increase in acrosome reaction with concentrations higher than 1000 ng/mL and conclude, also in agreement with the present study, that progesterone induce acrosome reaction without causing decrease on the sperm viability of fresh stallion semen.

After 24-hours in contact with the semen progesterone demonstrated significant ($P < 0,05$) a protective membrane effect at the 5 ng/mL concentration (superior than the control) and showed no significant differences among the exposition times for the same concentration.

It is also notable by data collected from flow cytometry during the toxicity phase that progesterone avoided spermatozoa lipid peroxidation on 24-hour contact at the concentrations of 1, 10, 100 and 1000 ng/mL when compared to the control group, behaving as an anti-oxidant tampon. There are basically two manners that the anti-oxidant substance can act; one

is preventing the ROS production and the second is capturing this reactive oxygen species avoiding membrane phospholipids peroxidation.

Since progesterone is a hormone derived from the cholesterol, the authors believe that it may act kidnapping the reactive oxygen species produced by the cellular metabolism and reacting with them instead of oxidizing the phospholipids presented in the spermatozoa membrane. This anti-oxidant effect is also observed at the cryopreservation phase, once the values of ROS Total and ROS Viable at the concentrations of 5, 10 and 100 ng/mL are significantly lower than the values found in the 0 ng/mL.

[16] demonstrated in his experiment that the addition of progesterone before cryopreservation of human semen has no benefit effect on the sperm cell, instead it showed a significant reduction in sperm motility, curvilinear velocity and linearity. Similar data in the present study revealed low values of total and progressive motility encountered after thawing; it may be related to the hyperactivation phenomenon promoted by progesterone contact during all the period of cooling and stabilizing the sperm cell before cryopreservation.

6. Conclusion

After all data analyzed we were able to conclude that the addition of progesterone is beneficial to the utilization on fresh and cooled semen once it promotes sperm acrosome reaction, hyperactivation, and anti-oxidant effect. The addition of this steroid could be used immediately before the artificial insemination process with the intent to increase the fertility rate. However, it is not recommended the utilization of progesterone on the freezing medium to cryopreserved stallion semen.

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Table 1: Kinect sperm values of total motility analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control 0 ng/mL	$67,0 \pm 2,14^{ABa}$	$68,9 \pm 2,1^{Aa}$	$48,2 \pm 2,0^{Ab}$
1 ng/mL	$70,2 \pm 1,9^{Aa}$	$67,9 \pm 1,6^{ABa}$	$47,9 \pm 2,1^{Ab}$
2,5 ng/mL	$69,4 \pm 2,0^{ABa}$	$62,8 \pm 2,1^{BCb}$	$51,3 \pm 2,0^{Ac}$
5 ng/mL	$70,1 \pm 1,9^{ABa}$	$67,9 \pm 2,1^{ABa}$	$48,0 \pm 2,7^{Ab}$
10 ng/mL	$68,9 \pm 2,4^{ABa}$	$67,3 \pm 1,6^{ABa}$	$47,3 \pm 2,5^{Ab}$
100 ng/mL	$67,2 \pm 1,9^{ABa}$	$60,3 \pm 2,3^{Cb}$	$50,2 \pm 2,3^{Ac}$
1000 ng/mL	$64,4 \pm 2,0^{Ba}$	$59,7 \pm 1,9^{Ca}$	$47,4 \pm 2,0^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 2: Kinect sperm values of progressive motility analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$52,9 \pm 2,6^{Aa}$	$56,4 \pm 2,2^{Aa}$	$30,0 \pm 1,9^{ABb}$
1 ng/mL	$52,2 \pm 2,4^{Aa}$	$53,4 \pm 2,1^{ABa}$	$32,3 \pm 2,5^{ABb}$
2,5 ng/mL	$55,1 \pm 2,4^{Aa}$	$48,0 \pm 2,0^{BCb}$	$35,0 \pm 2,1^{Ac}$
5 ng/mL	$57,3 \pm 2,3^{Aa}$	$53,1 \pm 2,7^{ABa}$	$28,2 \pm 2,6^{Bb}$
10 ng/mL	$55,7 \pm 2,7^{Aa}$	$51,6 \pm 1,6^{ABa}$	$32,0 \pm 2,5^{ABb}$
100 ng/mL	$53,0 \pm 2,4^{Aa}$	$44,1 \pm 2,7^{Cb}$	$31,5 \pm 2,2^{ABc}$
1000 ng/mL	$51,7 \pm 2,2^{Aa}$	$43,6 \pm 2,3^{Cb}$	$29,2 \pm 2,1^{ABc}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 3: Kinect sperm values of DAP (distance average path, μm) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$34,0 \pm 0,6^{Aa}$	$34,5 \pm 0,7^{Ca}$	$32,8 \pm 0,9^{ABa}$
1 ng/mL	$32,3 \pm 0,6^{ABb}$	$35,8 \pm 0,7^{BCa}$	$31,8 \pm 0,9^{ABb}$
2,5 ng/mL	$32,6 \pm 0,6^{ABb}$	$35,6 \pm 0,9^{BCa}$	$34,1 \pm 1,2^{Aab}$
5 ng/mL	$33,9 \pm 0,7^{Ab}$	$37,2 \pm 0,7^{ABa}$	$31,3 \pm 0,7^{Bc}$
10 ng/mL	$33,6 \pm 0,7^{Aa}$	$34,1 \pm 0,7^{Ca}$	$33,1 \pm 0,8^{ABa}$
100 ng/mL	$33,2 \pm 0,6^{ABa}$	$35,1 \pm 0,8^{Ca}$	$32,9 \pm 1,9^{ABa}$
1000 ng/mL	$31,6 \pm 0,8^{Bb}$	$38,3 \pm 0,8^{Aa}$	$31,6 \pm 0,9^{ABb}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 4: Kinect sperm values of DCL (distance curved line, μm) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$67,6 \pm 1,3^{Aa}$	$66,8 \pm 1,4^{Ca}$	$66,0 \pm 1,8^{Aa}$
1 ng/mL	$64,0 \pm 1,1^{BCb}$	$70,5 \pm 1,5^{BCa}$	$63,9 \pm 2,0^{Ab}$
2,5 ng/mL	$64,3 \pm 1,2^{ABCb}$	$70,4 \pm 1,8^{BCa}$	$67,2 \pm 2,0^{Aab}$
5 ng/mL	$66,2 \pm 1,3^{ABb}$	$72,0 \pm 1,2^{Ba}$	$63,4 \pm 1,6^{Ab}$
10 ng/mL	$67,0 \pm 1,3^{ABa}$	$67,1 \pm 1,3^{Ca}$	$66,0 \pm 1,7^{Aa}$
100 ng/mL	$65,0 \pm 1,2^{ABCa}$	$68,5 \pm 1,6^{BCa}$	$66,0 \pm 2,2^{Aa}$
1000 ng/mL	$62,1 \pm 1,5^{Cb}$	$76,9 \pm 1,5^{Aa}$	$63,9 \pm 2,1^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 5: Kinect sperm values of DSL (distance straight line, μm) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control 0 ng/mL	$23,0 \pm 0,5^{Aab}$	$23,1 \pm 0,6^{Ca}$	$21,5 \pm 0,6^{Ab}$
1 ng/mL	$22,1 \pm 0,5^{Aa}$	$23,2 \pm 0,5^{Ca}$	$20,4 \pm 0,6^{Ab}$
2,5 ng/mL	$22,4 \pm 0,4^{Aa}$	$23,2 \pm 0,6^{Ca}$	$20,9 \pm 0,6^{Ab}$
5 ng/mL	$23,2 \pm 0,5^{Ab}$	$25,1 \pm 0,7^{ABa}$	$20,4 \pm 0,6^{Ac}$
10 ng/mL	$22,9 \pm 0,5^{Aa}$	$22,5 \pm 0,6^{Ca}$	$20,6 \pm 0,5^{Ab}$
100 ng/mL	$23,0 \pm 0,6^{Aa}$	$23,8 \pm 0,6^{BCa}$	$20,8 \pm 0,6^{Ab}$
1000 ng/mL	$21,8 \pm 0,7^{Ab}$	$25,5 \pm 0,5^{Aa}$	$20,5 \pm 0,6^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 6: Kinect spermvalues of VAP (velocity average path, μm/s) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$78,5 \pm 1,4^{Aa}$	$80,0 \pm 1,7^{Ca}$	$75,4 \pm 2,1^{Aa}$
1 ng/mL	$75,2 \pm 1,4^{ABb}$	$83,1 \pm 1,8^{ABCa}$	$72,5 \pm 2,1^{Ab}$
2,5 ng/mL	$75,4 \pm 1,6^{ABb}$	$81,9 \pm 2,0^{BCa}$	$76,8 \pm 2,6^{Aab}$
5 ng/mL	$78,2 \pm 1,7^{Ab}$	$86,3 \pm 1,7^{ABa}$	$72,1 \pm 1,7^{Ac}$
10 ng/mL	$76,9 \pm 1,7^{Aa}$	$79,3 \pm 1,5^{Ca}$	$75,1 \pm 1,9^{Aa}$
100 ng/mL	$76,9 \pm 1,4^{Aab}$	$81,3 \pm 1,8^{Ca}$	$75,0 \pm 2,6^{Ab}$
1000 ng/mL	$72,2 \pm 1,8^{Bb}$	$87,2 \pm 1,8^{Aa}$	$72,3 \pm 2,1^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 7: Kinect spermvalues of VCL (velocity curved line, μm/s) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$155,2 \pm 2,8^{Aa}$	$154,5 \pm 3,2^{Ca}$	$150,6 \pm 4,3^{Aa}$
1 ng/mL	$147,9 \pm 2,5^{ABb}$	$162,8 \pm 3,6^{BCa}$	$144,9 \pm 4,5^{Ab}$
2,5 ng/mL	$148,0 \pm 2,9^{ABb}$	$161,2 \pm 4,0^{BCa}$	$150,8 \pm 4,4^{Aab}$
5 ng/mL	$152,1 \pm 3,0^{Ab}$	$166,2 \pm 2,9^{ABa}$	$145,3 \pm 3,6^{Ab}$
10 ng/mL	$153,0 \pm 3,0^{Aa}$	$155,3 \pm 2,9^{Ca}$	$148,7 \pm 3,7^{Aa}$
100 ng/mL	$149,9 \pm 2,9^{Aa}$	$158,2 \pm 3,6^{BCa}$	$149,9 \pm 4,8^{Aa}$
1000 ng/mL	$141,3 \pm 3,4^{Bb}$	$174,6 \pm 3,4^{Aa}$	$145,5 \pm 4,6^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 8: Kinect spermvalues of VSL (velocity straight line, μm/s) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$53,4 \pm 1,1^{Aa}$	$53,8 \pm 1,3^{Ba}$	$49,6 \pm 1,4^{Ab}$
1 ng/mL	$51,6 \pm 1,1^{ABa}$	$54,1 \pm 1,2^{Ba}$	$46,9 \pm 1,5^{Ab}$
2,5 ng/mL	$52,0 \pm 1,0^{ABa}$	$53,7 \pm 1,3^{Ba}$	$47,3 \pm 1,5^{Ab}$
5 ng/mL	$53,6 \pm 1,1^{Ab}$	$58,5 \pm 1,5^{Aa}$	$47,1 \pm 1,4^{Ac}$
10 ng/mL	$52,5 \pm 1,2^{ABa}$	$52,6 \pm 1,2^{Ba}$	$46,7 \pm 1,2^{Ab}$
100 ng/mL	$53,4 \pm 1,3^{Aa}$	$55,5 \pm 1,4^{ABa}$	$47,8 \pm 1,4^{Ab}$
1000 ng/mL	$49,9 \pm 1,4^{Bb}$	$58,4 \pm 1,1^{Aa}$	$47,0 \pm 1,4^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 9: Kinect spermvalues of STR (straightness, VSL/VAP, %) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$0,6765 \pm 0,008^{Aa}$	$0,6693 \pm 0,0112^{Aa}$	$0,6570 \pm 0,0101^{Aa}$
1 ng/mL	$0,6828 \pm 0,007^{Aa}$	$0,6545 \pm 0,6135^{Aab}$	$0,6486 \pm 0,0135^{Ab}$
2,5 ng/mL	$0,6898 \pm 0,007^{Aa}$	$0,6594 \pm 0,0147^{Aab}$	$0,6324 \pm 0,0172^{Ab}$
5 ng/mL	$0,6869 \pm 0,0101^{Aa}$	$0,6733 \pm 0,0132^{Aab}$	$0,6481 \pm 0,0106^{Ab}$
10 ng/mL	$0,6802 \pm 0,008^{Aa}$	$0,6610 \pm 0,0105^{Aa}$	$0,6250 \pm 0,0129^{Aa}$
100 ng/mL	$0,6873 \pm 0,008^{Aa}$	$0,6791 \pm 0,009^{Aa}$	$0,6431 \pm 0,009^{Ab}$
1000 ng/mL	$0,6849 \pm 0,008^{Aa}$	$0,6734 \pm 0,0117^{Aab}$	$0,6514 \pm 0,0115^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 10: Kinect spermvalues of LIN (Linearity, VSL/VCL, %) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$0,3406 \pm 0,004^{Aab}$	$0,3442 \pm 0,006^{Aa}$	$0,3268 \pm 0,005^{Ab}$
1 ng/mL	$0,3444 \pm 0,004^{Aa}$	$0,3326 \pm 0,008^{Aab}$	$03226 \pm 0,007^{ABb}$
2,5 ng/mL	$0,3476 \pm 0,003^{Aa}$	$0,3327 \pm 0,008^{Aab}$	$0,3160 \pm 0,009^{ABb}$
5 ng/mL	$0,3502 \pm 0,006^{Aa}$	$0,3464 \pm 0,007^{Aa}$	$0,3178 \pm 0,004^{ABb}$
10 ng/mL	$0,3381 \pm 0,004^{Aa}$	$0,3351 \pm 0,005^{Aa}$	$0,3113 \pm 0,005^{Bb}$
100 ng/mL	$0,3508 \pm 0,004^{Aa}$	$0,3471 \pm 0,005^{Aa}$	$0,3172 \pm 0,003^{ABb}$
1000 ng/mL	$0,3475 \pm 0,004^{Aa}$	$0,3325 \pm 0,005^{Ab}$	$0,3212 \pm 0,005^{ABb}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 11: Kinect spermvalues of WOB (Wobble, VAP/VCL, %) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$0,5020 \pm 0,002^{ABb}$	$0,5137 \pm 0,003^{Aa}$	$0,4978 \pm 0,004^{Ab}$
1 ng/mL	$0,5038 \pm 0,003^{ABa}$	$0,5064 \pm 0,003^{ABa}$	$0,4981 \pm 0,005^{Aa}$
2,5 ng/mL	$0,5034 \pm 0,002^{ABa}$	$0,5037 \pm 0,003^{Ba}$	$0,5029 \pm 0,006^{Aa}$
5 ng/mL	$0,5090 \pm 0,003^{Aa}$	$0,5140 \pm 0,003^{Aa}$	$0,4933 \pm 0,006^{Ab}$
10 ng/mL	$0,4969 \pm 0,003^{Ba}$	$0,5066 \pm 0,003^{ABa}$	$0,5031 \pm 0,008^{Aa}$
100 ng/mL	$0,5084 \pm 0,002^{Aa}$	$0,5097 \pm 0,002^{ABa}$	$0,4952 \pm 0,005^{Ab}$
1000 ng/mL	$0,5056 \pm 0,002^{Aa}$	$0,4937 \pm 0,003^{Cb}$	$0,4958 \pm 0,006^{Aab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 12: Kinect spermvalues of ALH (amplitude of lateral head displacement, μm) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$4,1 \pm 0,1^{Aa}$	$4,1 \pm 0,1^{Ba}$	$4,3 \pm 0,1^{Aa}$
1 ng/mL	$4,1 \pm 0,1^{Ab}$	$4,4 \pm 0,1^{Aa}$	$4,1 \pm 0,1^{Ab}$
2,5 ng/mL	$4,0 \pm 0,1^{ABb}$	$4,3 \pm 0,1^{ABa}$	$4,0 \pm 0,1^{Aab}$
5 ng/mL	$4,0 \pm 0,1^{Ab}$	$4,2 \pm 0,1^{ABa}$	$4,2 \pm 0,1^{Aa}$
10 ng/mL	$4,0 \pm 0,1^{Ab}$	$4,2 \pm 0,1^{ABa}$	$4,1 \pm 0,1^{Aab}$
100 ng/mL	$4,0 \pm 0,1^{ABb}$	$4,2 \pm 0,1^{ABa}$	$4,2 \pm 0,1^{Aa}$
1000 ng/mL	$3,8 \pm 0,1^{Bc}$	$4,4 \pm 0,1^{Aa}$	$4,1 \pm 0,1^{Ab}$

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 13: Kinect spermKinect sperm values of BCF (beat cross frequency,Hz) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone.

P4	Exposition Time		
	0,5 h	**3 h**	**24 h**
Control - 0 ng/mL	$31,4 \pm 0,4^{Aa}$	$30,8 \pm 0,5^{Ba}$	$28,7 \pm 0,3^{Ab}$
1 ng/mL	$31,2 \pm 0,3^{ABa}$	$31,0 \pm 0,4^{Ba}$	$27,5 \pm 0,4^{Bb}$
2,5 ng/mL	$31,3 \pm 0,3^{Aa}$	$30,6 \pm 0,4^{Ba}$	$28,2 \pm 0,4^{ABb}$
5 ng/mL	$31,7 \pm 0,3^{Aa}$	$32,2 \pm 0,5^{Aa}$	$28,3 \pm 0,3^{ABb}$
10 ng/mL	$31,1 \pm 0,3^{ABa}$	$31,3 \pm 0,4^{ABa}$	$27,7 \pm 0,3^{Bb}$
100 ng/mL	$32,0 \pm 0,5^{Aa}$	$31,3 \pm 0,5^{ABa}$	$27,8 \pm 0,3^{ABb}$
1000 ng/mL	$30,3 \pm 0,4^{Ba}$	$31,2 \pm 0,4^{ABa}$	$27,6 \pm 0,4^{Bb}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 14: Acrosome reaction (ACRO), membrane fluidity (FLU), values analyzed by Attune Acoustic Focusing Cytometer® (Applied Biosystems) of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone (0,5; 3 and 24-hour).

		Progesterone Concentration (ng/mL)						
	Exposition Time	**Control 0 ng/mL**	**1 ng/mL**	**2,5 ng/mL**	**5 ng/Ml**	**10 ng/mL**	**100 ng/mL**	**1000 ng/mL**
ACRO	**0,5 h**	$3,9 \pm 1,9^{Ba}$	$2,7 \pm 1,2^{Ba}$	$2,6 \pm 1,1^{Ba}$	$2,1 \pm 1,0^{Bb}$	$1,5 \pm 0,6^{Ba}$	$3,3 \pm 1,5^{Ba}$	$17,0 \pm 7,8^{Aa}$
	3 h	$4,1 \pm 1,7^{Aa}$	$3,2 \pm 1,2^{Aa}$	$3,2 \pm 1,3^{Aa}$	$4,2 \pm 1,5^{Aab}$	$2,0 \pm 0,7^{Aa}$	$1,8 \pm 0,7^{Aa}$	$2,1 \pm 1,0^{Ab}$
	24 h	$5,3 \pm 2,0^{ABa}$	$3,8 \pm 1,2^{ABa}$	$3,5 \pm 1,1^{Ba}$	$8,1 \pm 2,6^{Aa}$	$4,0 \pm 1,8^{ABa}$	$2,7 \pm 0,8^{Ba}$	$1,7 \pm 0,6^{Bb}$
FLU	**0,5 h**	$34,6 \pm 4,0^{Aa}$	$35,2 \pm 4,5^{ABa}$	$31,2 \pm 3,6^{ABab}$	$28,1 \pm 3,5^{Bb}$	$30,1 \pm 3,6^{ABa}$	$39,8 \pm 3,9^{Aa}$	$32,0 \pm 3,0^{ABa}$
	3 h	$38,7 \pm 5,1^{Aa}$	$42,2 \pm 4,4^{Aa}$	$40,3 \pm 3,9^{Aa}$	$37,8 \pm 3,4^{Aab}$	$33,5 \pm 4,4^{Aa}$	$31,7 \pm 3,0^{Aa}$	$34,6 \pm 3,3^{Aa}$
	24 h	$39,1 \pm 4,5^{Aa}$	$41,5 \pm 3,2^{Aa}$	$27,7 \pm 3,8^{Bb}$	$41,6 \pm 3,8^{Aa}$	$32,9 \pm 4,5^{ABa}$	$36,2 \pm 3,9^{ABa}$	$37,6 \pm 4,2^{ABa}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

T**able 15:**Membrane integrity (MEM) and viability (VIA) values analyzed by Attune Acoustic Focusing Cytometer® (Applied Biosystems) of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone (0,5; 3 and 24-hour).

		Progesterone Concentration (ng/mL)						
	Exposition Time	**Control 0 ng/mL**	**1 ng/mL**	**2,5 ng/mL**	**5 ng/Ml**	**10 ng/mL**	**100 ng/mL**	**1000 ng/mL**
MEM	**0,5h**	$30,9 \pm 5,5^{Aa}$	$33,8 \pm 4,3^{Aa}$	$38,0 \pm 4,6^{Aa}$	$33,7 \pm 4,7^{Aa}$	$35,7 \pm 4,7^{Aa}$	$33,7 \pm 4,7^{Aa}$	$32,0 \pm 3,6^{Aa}$
	3 h	$29,4 \pm 3,6^{Aa}$	$26,2 \pm 3,2^{Aab}$	$23,7 \pm 3,7^{Ab}$	$29,4 \pm 3,5^{Aa}$	$26,6 \pm 3,7^{Aab}$	$29,1 \pm 3,8^{Aa}$	$20,9 \pm 3,8^{Ab}$
	24 h	$21,5 \pm 2,6^{Ba}$	$20,1 \pm 2,8^{Bb}$	$26,1 \pm 2,6^{ABb}$	$33,5 \pm 2,8^{Aa}$	$25,0 \pm 2,8^{Bb}$	$26,0 \pm 3,1^{ABa}$	$26,8 \pm 3,1^{ABab}$
VIA	**0,5 h**	$72,6 \pm 6,1^{Aab}$	$77,7 \pm 3,7^{Aa}$	$75,4 \pm 3,3^{Aa}$	$75,6 \pm 3,9^{Aa}$	$73,6 \pm 3,5^{Aa}$	$75,9 \pm 3,8^{Aa}$	$76,4 \pm 3,5^{Aa}$
	3h	$77,8 \pm 3,5^{Aa}$	$73,9 \pm 3,^{9Aa}$	$74,8 \pm 3,8^{Aa}$	$76,2 \pm 4,2^{Aa}$	$74,4 \pm 4,2^{Aa}$	$75,9 \pm 4,0^{Aa}$	$69,0 \pm 6,1^{Aab}$
	24h	$64,5 \pm 3,8^{ABb}$	$59,0 \pm 4,7^{Bb}$	$71,0 \pm 3,4^{Aa}$	$68,8 \pm 4,0^{ABa}$	$69,5 \pm 3,3^{ABa}$	$64,2 \pm 4,5^{ABb}$	$66,9 \pm 3,6^{ABb}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 16: Mitochondrial functionality(MITO), lipid peroxidation (LPO) analyzed by Attune Acoustic Focusing Cytometer® (Applied Biosystems) of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone (0,5; 3 and 24-hour).

		Progesterone Concentration (ng/mL)						
	Exposition Time	**Control 0 ng/mL**	**1 ng/mL**	**2,5 ng/mL**	**5 ng/Ml**	**10 ng/mL**	**100 ng/mL**	**1000 ng/mL**
MITO	**0,5 h**	$49,7 \pm 5,8^{ABa}$	$51,8 \pm 4,9^{ABa}$	$58,6 \pm 3,2^{ABa}$	$56,9 \pm 3,1^{ABa}$	$61,0 \pm 2,6^{Aa}$	$56,2 \pm 4,4^{ABa}$	$48,0 \pm 5,6^{Ba}$
	3 h	$53,8 \pm 4,2^{Aa}$	$55,1 \pm 4,2^{Aa}$	$55,1 \pm 3,8^{Aa}$	$54,5 \pm 4,0^{Aa}$	$45,2 \pm 5,2^{Ab}$	$52,8 \pm 3,9^{Aa}$	$54,2 \pm 3,5^{Aa}$
	24 h	$44,4 \pm 3,8^{ABa}$	$50,3 \pm 3,7^{ABa}$	$49,7 \pm 3,5^{ABa}$	$47,8 \pm 3,8^{ABa}$	$41,6 \pm 4,4^{Bb}$	$50,8 \pm 3,6^{ABa}$	$52,3 \pm 3,6^{Aa}$
LPO	**0,5 h**	$43,0 \pm 3,5^{Bba}$	$40,1 \pm 2,0^{Ba}$	$41,9 \pm 1,9^{Bb}$	$45,4 \pm 1,5^{Ba}$	$47,3 \pm 5,3^{ABa}$	$48,4 \pm 4,7^{ABab}$	$57,9 \pm 5,2^{Aa}$
	3 h	$43,8 \pm 4,1^{ABa}$	$43,7 \pm 2,6^{ABa}$	$45,7 \pm 1,8^{ABab}$	$48,7 \pm 3,3^{ABa}$	$40,8 \pm 3,0^{Ba}$	$50,4 \pm 2,1^{Aa}$	$50,6 \pm 3,3^{Aab}$
	24 h	$51,4 \pm 2,4^{Aa}$	$38,7 \pm 3,7^{Ca}$	$50,7 \pm 2,1^{ABa}$	$43,4 \pm 0,9^{ABCa}$	$39,3 \pm 2,4^{Ca}$	$37,5 \pm 5,2^{Cb}$	$43,0 \pm 1,0^{BCb}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$). ^{a,b}Different letters in the same row have statistical difference test LSD ($P < 0.05$) (n=10 ejaculates).

Table 17: Reactive oxygen species (ROS) and DFI (DNA fragmentation index) analyzed by Attune Acoustic Focusing Cytometer® (Applied Biosystems) of Control (0 ng / mL); 1 ng / mL; 2,5 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL and 1000 ng/ mL in three different times of exposition to progesterone (0,5; 3 and 24-hour).

		Progesterone Concentration (ng/mL)						
	Exposition Time	**Control 0 ng/mL**	**1 ng/mL**	**2,5 ng/mL**	**5 ng/Ml**	**10 ng/mL**	**100 ng/mL**	**1000 ng/mL**
ROS Viable	**0,5 h**	479,6 ± 82,3^{Aba}	367,4 ± 72,2^{Aa}	384,8 ± 54,3^{Aa}	441,6 ± 70,4^{Aa}	420,7 ± 64,6^{Aa}	358,5 ± 70,1^{Aa}	493,9 ± 73,5^{Aa}
	3 h	355,6 ± 79,4^{Aa}	443,7 ± 81,4^{Aa}	475,4 ± 76,3^{Aa}	449,0 ± 74,8^{Aa}	426,7 ± 66,9^{Aa}	411,8 ± 71,6^{Aa}	251,6 ± 61,8^{Ab}
	24 h	304,8 ± 64,3^{Aa}	387,9 ± 32,4^{Aa}	352,0 ± 64,4^{Aa}	371,5 ± 55,0^{Aa}	341,1 ± 72,8^{Aa}	398,0 ± 71,3^{Aa}	332,8 ± 69,1^{Aab}
DFI	**24H**	0,00520 ± 0,001^{A}	0,00519 ± 0,009^{A}	0,00538 ± 0,002^{A}	0,0521 ± 0,002^{A}	0,0505 ± 0,001^{A}	0,0520 ± 0,001^{A}	0,0521 ± 0,001^{A}

^{A,B}Different letters in the same column have statistical difference test LSD (P <0.05). ^{a,b}Different letters in the same row have statistical difference test LSD (P <0.05) (n=10 ejaculates).

Table 18: Kinect sperm values after thawing of total motility, progressive motility, DAP (distance average path, μm), DCL (distance curved line, μm), DSL (distance straight line, μm) and VAP (velocity average path, μm/s) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL.

	Progesterone Concentration (ng/mL)				
Analisys	**Control**	**1 ng/mL**	**5 ng/mL**	**10 ng/mL**	**100 ng/mL**
Total Motility	$22,1 \pm 2,0^{A}$	$19,1 \pm 1,4^{AB}$	$15,5 \pm 1,4^{B}$	$21,1 \pm 1,8^{A}$	$18,0 \pm 1,8^{AB}$
Prog. Motility	$13,8 \pm 1,4^{A}$	$11,7 \pm 1,1^{AB}$	$10,3 \pm 1,2^{B}$	$12,8 \pm 1,1^{AB}$	$11,5 \pm 1,3^{AB}$
DAP	$36,7 \pm 0,6^{A}$	$35,8 \pm 0,8^{A}$	$35,5 \pm 0,8^{A}$	$35,7 \pm 0,7^{A}$	$37,1 \pm 0,9^{A}$
DCL	$70,7 \pm 1,4^{A}$	$68,9 \pm 1,5^{A}$	$70,4 \pm 1,7^{A}$	$70,6 \pm 1,5^{A}$	$73,0 \pm 1,9^{A}$
DSL	$29,6 \pm 0,5^{A}$	$27,8 \pm 0,8^{BC}$	$27,9 \pm 0,6^{BC}$	$27,6 \pm 0,5^{C}$	$29,5 \pm 0,6^{AB}$
VAP	$78,8 \pm 1,4^{A}$	$77,2 \pm 1,7^{A}$	$76,2 \pm 1,6^{A}$	$77,4 \pm 1,6^{A}$	$79,9 \pm 1,8^{A}$

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$).

Table 19: Kinect sperm values after thawing of VCL (velocity curved line, μm/s), VSL (velocity straight line, μm/s), STR (straightness, VSL/VAP, %), LIN (Linearity, VSL/VCL, %), WOB (Wobble, VAP/VCL, %), ALH (amplitude of lateral head displacement, μm) and (beat cross frequency,Hz) analyzed by AndroVision®: CASA Software of Control (0 ng / mL); 1 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL.

[A,B]Different letters in the same column have statistical difference test LSD (P <0.05).

	Progesterone Concentration (ng/mL)				
Analisys	**Control**	**1 ng/mL**	**5 ng/mL**	**10 ng/mL**	**100 ng/mL**
VCL	151,9 ± 3,0^{A}	148,5 ± 3,3^{A}	150,6 ± 3,5^{A}	152,9 ± 3,5^{A}	156,8 ± 4,0^{A}
VSL	63,6 ± 1,1^{A}	60,1 ± 1,6^{AB}	60,0 ± 1,4^{B}	60,1 ± 1,1^{AB}	63,8 ± 1,3^{A}
STR	0,805 ± 0,008^{A}	0,744 ± 0,010^{B}	0,785 ± 0,010^{AB}	0,778 ± 0,010^{B}	0,798 ± 0,010^{AB}
LIN	0,417 ± 0,006^{A}	0,404 ± 0,009^{AB}	0,398 ± 0,007^{AB}	0,394 ± 0,006^{B}	0,409 ± 0,009^{AB}
WOB	0,517 ± 0,004^{A}	0,518 ± 0,006^{A}	0,505 ± 0,005^{A}	0,504 ± 0,004^{A}	0,509 ± 0,006^{A}
ALH	3,2 ± 0,1^{A}	3,1 ± 0,1^{A}	3,2 ± 0,1^{A}	3,1 ± 0,1^{A}	3,2 ± 0,1^{A}
BCF	41,5 ± 0,4^{A}	38,2 ± 0,9^{C}	39,7 ± 0,7^{ABC}	39,5 ± 0,7^{BC}	40,8 ± 0,7^{AB}

Table 20: ROS Total (reactive oxygen species), ROS Viable, DFI (DNA Fragmentation Index), Lipid peroxidation (LPO), Mitochondrial functionality (MITO), viability (VIA), Membrane Fluidity and Acrosome Reaction (ACRO) values analyzed by Attune Acoustic Focusing Cytometer® (Applied Biosystems) of Control (0 ng / mL); 1 ng / mL; 5 ng / mL; 10 ng / mL; 100 ng / mL after thawing.

	Progesterone Concentrations (ng / mL)				
Analisys	**Control**	**1 ng / mL**	**5 ng / mL**	**10 ng / mL**	**100 ng / mL**
ROS Total	66024,0 ± 1859,0^{A}	38464,0 ± 9358,3^{AB}	27402,0 ± 5833,0^{B}	18816,0 ± 4952,5^{B}	14353,0 ± 3268,6^{B}
ROS Viable	87102,0 ± 32528,0^{A}	55291,0 ± 17124,0^{AB}	42145,0 ± 10355,0^{AB}	26714,0 ± 8529,7^{B}	15269,0 ± 5848,1^{B}
DFI	2,0 ± 0,5^{A}	2,2 ± 0,5^{A}	2,3 ± 0,6^{A}	1,7 ± 0,4^{A}	1,9 ± 0,5^{A}
LPO	46,0 ± 4,2^{A}	45,9 ± 4,4^{A}	51,8 ± 3,5^{A}	46,3 ± 4,4^{A}	51,2 ± 3,8^{A}
Func Mito	51,0 ± 5,6^{A}	60,7 ± 3,9^{A}	64,4 ± 4,6^{A}	58,1 ± 5,2^{A}	52,7 ± 5,6^{A}
Viability	38,7 ± 4,8^{A}	33,2 ± 4,0^{A}	33,1 ± 4,8^{A}	38,0 ± 4,4^{A}	41,4 ± 4,6^{A}
Fluidez Ne	69,1 ± 6,5^{AB}	74,3 ± 6,5^{AB}	76,4 ± 3,9^{AB}	63,8 ± 6,2^{B}	81,9 ± 4,2^{A}
Reac Acro2	19,9 ± 2,2^{AB}	24,2 ± 2,2^{A}	23,7 ± 2,1^{A}	21,6 ± 2,1^{A}	15,3 ± 2,2^{B}

^{A,B}Different letters in the same column have statistical difference test LSD ($P < 0.05$).

3 Considerações Finais

Os dados obtidos a partir da adição de quercetina ao meio espermático de congelamento não demonstraram melhora ou piora estatisticamente significativa em relação aos parâmetros seminais analisados. Entretanto, a suplementação de progesterona ao sêmen fresco resfriado demonstrou melhoras em parâmetros como velocidade média, distância percorrida, reação acrossomal e diminuição dos valores referentes a peroxidação lipídica, promovendo hiperativação, capacitação e efeito antioxidante. Já a adição de progesterona ao meio diluente anterior ao congelamento não demonstrou ser benéfico aos parâmetros espermáticos, uma vez que diminuiu valores como motilidade total e progressiva.

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