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Research Article/Artículo de Investigación

USE OF COMBINATION OF FLUORESCENT PROBES TO IDENTIFY SPERM SUBPOPULATIONS FOR QUALITY ASSESSMENT OF FRESH AND CRYOPRESERVED CANINE SEMEN. PRELIMINARY RESULTS

USO DE UNA COMBINACIÓN DE PRUEBAS FLUORESCENTES PARA IDENTIFICAR SUBPOBLACIONES ESPERMÁTICAS PARA MEDIR LA CALIDAD DE SEMEN CANINO FRESCO Y DESCONGELADO. RESULTADOS PRELIMINARES

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ABSTRACT

The use of fluorescent markers in the evaluation of sperm morphophysiology allows a better accuracy, compared to the subjective nature of some routine tests in semen qualification. In this study was used the combination of fluorescence probes: propionate iodide, Hoechst 33342 and FITC-PSA in fresh and thawed dog semen, to the identification of the following morphological subpopulations: II (intact plasma and acrosomal membranes), IL (intact plasma membrane and lesioned acrosomal membrane), LI (lesioned plasma membrane and intact acrosomal membrane) and LL (both membranes lesioned). When comparing the results obtained with the results of the tests used conventionally in semen evaluation (sperm motility and vigor, hypoosmotic test and morphological alterations), little correlation was observed. The II population declined from fresh semen to thawed, while LL population increased ($p < 0.05$). The IL population was composed of extremely small numbers of cells but increased ($p < 0.05$) from fresh semen to thawed semen. In the thawed semen the major defects had a positive correlation with the LL population ($p < 0.01$). For the thawed semen, the results of the hypoosmotic test (number of cells that reacted to the medium) correlated positively with population II ($p < 0.025$), that is, different from that observed in fresh semen. Although all tests were able to detect decrease in sperm quality post-thawing ($p < 0.05$). The use of this fluorescent probe association allowed qualification and more accurately quantification of plasma membrane and acrosomal insults mediated by cryopreservation.

Keywords: canine semen, propionate iodide, Hoechst 33342, FITC-PSA, cryopreservation.
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RESUMEN

El uso de marcadores fluorescentes en la evaluación de la morfofisiología espermática permite una mayor precisión, comparada con la naturaleza subjetiva de algunas pruebas de rutina en la valoración del semen. En este estudio se usó la combinación de pruebas fluorescentes: yoduro de propidio; Hoechst 33342 y FITC-PSA en semen fresco y descongelado de perro, para la identificación de las siguientes subpoblaciones morfológicas: II (membranas plasmática y acrosomal intactas), IL (membrana plasmática intacta y membrana acrosomal dañada), LI (membrana plasmática dañada y membrana acrosomal intacta) y LL (ambas membranas dañadas). Cuando se comparan los resultados obtenidos con los resultados de las pruebas usadas convencionalmente en la evaluación seminal (motilidad y vigor espermáticos, prueba hipoosmótica y alteraciones morfológicas), se observó poca correlación. La población II disminuyó desde el semen fresco al descongelado, mientras que la población LL se incrementó ($p<0.05$). la población IL estuvo compuesta por un número extremadamente pequeño de células, pero incremento ($p<0.05$) desde el semen fresco al descongelado. En el semen descongelado los defectos mayores tuvieron una correlación positiva con la población LL ($p<0.01$). En el semen descongelado, los resultados de la prueba hipoosmótica (número de células que reaccionan al medio) se correlacionaron positivamente con la población II ($p<0.05$). El uso de esta asociación de pruebas fluorescentes permitió la valoración y la cuantificación más precisa de los daños a la membrana plasmática y acrosomal mediados por la criopreservación.

Palabras clave: semen canino, yoduro de propidio, Hoechst 33342, FITC-PSA, criopreservación.
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INTRODUCTION

The freezing and thawing process causes damage to the integrity of the sperm cell and is responsible for the decrease in longevity and the fertilizing capacity of the cell. In the case of canine spermatozoa, such damage is responsible for the reduced ability for fertilize (Hay et al., 1997; Rota et al., 1999). During the cooling and freezing process, changes in the lipid structure of the membrane occur, which leads to a change in the fluidity of the phospholipid bilayer and, consequently, to its selective permeability (Watson, 2000). In this context, it has become important to develop tests to quantify damage caused by the freeze / thaw process. Thus, it is possible to measure the degree of damage caused, allowing the comparison of the efficiency of different cryopreservation protocols of the semen.

Among the tests routinely used, the following stand out: vigor and motility, hypoosmotic (HOST), thermoresistance (TTR), supravital staining and evaluation of the degree of sperm defects (Cunha, 2008). However, in the literature, studies point to the non-correlation between these tests (Rijsselaere et al., 2005, Eilts 2005, Graham & Mocé 2005, Mascarenhas, 2008). Silva (2005) reports the lack of studies on these relationships in dogs, noting the importance of this knowledge for predicting fertility in vitro and in vivo. For this reason, more specific tests for the evaluation of sperm cell integrity have been studied.

Fluorescent probes have proven to be an important tool for evaluating the viability of various compartments of the sperm cell. These probes are accurate indicators of metabolic changes in the cell (Haugland, 2001). The probes bind to specific molecules under specific conditions, and thus conjugate will emit fluorescence, if excited by a suitable wavelength of light (Celehini, 2005). In this sense, these tests can be performed on fresh semen or preserved samples. In the former, it would allow to increase the ability to predict sperm quality, the selection of donor/sperm for cryopreservation and to assess infertility causes. In the latter it could be of utmost interest to study the sperm response to preservation trials, such as the design of a new extender (Payan-Carreira et al., 2013).

Several compartments and organelles of the sperm cell can be evaluated through the use of fluorescent probes (Peterson et al., 1974). Propidium iodide binds to cell DNA, but can only access genetic material if the plasma membrane is injured (Rijsselaere et al., 2005). This molecule conjugates to the DNA emitting red fluorescence (Maxwell et al., 1997). Other probes, such as Hoechst 33342 also have affinity for the genetic material, but are able to transpose only the intact and functional plasma membrane, thus acting as a propidium iodide counter-dye (Maxwell et al., 1997), in a more recent function Hoeschst 33342 is fundamental for some semen sexing techniques (Rodenas et al., 2014). The acrosomal membrane is also able to be evaluated by the use of fluorescent probes. The fluorophore to these probes, however, needs to be associated with antibodies or other molecules such as agglutinins (Baccettiet al., 1999). In the latter case, fluoresceins such as fluorescein isothiocyanate should be conjugated to agglutinins with affinity for membrane glycoproteins or acrosomal matrix, such as Pisum Sativum Aglutinin (PSA). As an example of this probe is FITC-PSA, capable of differentiating acrosomal membranes that have undergone or initiated the acrosome reaction and intact acrosomal membranes (Farlin et al., 1992, Rijsselaere et al., 2005).

According to a literature search, the association of fluorescence probes Hoechst 33342 and propidium iodide, for plasma membrane integrity and FITC-PSA for acrosomal membrane integrity, has not yet been tested for the domestic dog (Neves et al. 2009, Garner 2009, Rodenas et al., 2014). In this sense, the objective of the present study was to evaluate the effect of cryopreservation on sperm membranes using a combination fluorescence probes Hoechst 33342, propidium iodide and FITC-PSA compared to routine tests (HOST, sperm defects, vigor and motility).

MATERIALS AND METHODS

An adult Pitbull male dog of 7.5 years and 26 kg, was used. Kept by a private owner. The animal was healthy during the experimental period. The dog used showed good seminal quality and good results after thawing in pilot tests, besides having proven fertility in vivo.

The fraction of ejaculate rich in spermatozoa, was collected by penile manipulation in pre-heated falcon tube at 38ºC. The semen was homogenized with the help of a micropipette and aliquots were taken for: sperm concentration in Newbauer chamber, vigor and motility analysis, hypoosmotic test (HOST) and analysis of sperm pathologies. The remainder of the ejaculate was standardized to a concentration of $100x10^6$ sperm/ml by pre-dilution in TRIS-Citrate medium with 20% egg yolk without further additives. A 1 ml portion of this pre-diluted semen had its volume doubled by adding TRIS-Citrate medium with 20% egg yolk containing 12% glycerol and 1% Paste® STM Equex, thereby obtaining a final concentration of $50x10^6$ sperm/mL with 6% glycerol and 0.5% Paste® STM Equex, this was then cooled and frozen according to methodology described by Mascarenhas (2008). Thus, it was packed in 0.25 ml straws and cooled at a rate of 0.56°C/min for one hour to the temperature of 4°C. For this the vats were packed in a closed test tube inside a container of glass containing 800ml of water at 38ºC, packed in a closed isothermal box (24cm x 19.5cm x 28.5cm) wrapped in 6,67L of ice and water (1:1). After this step, the semen was kept at

4ºC for an additional hour as the equilibration time. Soon after, the straws were frozen in liquid nitrogen vapor. For this, they were transferred to a floating artifact, in an isothermal box containing a 3 cm high liquid nitrogen (1.68L), the artifact kept the straws horizontally at a height of 10cm of the liquid nitrogen. After 15 minutes the straws were immersed in liquid nitrogen and stored. After 24 hours, the straws were thawed for 7 seconds at 38ºC and subjected to the same analyzes as for fresh semen.

With samples of the two treatments, the incubation was performed at 38ºC with the fluorescent probes. 40μL of semen from each treatment, 4μL of propidium iodide, 40μL of Hoechst 33342 and 100μL of FITC-PSA were added and incubated for 20 minutes, according to Celeghini, 2005.

The sperm evaluation procedures were performed as described below:
- Sperm vigor and motility: The parameters were evaluated from a drop of semen diluted between lamina and cover slip heated at 38ºC and observed under a light microscope (Olimpus®). The values of vigor (intensity of movement) were established between 0 and 5 and progressive rectilinear sperm motility between 0 and 100%, according to the norms established by the Brazilian College of Animal Reproduction (CBRA, 1998). A 400-fold increase was used.

- Morphology: An aliquot of semen was added in Karnovsky fixative until the mixture became turbid. Subsequently, slides were made by the moist chamber method, where 200 cells were evaluated using a phase contrast microscope (Nikon®) for the presence of sperm defects. The sperm defects were subdivided into major and minor ones according to CBRA, 1998.

-Hypoosmotic test (HOST): A 20μL aliquot of semen was added to 250μL sucrose solution at 150mOsmol/Kg. After 30 min Incubation at 38ºC was added 0.5mL of the Karnovsky fixative. 200 cells were counted at 1000-fold increase. Cells with folded or heavily folded tail and coiled or heavily curled tail were considered reactive to the hypoosmotic test, the result being expressed as a percentage. The similar pathologies found in the morphological analysis were subtracted from this result. The counting was performed under a phase contrast microscope (Nikon®) in a wet preparation.

-Fluorescent probes: After the incubation protocol (Celeghini, 2005), one drop of the semen was deposited between the lamina and the cover plate preheated at 38ºC and evaluated under an epifluorescence microscope (Nikon®) equipped with excitation filter (365nm) and barrier filter (410nm) in increases of 400 and 1000 times. 100 cells were counted per analysis. The results were expressed as percentage of stained cells. Two samples were read for each treatment, obtaining the mean between the samples.

Statistical analysis
For statistical analysis, the behavior of the variables between treatments was submitted to analysis by the Lilliefors test, in order to verify whether the variables were normal or not. It was also analyzed by the Cochran and Bartlett tests, to verify the homogeneity of the variances.

For the comparison of the averages of the data obtained in the semen analysis between the fresh and thawed treatments, the T test at 5% of significance was used, when they fulfilled the premises for performing parametric analysis. The variables that did not allow the parametric analysis and those of qualitative character (vigor) were analyzed by the Wilcoxon nonparametric test, also at 5% of significance. Correlations of the characteristics evaluated between and within the fresh and thawed semen treatments were obtained by Pearson's Simple Correlation at 5% significance.

The option of using only one donor animal was due to the fact that there was a large individual variation of the dogs in the response of the semen to the freezing / thawing process, so as the objective of this work was to test the efficiency of the technique there was a need for greater standardization of the samples by removing the male effect (Yu et al., 2002; Mascarenhas, 2008; Rodenas et al., 2014). Five replicates were performed, where the collected semen was evaluated fresh and after thawing. Fresh, thawed and unfeasible semen were considered as treatments. All analyzes were performed using the SAEG 9.1 software (2007).

RESULTS AND DISCUSSION

With the use of fluorescent probes, it was possible to distinguish four populations in the ejaculates analyzed (Table 1). The spermatozoa with both intact membranes showed the blue-stained nucleus by the Hoechst 33342 probe. Cells with intact plasma membrane and acrosomal membrane lesioned had the nucleus stained blue and the region corresponding to the acrosomal hood emitting green/yellow fluorescence due

to the probe FITC-PSA. Sperm with lesioned plasma membrane and intact acrosomal membrane had the nucleus stained red by propidium iodide and those with both membranes damaged, the nucleus in red and acrosome in green/yellow.

Table 1. Populations of dog spermatozoa identified by staining by association of IP, H342 and FITC-PSA probes.

Populations	H342	PI	FITC-PSA
Plasma and acrosomal membrane intact (II)	+	-	-
Plasma membrane Integral and acrosomal lesioned (IL)	+	-	+
Plasma membrane lesioned and acrosomal intact (LI)	-	+	-
Plasma and acrosomal membrane lesioned (LL)	-	+	+

H342: Hoechst 33342; IP: Propidium iodide; FITC-PSA: Fluorescein isothiocyanate conjugated to *Pisum sativum*

From the variation observed between the proportions of these populations, the association of the probes also allowed to quantify and to qualify specific damages caused by the process of freezing / thawing. Structural damages and loss of functionality of the plasma and acrosomal membrane, and the early occurrence of the acrosome reaction could be quantified by the association of probes tested. This association of fluorescent probes was also able to distinguish the same populations in bovine semen (Celeghini, 2005).

The difference of the averages between the fresh and thawed treatments were evaluated by the T test at 5% significance. Only the Minor Defects and IL population variables did not present a distribution that allowed parametric analysis, being therefore analyzed by the Wilcoxon test at 5% of significance. In this study, the results of all tests were significant to identify damage to the sperm cell by the freezing/thawing process (Table 2).

Table 2. Means of the parameters evaluated followed by the standard deviation and coefficient of variation between fresh and thawed treatments of dog semen.

	Fresh	CV Fresh	Thawed	CV Thawed
Vigor*	4,6 0± 0,22 [a]	4,86	2,60 ± 0,41 [b]	16,08
Motility %	95,00 ± 0,00 [a]	-	34,20 ± 20,32 [b]	59,43
Defects %	24,00 ± 8,86 [a]	36,91	44,00 ± 10,68 [b]	24,27
DM %	21,00 ± 1,87 [a]	8,90	64,40 ± 25,55 [b]	39,68
Dm %*	26,80 ± 16,33 [a]	60,93	23,60 ± 18,83 [b]	79,81
HOST %	77,80 ± 10,01 [a]	12,86	47,30 ± 15,92 [b]	33,65
II %	68,46 ± 7,13 [a]	10,42	8,80 ± 10,08 [b]	114,59
IL %*	0,06 ± 0,14 [a]	223,60	5,50 ± 9,84 [b]	179,07
LI %	25,86 ± 6,88 [a]	26,61	39,40 ± 24,74 [b]	62,80
LL %	8,00 ± 6,59 [a]	82,44	53,90 ± 16,18 [b]	30,03
FITC-PSA	8,06 ± 6,57 [a]	81,56	59,40 ± 16,51 [b]	27,80

Values followed by different letters on the same line differ by the T test at 5% significance. * Variables analyzed by the Wilcoxon test at 5% significance. Values followed by different letters on the same line differ statistically. CV: Coefficient of Variation / DM: Major Defects / Dm: Minor Defects / HOST: hypoosmotic / II test: both intact membranes / IL: intact plasma membrane and acrosomal lesioned / LI: Lesioned plasma membranes and acrosomal intact / LL: both membranes lesioned/ FITC-PSA: fluorescein isothiocyanates conjugated to *Psium sativum*.

Sperm vigor and motility are subjectively assessed characteristics, and therefore more subject to variation among observers, thus being difficult to compare between studies (Cunha, 2008). In this study there was a fall ($p < 0.05$) in both parameters in thawed semen. However, although the relationships between motility and fertility in vivo are conflicting, this parameter is widely used, given its practicality and being a good indicator of cellular integrity and functionality (Tardifet al., 1999), an indispensable factor for fecundation (Hafez and Hafez, 2004). Therefore, these parameters should be associated to other more specific, objective and complementary tests, in order to better predict ejaculate quality.

Cryopreservation increased the total defects ($p < 0.05$), according to Oettlé (1993), the degree of sperm defects shows a good correlation with fertility. In their study, ejaculates with a degree of defects lower than 60% had a 61% fertility rate, whereas ejaculates with a degree of defects greater than 60% presented only 13% of fertility. However, in vivo fertility results suffer from female variables such as age, nutritional status, and oocyte quality, for example (Rota et al., 1999; Santos, 2010), which greatly impairs the interpretation of results, particularly when a small number of females are used.

In this study, the total defects were divided into major and minor defects, and both showed a difference ($p < 0.05$) in fresh treatment compared to thawed treatment. Minors are defects which, although they may cause infertility, are not specifically associated with it (Blom, 1973). In the present experiment after the freezing/thawing process there was a significant increase in only certain pathologies, especially a strongly folded tail, considered as a major defect by the CBRA, 1998. According to Blom (1973), the tail is strongly bent in bulls to defects associated with epididymal transit, and of genetic origin. In the present study it was associated with the freezing / thawing process. Other pathologies, including all minor defects, did not contribute significantly to the final semen qualification. In this study, most minor defects were due to tail folding.

The hypoosmotic test is used to check the functionality and integrity of the plasma membrane. The spermatozoid exposed to a hypoosmotic medium responds to the medium by gaining water, which results in winding and tail bending (England and Pummer, 1993). During the freezing / thawing process destabilization of the phospholipid bilayer composing the plasma membrane occurs (Kim et al., 2010). In this study there was a decrease ($p < 0.05$) in the number of reactive cells in the hypoosmotic medium of the fresh treatment for thawing. Other authors, using compositions of different hypoosmotic medium, also obtained significant results between fresh and thawed treatments (Silva, 2005; Mascarenhas, 2008), however, the different media used, as well as different incubation times, made the comparison of results unfeasible.

The IL population was composed of extremely small numbers of cells, but increased ($p < 0.05$) from fresh semen to thawed semen. The association between intact plasma membrane and injured (reacted) acrosomal membrane was a rare combination to be observed, since the acrosomal membrane is somehow protected by the presence of an intact plasma membrane. The acrosome reaction depends on a previous destabilization of the plasma membrane, which allows the influx of substances that contribute to the occurrence of the reaction (Szász et al., 2000; Almadaly et al., 2012).

The II population declined from fresh semen to thawed, while LL population increased ($p < 0.05$). This result shows sensitivity of the probes to detect deleterious changes of the freeze / thaw process in plasma and acrosomal membranes. Celeghini, 2005, using two distinct bovine semen freeze protocols, used fluorescent probes to check membrane integrity also found significant results between fresh and thawed treatments. In this study, also there was an increase in lesioned membranes and a consequent decrease in intact membranes. In sheep semen, the use of propidium iodide probes and FITC-PSA was also effective in measuring damage to the membranes by freezing (Celeghini et al., 2010).

The results obtained with the FITC-PSA probe showed a large increase of damaged acrosome membranes in the thawed semen relative to the fresh semen. This injury may due to the process known as cryocapacitation. This term was used by Cormier and Bailey (2003) to describe the changes caused by the freezing / thawing process to bovine acrosomal membrane. In this process there is still a destabilization of the phospholipid bilayer of the plasma membrane, which allows an increase of the calcium influx in the sperm cell, thus increasing its activity. Another effect of this destabilization is the creation of a favorable environment for the beginning of the acrosome reaction (Szász et al., 2000). The process of induction to early sperm capacitation due to cryopreservation in dogs is reported by Rota et al. (1999).

In fresh dog semen the percentage of total defects was positively correlated with minor defects, being these the predominant defect in fresh semen. The percentages of total defects and minor defects correlated negatively with the number of reactive cells to the hypoosmotic test ($r = -0.9781$, $p = 0.0110$ and $r = -0.9906$, $p = 0.0001$ respectively). Among the minor defects, the most observed in the samples analyzed was the folded tail. In this study, the number of cells reactive to the hypoosmotic medium in dogs was not correlated with motility, which happened in other studies (Rodriguez-Gil et al., 1994; Pinto and Kozink, 2008).

According to Hafez and Hafez (2004), the integrity of the plasma membrane prevents the acrosome reaction. The change in conformation of the plasma membrane favors the influx of calcium ions, increasing the cellular activity and creating an environment conducive to the acrosome reaction (Cormier and Bailey, 2003; Hafez et al. Hafez, 2004). Thus, in an ejaculate with predominance of cells with integral plasma membrane, a small number of reacted cross-reactive membranes is expected. In the present study, with fresh semen, as expected, population II showed a negative correlation with the LL population ($r = -0.8360$, $p = 0.0366$). These data reflect a satisfactory behavior of the combination of fluorescent probes tested. On the other hand, although the HOST test is a measure of plasma membrane integrity, no correlation was observed with populations stained as intact by fluorescent probes ($r = -0.2378$, $p = 0.3811$). In this sense, under the conditions tested, there is a greater specificity of the use of fluorescent probes in detriment of the HOST test in the identification of the plasma membrane integrity of the spermatozoa in fresh semen.

In the samples of thawed semen, a positive correlation ($r = 0.9675$, $p = 0.0162$) between vigor and motility was observed. According to Oettlé (1993) and Peña-Martinez (2004), cellular integrity is associated with fertility, being these directly reflected in the values of vigor and motility. In the thawed semen the major defects had a positive correlation with the LL population ($r = 0.9429$, $p = 0.0285$), and the largest defect found in the thawed semen was the strongly folded tail. This correlation suggests that there is a direct association between plasma membrane damage and the presence of major defects. This same correlation was not observed in fresh semen, which allows us to suppose that the freezing protocol favored the appearance of major defects in direct relation to the increase in the population of damaged plasma membrane cells. According to CBRA,1998, these pathologies are associated with an evident decrease in the fertilization potential of the cell.

Also for the thawed treatment, the results of the hypoosmotic test (number of cells that reacted to the medium) correlated positively with population II ($r = 0.8807$, $p = 0.0246$). That is, different from that observed in fresh semen, the hypoosmotic test in thawed semen presents a real predictive value in the detection of plasma membrane integrity under the conditions tested. Neves et al. (2009), working with thawed canine semen, also obtained a similar correlation, although with the use of carboxyfluorescein diacetate.

The total defects of thawed semen correlated positively with the defects and minor defects of fresh semen. Minor defects decreased ($p < 0.05$) in fresh treatment compared to thawed, while larger defects increased ($p < 0.05$). The defect present in greater quantity in the fresh semen was the folded tail whereas in the thawed semen the tail was heavily folded. Possibly, cells exhibiting folded tail, in fresh semen, evolved to a strongly folded tail during the freezing process. Farstad (1996) names this type of defect as secondary because it is associated with the process of manipulation, freezing and thawing.

CONCLUSIONS

The association of fluorescence probes: propidium iodide, Hoechst 33342 and FITC-PSA, was shown to be effective in distinguishing different populations from the integrity of plasma and acrosomal membranes to evaluate the effect of cryopreservation. In this work Hoechst 33342 proved to be efficient in the role of propidium iodide counter-dye in the measurement of plasma membrane integrity. The FITC-PSA probe was effective in detecting the integrity of acrosome membrane. Little correlation was observed between staining by fluorescent probes and the routine tests used in the evaluation of canine semen, which suggests the inclusion of probes in semen analysis protocols, since they are more accurate indicators of sperm cell injury. The use of this combination allowed to quantify and qualify damages caused by the freezing/thawing process in the semen in this preliminary study, and further studies with higher number of dogs will be necessary

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