A triple stain method in conjunction with an in-depth screening of cryopreservation effects on post-thaw sperm in dogs

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Abstract

In order to accurately analyze the possible side effects of sperm cryopreservation, an in-depth screening of post-thaw sperm status is necessary. Thus, this study aimed to identify thorough effects of sperm cryopreservation, by evaluating the integrity of all specific structures of the canine spermatozoa. Thirteen \( n=13 \) mature dogs of different breeds were selected. Six dogs \( n=6 \) were subjected to sperm cryopreservation, whereas seven dogs \( n=7 \) were used as semen donors to validate a simultaneous assessment of sperm plasmatic, acrosomal, and mitochondrial membranes (triple stain) by fluorescent probes. Fresh and post-thaw semen samples were evaluated through a computer-assisted analysis of sperm motility, sperm morpho-functional evaluation, triple stain and sperm DNA integrity. Post-thaw semen samples had lower total and progressive motility, as well as higher percentage of minor and major defects. Moreover, post-thaw samples had higher percentage of sperm with plasma membrane and mitochondrial damage but intact acrosome, and also sperm with simultaneous damaged plasma, acrosomal and mitochondrial membranes. Furthermore, post-thaw sperm had higher protamination deficiency and DNA fragmentation. In conclusion, cryopreservation has a broad impact in sperm morphology and function, altering motility patterns, plasma, acrosome and mitochondrial membranes integrity, as well as sperm DNA.

Keywords: DNA fragmentation, DNA protamination, triple stain, CASA, canine.
1. Introduction

Sperm cryopreservation is a pivotal reproductive process to maintain breeding lineages for long periods of time, aiming to multiply the genetic material even post mortem [39]. Canine sperm cryopreservation is employed mainly for dogs with high zootechnical and genetic value or unique dogs for pet owners [39]. Taking such importance into account, cryopreservation process has to guarantee semen quality after thawing and, thus achieve feasible fertility rates. However, canine sperm cryopreservation is still known to cause critical sperm outcomes due to formation of intracellular ice crystal, osmotic modification and cold shock [44], damaging sperm structure and function, ultimately compromising fertilization potential [45].

Cryopreservation is recognized to reduce the integrity of sperm plasmatic, acrosomal, and mitochondrial membranes at the same time, leading to negative impact to fertility rates [3, 41]. Hence, simultaneous evaluation of sperm plasma and acrosome membrane integrity, as well as mitochondrial function using combined dyes is a feasible and accurate manner to assess sperm fertilization potential in a practical point of view [7]. In fact, bovine sperm attested with intact plasma and acrosome membrane, and high mitochondrial function well correlate with high fertility rates [27]. The combination of the fluorescent dyes propidium iodide (PI), fluorescein isothiocyanate–conjugated Pisum sativum agglutinin (FITC), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetramethyl benzimidazol carbocyanine iodide (JC-1) has been previously validated for bovine, ovine, equine and pig spermatozoa [7, 1, 9, 10]. However, the use of a triple stain that analyses at the same time all sperm structures (plasma, acrosome and
mitochondrial membranes) has not yet proven valuable for canine sperm or systematically employed as a method for sperm quality routine work in dogs.

In addition to sperm morpho-functional changes, cryopreservation process may also disrupt sperm DNA [46], albeit there is no consensus whether canine sperm DNA undergoes fragmentation during cryopreservation. Prinosilova et al. [30] and Urbano et al. [40] have not evidenced differences on sperm DNA damage between fresh and post-thaw canine semen, whereas Kim et al. [16] showed high DNA fragmentation rates post-freezing. However, such controversial findings may be due to different techniques to identify sperm DNA damage, such as sperm chromatin structure assay (SCSA) and sperm chromatin dispersion test (SCDt). Nevertheless, distinct tests allow for a target sperm DNA analysis, enabling to infer a causative factor for the sperm chromatin damage [34]. For example, screening sperm DNA fragmentation directly by SCDt or indirectly by SCSA indicates a post-testicular etiology. In addition, the Terminal Transferase-mediated dUTP end Labeling (TUNEL) assay can directly evaluate both single- and double-stranded DNA breaks with high accuracy and specifically detect apoptosis-induced sperm DNA fragmentation [36]. The chromomycin A3 (CMA3) test evaluates sperm DNA protamination, which is a physiological process that leads to histone-to-protamine transition within the testicle during spermatogenesis, protecting sperm DNA against fragmentation [31]. Hence, an in-depth screening of sperm DNA integrity can be more accurate and sensible in detecting less obvious DNA damage and, thus, predicting the fertilizing ability of post-thaw sperm samples.
In this context, the aim of this study was to validate the triple stain (fluorescent probes) for canine sperm and propose a thorough method to analyze sperm quality considering the integrity of all specific structures of the spermatozoa, i.e., plasmatic, acrosomal and mitochondrial membranes (triple stain) and sperm DNA.

2. Materials and Methods

This study was conducted according the Ethics Committee of the Faculty of Veterinary Medicine of Ghent University and of the School of Veterinary Medicine and Animal Science—University of São Paulo (protocol number 7122171213). In addition, all owners were aware of the methodological procedures prior to entering the study and agreed with all sperm analysis.

2.1. Animals and Experimental Study

Thirteen (n=13) clinically healthy and sexually mature dogs (aged from 1 to 7 years) of different breeds and body weights were selected, considering the inter-individual variability of the canine species. All dogs were stud animals, with a history of positive reproductive performance. Six dogs (n=6), with mean age of 4.8 years, were subjected to sperm cryopreservation. The remaining seven dogs (n=7), with mean age of 3.6 years, were used as semen donors for the validation of a triple stain by fluorescent probes (simultaneous assessment of plasmatic, acrosomal, and mitochondrial membranes) in dogs.
To assure the appropriate sample size, an analysis was conducted with the SAS Power and Sample Size 12 (SAS Institute Inc., Cary, NC, EUA). A retrospective analysis of the data indicated a power of >0.99, which is considered an acceptable statistical power (at least 0.8). Hence, a minimum of 6 dogs were sufficient to demonstrate significant differences in the data.

2.2. Experiment 1

2.2.1. Semen Processing

Semen collection was performed by digital manipulation of the penis directly into plastic tubes through funnels. Only the sperm-rich fraction was collected by means of visual inspection of the ejaculate.

After collection, samples were immediately analyzed macroscopically (appearance, color and volume) and microscopically for sperm motility (conventional and subjective analysis of motility) and sperm concentration (Bürker counting chamber - Merck, Leuven, Belgium - after a 1:40 dilution with water). Only sperm samples with normal appearance and total motility of >70% and sperm count >200 million cells per mL were used.

Subsequently after semen analysis, fresh samples were subjected to a two-step freezing procedure, previously described by Rota et al. [32]. Extender without cryoprotectant (Fraction A) was added to the semen samples at 37°C kept in water bath and refrigerated until 5°C within approximately 2 hours [13]. Then, extender with 5% glycerol (Fraction B) at 5°C was added and allow for glycerolization for approximately 1 hour. Samples were placed in 0.5 mL labeled straws, at a final sperm
concentration of 100 million sperm per straw. Then, each straw was exposed to liquid nitrogen vapor for 20 minutes (4 cm above the liquid nitrogen column) and, finally, immersed into liquid nitrogen [13]. At least one month after cryopreservation, semen samples were thawed in 37°C water-bath during 30 seconds.

2.2.2 Computer-assisted analysis of sperm motility

Samples were analyzed using a computer-assisted semen analyzer (CASA), the HTR Ceros 12.1 (Hamilton-Thorne Research, Beverly, MA, USA) according to previous protocol for dogs [12]. For each sample, an aliquot (6 μL) was placed on microscope slides pre-warmed at 37°C and covered by coverslip. Five fields of view were selected randomly and the following parameters were evaluated: pathway velocity (VAP - μm s⁻¹), curvilinear velocity (CSL/VCL - μm s⁻¹), straight line velocity (VSL - μm s⁻¹), amplitude of lateral head displacement (ALH – μM/s), beat cross-frequency (BCF - Hz), straightness (STR - %), linearity (LIN - %), total motility (%), progressive motility (%), rapid motility (> 50 μm/s; %), medium motility (25–50 μm/s; %), slow motility (< 25 μm/s; %), static (%) and path wobble (departure of actual sperm track from average path, WOB = VAP/VCL; %) (Supplementary Material).

2.2.3 Sperm morpho-functional evaluation

The vital staining (eosin/nigrosine stain) was used to evaluate sperm plasma membrane integrity, according to the adapted protocol for dogs [2]: 5 μL of semen and 5 μL of the eosin/nigrosine stain were placed in a warmed (37°C) slide, mixed and smeared. Sperm smears were evaluated under light microscopy at 1000×
magnification, using immersion oil, counting 200 cells. Damaged sperm (plasma membrane lesion) were considered as pink colored cells, while intact sperm (plasma membrane integrity) presented no stain. In addition, sperm morphological evaluation was performed under light microscopy at 1000× magnification by counting 200 cells. Results were expressed as percentage (%) of sperm defects, classified as major (observed: proximal droplet, knobbed acrosome, midpiece abnormality, abnormal contour of the head, hard coiled tail, double tail and hard bent tail), minor (observed: distal droplet, detached head, coiled tail and bent tail only in terminal portion) and total (sum of minor and major defects) according to Barth and Oko, [4].

2.2.4. Sperm DNA integrity by real detection of DNA strand breaks (TUNEL - Terminal dUTP Nick End Labelling)

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Boehringer, Mannheim, Germany), which identifies the presence of free 3’-OH termini in single and double-stranded sperm DNA, according to Filiers et al. [14]. Briefly, sperm samples were diluted in PVP solution (1 mg/ml in PBS) to a final concentration of 2.5 × 10⁶ sperms. After fixation with 4% paraformaldehyde in PVP solution (pH 7.4) and permeabilization with 0.5% (v/v) Triton X-100 in PBS, sperm were incubated in TUNEL-mixture (fluorescein-dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37°C in the dark. Positive (1 mg/ml DNAse I) and negative controls (nucleotide mixture in the absence of transferase) were included in each replicate. We used Hoechst 33342 to stain sperm DNA. Images of the slides were
captured using fluorescence microscopy (Leica DMR, 400 x in oil immersion) and 200 sperm cells per slide were evaluated. Results were expressed in percentage (%).

2.2.5. Sperm DNA integrity by assessment of sperm chromatin protamination

In order to identify sperm protamination deficiency, the chromomycin A3 technique was used, following previously described protocol [31, 38]. For the positive control, we induced DNA protamination with 0.001% Triton X-100, 5 mM DTT in 200 µL of PBS for 15 minutes and then 1 M NaCl and 5 mM DTT in H₂O distilled for 2 h at room temperature in a plastic tube. Subsequently, the positive control and sperm samples were washed in PBS and fixed in Carnoy's solution (3:1 methanol : acetic acid; Merck, Darmstadt, Germany) at 4°C for 10 min. Smears were treated for 20 min with 12.5 µL of CMA3 solution (0.25 mg/mL in 1000 µL of McIlvaine buffer (7 mL of 0.1 M citric acid + 32.9 mL of Na₂ HPO₄.7H₂O, 2 M, pH 7.0, containing 10 mM MgCl₂). Semen cells were stained in Hoechst 33342 solution (5 mg/mL) for 2-5 min semen, then, slides were washed in PBS. Microscopy images of the slides were captured using fluorescence microscopy (Leica DMR, 400 x with oil immersion) with appropriate filter (460-470 nM), evaluating 200 cells per slide. Results were expressed in percentage (%).

2.3. Experiment 2

2.3.1. Sperm triple stain evaluation and validation of a simultaneous assessment of plasmatic, acrosomal and mitochondrial membranes by fluorescent probes
For the purpose of optimizing the triple stain for dog sperm, semen samples of 7 dogs were collected and diluted in TALP sperm medium, in order to reach the final concentration of 25 million cells per mL [8]. Diluted samples were divided into two aliquots: sperm kept at 37°C (considered as intact sperm) and sperm submitted to snap-freezing (considered as damaged sperm). Snap-freezing was performed by three consecutive cycles (30 seconds each) of liquid nitrogen immersion and thawing, thus damaging mitochondria, plasma and acrosome membranes. This samples were checked and no moving were observed in the sperm cells. The two aliquots (intact and damaged sperm) were then mixed in progressive and known proportions of damaged sperm (0%, 50% and 100%). Staining procedure was performed according to Celeghini et al. [8]: 150 μL of each mixture (0%, 50% and 100%) at 37°C were added to 12 μL of PI (0.5 mg/mL), 2 μL of JC-1 (153 μM) and 50 μL of FITC-PSA (100 μg/mL). Samples were incubated for 8 minutes at 37°C and then placed in a warmed slide covered by a coverslip and evaluated using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 1000×. Two hundred sperm cells per slide were examined and spermatozoa were classified in eight different classes according to the fluorescence emitted from each dye: simultaneous assessment of plasma (propidium iodide – PI), acrosomal (Pisum sativum agglutinin – FITC/PSA), and mitochondrial (JC-1) membranes (Table 1 and Figure 1).

For the fresh and post-thaw samples, the triple stain was performed as formerly described. In brief, 200 sperm cells were evaluated and classified as stated in Table 1. Results were expressed as percentage (%).
2.4. Statistical analysis

All data were evaluated using the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). For the triple stain validation, linear regression was performed with Guided Data Analysis by SAS. High linear regression coefficients were considered between the expected proportions of damaged sperm (plasma, acrosome and mitochondrial membranes). A p value <0.05 was considered statistically significant.

Differences between Fresh and Post-thaw sperm were analyzed using parametric (t test) and non-parametric (Wilcoxon) tests, according to the residual normality (Gaussian distribution) and variance homogeneity. The correlation analysis was also applied in parametric (Pearson) and nonparametric (Spearman) variables. Results were described as untransformed means ± SEM. Significant level was p<0.05.

3. Results

For the triple stain validation, we had a high linear regression coefficient (R²=0.9236) between expected percentage of integrity and spermatozoa exhibiting intact plasma membrane, intact acrosome and high mitochondrial potential (IPIAH - Class I) (Figure 2). Square roots values were higher than 0.9, thus showing that the triple stain technique has high accuracy and repeatability.

Fresh semen samples had higher total and progressive motility (88.5±3.8% and 32.2±6.7%, respectively), compared to post-thaw sperm (50.2±6.6% and 13.6±2.1%, respectively) (Figure 3A and 3B). Equally, sperm kinetics variables (rapid and medium sperm velocity, VAP, VCL and ALH) were higher in fresh semen (Table 2). On the
other hand, post-thaw samples had higher static sperm velocity compared to fresh semen (Table 2).

Regarding morpho-functional evaluations, we detected a lower percentage of minor and major defects in fresh semen compared to post-thaw sperm (Table 3). Conversely, fresh samples had higher percentage of sperm with plasma membrane integrity (eosin/nigrosine stain) compared to post-thaw samples (Table 3). Additionally, triple stain analysis revealed higher IPIAH (Class I) percentage in fresh samples (78.8±3.9%) compared to post-thaw sperm (40.1±3.6%) (Figure 4A). On the other hand, triple stain class VI and VIII sperm (plasma membrane and mitochondrial damage but intact acrosome and damaged plasma and acrosomal membrane low mitochondrial potential, respectively) were higher in post-thaw samples (20.8±4.2% and 20.5±2.5%, respectively), compared to fresh semen (5.3±1.9% and 4.6±1.9%, respectively) (Figure 4B and 4C).

Fresh semen samples had lower sperm protamination deficiency (0.85±0.26%) and sperm DNA fragmentation (0.80±0.45%) than post-thaw sperm (3±0.5% and 4.1±0.7%, respectively) (Figure 5A and 5B).

For post-thaw samples, there was a positive correlation between DNA fragmentation and triple stain class VIII (r = 0.68; P = 0.01) and a negative correlation between triple stain class I (IPIAH) and DNA fragmentation (r = -0.75; P = 0.0006).

### 4. Discussion

The present study aimed to validate a triple stain combination for dog sperm that simultaneously assessed plasma, acrosomal and mitochondrial membranes using...
fluorescent probes. Secondly, we evaluated the effects of cryopreservation on canine semen through an in depth screening of spermatozoa, including a close look on sperm DNA integrity. As we achieved a high linear regression coefficient of known proportions of damaged sperm, the triple stain combination can provide an accurate estimation of sperm cells with intact plasma, acrosomal and mitochondrial membranes (named IPIAH sperm). Similarly, Nagy et al. [24] have proven the triple stain feasible and repeatable for the precise evaluation of viability and acrosome integrity of bovine post-thaw sperm. Therefore, the triple stain allows systematic use as an effective method to evaluate canine sperm and, ultimately, identify male fertilization potential. The triple stain is easy to use, because it simultaneously shows damage at the level of the plasma membrane, acrosome and the potential of the inner mitochondrial membrane [27, 35, 21]. Thus, the triple stain can be a promising technique to select stud dogs with high fertility potential and evaluate the sperm quality after cryopreservation in routine assisted reproductive technologies (i.e. veterinary clinics with a microscope equipped with a mercury lamp and appropriate filters). However more studies must be performed observing the fertilizing ability of the triple-stained sperm after cell sorting in an in vitro or in vivo environment.

It is well known that cryopreservation decreases sperm quality, due to cold shock, osmotic stress, and generated oxidative stress, which ultimately reduces sperm fertilization ability [21, 18]. Despite this is widely know our data also showed that cryopreservation negatively affects sperm motility (total and progressive motility) and sperm membrane integrity, while increasing sperm morphological defects. These
results are not innovative and have been previously observed by several authors as classical examples of sperm cryopreservation damage [21, 5, 11]. Nonetheless, we were able to simultaneously detect altered post-thaw sperm functionalities in dogs, through the novelty triple stain assay, that can explain the poor fertility rates observed in vivo [22, 29]. However we highlighted that preparation and evaluation is can be far complicated for a routine procedure, thus the enhancement in scientific studies must be performed firstly.

Cryopreservation process is recognized to reduce mitochondrial membrane potential of the spermatozoa [37], causing pore formation and the release of proapoptotic factors into the cytoplasm, damaging plasma and acrosomal membranes [25, 26]. In addition, sperm structural damage has a pivotal role in generating increased reactive oxygen species (ROS) that triggers an oxidative environment, ultimately leading to damage of sperm DNA [15]. Indeed, we observed a positive correlation between sperm DNA fragmentation and the percentage of triple stain class VIII spermatozoa, as well as a negative correlation between percentage of sperm triple stain class I (IPIAH sperm) and DNA fragmentation. Oxidative stress can provoke mitochondrial DNA damage and, in a loop manner, generate secondary ROS that leads to the activation of stress response genes, eventually followed by apoptosis [19]. We believe that sperm morphological defects in post-thaw samples trigger a cascade of events, which leads to sperm DNA damage, such as previously described [19]. Hence, post-thaw structural sperm damage has a close relationship with the origin of sperm DNA fragmentation in dogs. Taken these data together, the triple stain can indirectly estimate sperm DNA integrity in an ejaculate with high proportion of intact sperm
cells. Thus, we suggest using the triple stain as a screening method for candidate dogs further subjected to sperm cryopreservation.

Although the effects of cryopreservation on sperm motility, morphology and membrane integrity are well documented in dogs, probable alterations of sperm DNA remains unknown, mainly the mechanisms through which cryopreservation influences DNA integrity. Among the most reported causes of DNA damage, we can underline the post-testicular acute oxidative stress, which is considered an intrinsic consequence of sperm cryopreservation [34]. Recent studies suggest that sperm DNA cryodamage is derived primarily from oxidative stress and less attributable to the activation of apoptotic pathways [33, 47]. Additionally, melting of extracellular ice during sperm thawing leads to lower extracellular solute concentration, which may promote the formation of intracellular ice crystals, physically disrupting sperm structure [28, 23]. Such chemical-physical alterations can also lead to irreversible cell damage by histone exposure and sperm chromatin desproamination [28, 23]. In the present study, we observed higher rates of sperm DNA breaks (by TUNEL assay) and chromatin protamination deficiency (by chromomycin A3) in canine post-thaw samples, compared to fresh semen. These data directly denote modifications in sperm DNA structure. Thus, cryopreservation damages spermatozoa chromatin in dogs. Additionally, our data allow for the establishment of a sequence in sperm cryodamage process, by which physical and chemical factors cause disruption of sperm DNA protamination (as a primary chromatin damage) which, in turns, results in sperm DNA breaks through higher DNA accessibility. In other words, the destructive effect of cryopreservation to the integrity of the sperm membrane causes changes in osmotic
pressure, which itself leads to DNA damage [17]. Indeed, we observed a positive correlation between DNA fragmentation and triple stain class VIII sperm (damaged plasma membrane and acrosome low mitochondrial potential). Hyperosmotic stress can lead to an increased frequency of DNA double-strand breaks by mechanical strain on the DNA molecule, formation of free radicals and changes of chromatin compactness [17]. Relaxation of chromatin post-cryopreservation is the main cause of a physical cryoinjury of the tertiary structure of the DNA–protamine complex [20]. Some authors suggest that DNA degradation can also be a result of endogenous endonuclease activation in membrane-damaged sperm [42].

Despite significant differences between fresh and post-thaw samples, the percentage of post-thaw sperm DNA damage is low (3±0.5% and 4.1±0.7%, respectively for chromomycin A3 and TUNEL) in comparison to the overall assessment of sperm quality. In men, sperm DNA fragmentation ratio after cryopreservation can reach 36% by TUNEL assay [6]. Hence, it is not possible to estimate the actual impact of sperm DNA damage on in vivo fertilization rates, embryonic and fetal development, as oocyte and embryo have specialized mechanisms to repair sperm DNA fragmentation [43]. Thus, future studies should be undertaken to analyze the in vivo impact of post-thaw sperm samples on reproductive efficiency in dogs.

In conclusion, triple-stain method is feasible in detecting simultaneous plasma and acrosome membrane integrity and functional status of the mitochondria of dog sperm cells. In addition, as previously described canine sperm cryopreservation has a broad impact in sperm morphology and viability, since altered motility patterns,
lesions of plasma, acrosome and mitochondrial membranes, as well as sperm DNA damage occur during freezing.

5. Declaration of interest

The authors declare that there is no conflict of interest.

6. Funding

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7. Author Contribution Statement

D.S.R.A., L.C.B., N.L.L. and B.L. carried out the study. D.S.R.A. wrote the manuscript with support from C.I.V. Moreover, A.V.S. helped supervise the experiments. D.S.R.A., M.N. and A.V.S. conceived the original idea and A.V.S. supervised the project.

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Figure 1. Simultaneous assessment of sperm plasma (propidium iodide – PI), acrosomal (*Pisum sativum* agglutinin – FITC/PSA) and mitochondrial (JC-1) membranes by triple stain fluorescent probes in dogs. (A) Spermatozoa classified as Class I: intact plasma membrane, intact acrosome and high mitochondrial potential. (B) Spermatozoa classified as Class VI: damaged plasma membrane, intact acrosome low mitochondrial potential. (C) Spermatozoa classified as Class VIII: damaged plasma membrane and acrosome low mitochondrial potential.
Figure 2. Regression analysis of the Triple Stain Class I (IPIAH) - intact sperm plasma membrane, intact acrosome and high mitochondrial potential in dogs. Linear least square regression of proportions between intact/damaged spermatozoa structures (0%, 50% and 100%) was used to determine relationships between expected and measured percentages of structures integrity (acrosome and plasma membrane integrity and high mitochondrial potential).
Figure 3. Total (A) and progressive (B) motility by computer-assisted sperm analysis (CASA) of fresh and post-thaw sperm samples in dogs. *indicate significant differences (P<0.05).
Figure 4. Simultaneous assessment of sperm plasma, acrosomal and mitochondrial membranes by triple stain of fresh and post-thaw sperm samples in dogs. (A) Triple Stain Class I - Intact plasma membrane, intact acrosome and high mitochondrial potential. (B) Triple Stain Class VI - Damaged plasma membrane, intact acrosome and low mitochondrial potential. (C) Triple Stain Class VIII - Damaged plasma membrane, damage acrosome and low mitochondrial potential. *indicate significant differences (P<0.05).
Figure 5. Sperm protamination deficiency (A) and sperm DNA fragmentation (B) of fresh and post-thaw sperm samples in dogs. * indicate significant difference (P<0.05).
Table 1. Sperm classification by simultaneous assessment of plasma (propidium iodide – PI), acrosomal (*Pisum sativum* agglutinin – FITC/PSA) and mitochondrial (JC-1) membranes by triple stain fluorescent probes in dogs.

<table>
<thead>
<tr>
<th>Sperm Cell</th>
<th>PI</th>
<th>FITC-PSA</th>
<th>JC-1</th>
</tr>
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<tbody>
<tr>
<td>Intact plasma membrane, intact acrosome and high mitochondrial potential (IPIAH - Class I)</td>
<td>-</td>
<td>-</td>
<td>RED</td>
</tr>
<tr>
<td>Intact plasma membrane, intact acrosome low mitochondrial potential (Class II)</td>
<td>-</td>
<td>-</td>
<td>GREEN</td>
</tr>
<tr>
<td>Intact plasma membrane, damaged acrosome and high mitochondrial potential (Class III)</td>
<td>-</td>
<td>+</td>
<td>RED</td>
</tr>
<tr>
<td>Intact plasma membrane, damaged acrosome low mitochondrial potential (Class IV)</td>
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<td>+</td>
<td>GREEN</td>
</tr>
<tr>
<td>Damaged plasma membrane, intact acrosome and high mitochondrial potential (Class V)</td>
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<td>-</td>
<td>RED</td>
</tr>
<tr>
<td>Damaged plasma membrane, intact acrosome low mitochondrial potential (Class VI)</td>
<td>+</td>
<td>-</td>
<td>GREEN</td>
</tr>
<tr>
<td>Damaged plasma membrane, damaged acrosome and high mitochondrial potential (Class VII)</td>
<td>+</td>
<td>+</td>
<td>RED</td>
</tr>
<tr>
<td>Damaged plasma membrane, damaged acrosome low mitochondrial potential (Class VIII)</td>
<td>+</td>
<td>+</td>
<td>GREEN</td>
</tr>
</tbody>
</table>
PI positive (+) = red stained nucleus, FITC-PSA positive (+) = green acrosome region. PI negative (-) = unstained nucleus, FITC-PSA negative (-) = unstained acrosome.

Table 2. Mean and standard error (X ± SEM) and P value of computer-assisted sperm analysis (CASA) in fresh and post-thaw sperm samples in dogs.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fresh</th>
<th>Post-thaw</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Static Sperm (%)</td>
<td>10.8±3.3*</td>
<td>49.8±6.5</td>
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<td>Rapid Sperm Velocity (%)</td>
<td>18.7±2.3*</td>
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<td>Medium Sperm Velocity (%)</td>
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<td>9.3±1.9</td>
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<td>Slow Sperm Velocity (%)</td>
<td>45.6±7.7</td>
<td>30.1±5</td>
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<tr>
<td>Straightness (%)</td>
<td>80.8±6.7</td>
<td>87.5±0.6</td>
<td>0.296</td>
</tr>
<tr>
<td>Linearity (%)</td>
<td>53.2±7.9</td>
<td>61.3±1.6</td>
<td>0.368</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>64.6±4.3*</td>
<td>43.1±3.8</td>
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<tr>
<td>VSL (µm/s)</td>
<td>52.7±6.2</td>
<td>37.7±3.2</td>
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<td>VCL (µm/s)</td>
<td>100.9±6.2*</td>
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<td>BCF (Hz)</td>
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<td>ALH (µm/s)</td>
<td>3.9±0.2*</td>
<td>2.5±0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Wobble (%)</td>
<td>64.5±4.5</td>
<td>70±1.8</td>
<td>0.259</td>
</tr>
</tbody>
</table>
Table 3. Mean and standard error (X ± SEM) and P value of sperm morpho-functional evaluation in fresh and post-thaw samples.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fresh</th>
<th>Post-thaw</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor sperm defects (%)</td>
<td>3±0.72*</td>
<td>19.8±6.4</td>
<td>0.0211</td>
</tr>
<tr>
<td>Major sperm defects (%)</td>
<td>20.5±6.5*</td>
<td>5.6±1.0</td>
<td>0.0224</td>
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<tr>
<td>Total sperm defects (%)</td>
<td>25±6.3</td>
<td>25.8±7</td>
<td>0.9313</td>
</tr>
<tr>
<td>Normal spermatozoa (%)</td>
<td>76.4±6.6</td>
<td>74±6.9</td>
<td>0.8057</td>
</tr>
<tr>
<td>Plasma membrane integrity (%)</td>
<td>97±0.6*</td>
<td>72.1±5.9</td>
<td>0.0087</td>
</tr>
<tr>
<td>Triple Stain – Class II (%)</td>
<td>2±1.3</td>
<td>0.5±0.3</td>
<td>0.3304</td>
</tr>
<tr>
<td>Triple Stain – Class III (%)</td>
<td>2.6±1.4</td>
<td>2.1±0.7</td>
<td>0.7740</td>
</tr>
<tr>
<td>Triple Stain – Class IV (%)</td>
<td>0.6±0.3</td>
<td>0.1±0.1</td>
<td>0.2094</td>
</tr>
<tr>
<td>Triple Stain – Class V (%)</td>
<td>4.3±1.2</td>
<td>7.6±2.4</td>
<td>0.2512</td>
</tr>
<tr>
<td>Triple Stain – Class VII (%)</td>
<td>2.8±1.1</td>
<td>7.5±2.2</td>
<td>0.0876</td>
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</tbody>
</table>
Supplementary Material. Technical settings of the computer-assisted semen analyzer (CASA) as used in the present study.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cut-off value</th>
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</thead>
<tbody>
<tr>
<td>Depth of sample chamber (µm)</td>
<td>10</td>
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<tr>
<td>Temperature of analysis (°C)</td>
<td>38</td>
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<tr>
<td>Minimum cell size (µm)</td>
<td>5</td>
</tr>
<tr>
<td>Low APV cut-off (µm/s, LVV)</td>
<td>15</td>
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<tr>
<td>Medium APV cut-off (µm/s, MVV)</td>
<td>50</td>
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<tr>
<td>Frame rates (frames/sec)</td>
<td>25</td>
</tr>
<tr>
<td>Frames acquired (frames/object)</td>
<td>25</td>
</tr>
<tr>
<td>Minimal straightness (%; STR) for progressive fast sperm</td>
<td>75</td>
</tr>
<tr>
<td>Minimal linearity (%; LIN) for circular motility</td>
<td>50</td>
</tr>
<tr>
<td>Connectivity</td>
<td>14</td>
</tr>
</tbody>
</table>