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Original article

Effect of Tris-Citric- Fructose- Asolectin extender on mitochondrial activity and intracellular reactive oxygen species in cryopreserved dog semen

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Abstract

The aim of this study was to evaluate the cryoprotective capacity of Tris-Citrate-Fructose (TCF) extenders supplemented with soy lecithin (asolectin) at concentrations of 0.05% (Asol 0.05%) and 0.5% (Asol 0.5%), in comparison with the commonly used egg yolk-based extender (TCF-EY). Ten ejaculates, each obtained from a different dog, were included in the experiment. Both fresh and cryopreserved semen were assessed for total motility (TM) and progressive motility (PM) using computer-assisted sperm analysis (CASA). Plasma membrane integrity (LIVE), DNA fragmentation index (DFI), mitochondrial activity (MT−/PI+, MT+/PI+, MT+/PI−), and oxidative stress (CellROX+/DRD+, CellROX−/DRD−, CellROX+/DRD−) were measured by flow cytometry.

Significant differences ($p<0.05$) were found in TM (%) between the TCF-EY and Asol 0.5% groups (51.16±5.80 vs. 22.33±9.62). However, no significant differences were observed in LIVE sperm. The DFI remained below 5% in all examined groups. Compared with the control TCF-EY extender, both soy-supplemented groups showed a significant reduction in the population of viable sperm without oxidative stress. The use of asolectin-based extenders also significantly reduced the percentage of viable sperm with active mitochondria, compared to the TCF-EY extender. There were no significant differences between the tested two soy lecithin concentrations in any of the evaluated parameters..

In conclusion, the TCF-EY extender demonstrated superior efficacy in preserving semen quality after cryopreservation. Further research is needed to explore alternative phospholipid sources, including different types and concentrations of soy lecithin, and their effects on sperm fertilizing capacity.

Keywords: asolectin, dog, semen cryopreservation, sperm analysis

Introduction

Artificial insemination (AI) using frozen semen is a widely applied assisted reproductive technology (ART) in domestic dogs. The ability to preserve and internationally distribute genetic material facilitates the implementation of selective breeding strategies, allowing for the production of offspring with desirable phenotypic and behavioral characteristics (Peña et al. 2006, Lechner et al. 2022, Pulkowska-Bluj and Trzcińska 2025).

The cryopreservation process imposes significant biophysical and biochemical stress on sperm, mainly by destabilizing the plasma membrane during freezing and thawing. This can lead to intracellular ice formation and various subcellular changes, collectively termed cryoinjury (Gonzales et al. 2019). Key consequences include excessive production of reactive oxygen species (ROS), oxidative stress and lipid peroxidation, all of which compromise membrane integrity and reduce post-thaw sperm viability and fertilizing capacity. (Kim et al. 2010, Lechner et al. 2022, Foutouhi and Meyers 2022). Because of these challenges, performing only basic semen quality analyses (assessment of motility and morphology) is insufficient to predict the functional competence of sperm after thawing. In contrast, integrative assessments which focus on sperm membrane and chromatin integrity, mitochondrial function and ATP-dependent energy metabolism are considered key for determining sperm resilience to cryogenic stress (Schäfer-Somi et al. 2022).

Egg yolk is commonly used as a membrane-protective agent in extenders to minimize cryodamage during the freezing process. Its high effectiveness is associated with phospholipids such as lecithin, which stabilize the sperm membrane integrity and contribute to the maintenance of viability and motility (Farstad 2009, Mahiddine and Kim 2021). However, use of egg yolk in extenders has certain limitations. The spread of the avian influenza virus H5N1 since 2020 has raised biosecurity concerns for international semen exchange (Paz et al. 2024). Moreover, the exact composition of egg yolk is highly variable, depending on factors such as breed, diet, and environment of the laying hen. (Ledvinka et al. 2012). In addition, it can promote bacterial growth, leading to higher microbial loads than those observed in soy-based extenders (Pignataro et al. 2020, Ďuračka et al. 2024).

Soy-based alternatives such as AndroMed® and BioXcell® provide comparable or even superior post-thaw sperm quality in cattle, with improved motility and lower ROS levels (Miguel-Jimenez et al. 2012). Similar studies in dogs have shown that extenders containing relatively low concentrations of soy lecithin can effectively replace egg yolk, maintaining membrane integrity, mitochondrial function and motility. (Axnér and Lagerson 2016, Dalmazzo et al. 2018, Zakošek Pipan et al. 2020, Hermansson et al. 2021). To the best of our knowledge, this is the first publication addressing specifically the effect of asolectin, as a soybean-derived extender component, on the quality of cryopreserved dog semen.

The aim of the present study was to investigate the potential use of asolectin at concentrations of 0.5% and 0.05% in extenders for cryopreservation of canine semen. The post-thaw semen quality was compared with that obtained using widely used egg yolk-based extender.

Materials and Methods

Semen collection and inclusion criteria

The experiment was conducted using ten ejaculates (n=10), each obtained from a different stud dog (n=10) aged between 18 months and 7 years. The dogs represented various breeds, including Yakutian Laika (n=2), Polish Greyhound (n=2), Broholmer (n=1), Miniature American Shepherd (n=2), Miniature Schnauzer (n=2), and Polish Lowland Sheepdog (n=1). All animals were privately owned and registered with the Polish Kennel Club. The dogs were maintained on a complete, balanced commercial dry diet and had not received any antibiotic treatment for a minimum of 30 days prior to semen collection.

All semen collections were conducted between September and December in order to minimize the impact of seasonal variation, particularly the decline in sperm plasma membrane integrity and post-thaw motility that has been reported during certain periods of the year (Lechner et al 2022). Olfactory stimulation was performed using vaginal swabs obtained from bitches in estrus, facilitating the induction of physiological erection. Semen was collected via the manual massage technique, a method commonly used in canine reproductive studies.

A standardized manual stimulation of the *bulbus glandis* was performed for approximately one minute using sterile gloves. The second, sperm-rich fraction of the ejaculate was collected directly into a sterile 15 ml conical tube (Sarstedt AG and Co. KG, Nümbrecht, Germany).

Only ejaculates that met predefined quality criteria were included for further analysis. The inclusion thresholds were as follows:

Macroscopic evaluation: ejaculate appearance characterized by a greyish-white, white, or milky-white color, consistency classified as watery-milky or milky;

Table 1. Composition of extenders used for dog semen cryopreservation. All components are expressed per 100 ml of distilled water.

	TCF-EY		Asol 0.5%		Asol 0.05%	
Dilution	I	II	I	II	I	II
Tris (g)[a]	3.025	3.025	3.025	3.025	3.025	3.025
Citric Acid (g)[a]	1.7	1.7	1.7	1.7	1.7	1.7
Fructose (g)[b]	1.25	1.25	1.25	1.25	1.25	1.25
Benzylpenicillin (g)[b]	0.06	0.06	0.06	0.06	0.06	0.06
Streptomycin (g)[b]	0.1	0.1	0.1	0.1	0.1	0.1
Glycerol (ml)[b]	3	7	3	7	3	7
Egg yolk (ml)[b]	20	20	-	-	-	-
Equex Paste (ml)[c]	-	1	-	-	-	-
Asolectin from soy bean (g)[b]	-	-	0.5	0.5	0.05	0.05

[a] Pol-Aura, Dywity, Poland, [b] Sigma-Aldrich, St. Louis, MO, USA, [c] Minitube, Tiefenbach, Germany

absence of any visible contaminants (e.g., urine, blood, hair or debris).

Microscopic evaluation: total sperm motility ≥ 90%, sperm concentration ≥ 250 × 10^6/ml, and morphologically normal sperm ≥ 70%.

Semen cryopreservation protocol and extender preparation

Ejaculates were subjected to centrifugation at 720 × g for 4 minutes to separate sperm cells from seminal plasma. The resulting sperm pellet was then resuspended in a Tris-Citrate-Fructose (TCF) extender, prepared according to the protocol described previously (Rijsselaere et al. 2002). Each sample was subsequently divided into three experimental groups: a control group extended with a conventional egg yolk-based extender (TCF-EY) and two treatment groups using asolectin-based extenders at concentrations of 0.5% (Asol 0.5%, n=5) and 0.05% (Asol 0.05%, n=5) (Table 1). To prepare the asolectin-based extenders, soy lecithin powder was dissolved using a magnetic stirrer (1 hour at 400 rpm). The mixture was then centrifuged at 200 × g for 10 minutes to eliminate undissolved particles. The resulting supernatant was sequentially filtered through 20 µm and subsequently 0.22 µm membrane filters (Nalgene®, Thermo Fisher Scientific, Waltham, MA, USA) to ensure sterility and clarity of the extender.

Following the initial dilution to a concentration of 400 × 10^6 spermatozoa/ml, semen samples were placed in a water bath at 37°C and gradually cooled to 4°C over a period of 75 minutes. Upon reaching 4°C, an equal volume of Extender II was added, resulting in a final concentration of 200 × 10^6 spermatozoa/ml. The extended semen was then loaded into 0.5 ml plastic straws, each containing a final sperm concentration of 100 × 10^6 cells per straw.

Subsequently, the straws were exposed to liquid nitrogen vapor at -140°C for 10 minutes, followed by immersion in liquid nitrogen (-196°C) for long-term storage until post-thaw evaluation was performed. Assessment of sperm quality parameters

Sperm motility and concentration were assessed using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer [S.C.A.], version 5.1, Microptic, Barcelona, Spain). Both fresh and thawed semen samples were evaluated using pre-warmed Léja® counting chambers (20 µm depth) at 37°C, following dilution in Dulbecco's Phosphate-Buffered Saline (DPBS, Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 25 × 10^6 spermatozoa/ml. CASA motility module settings: frame rate of 25 fps and a minimum head size of 15 µm². Data were collected from five to seven fields (up to 2000 spermatozoa) to assess total motility.

Sperm morphology was evaluated by analyzing a minimum of 200 spermatozoa per sample. Smears were prepared from 10 µl aliquots of raw semen diluted in DPBS to a concentration of 25 × 10^6 spermatozoa/ml, and subsequently stained with the Diff-Quick® staining kit (RAL Diagnostics, Martillac, France), following the manufacturer's protocol. Morphological assessment was performed by a single trained observer using a light microscope (Nikon Eclipse 200, Tokyo, Japan) under oil immersion at 100× magnification. Sperm abnormalities were classified according to the morphological defect criteria described by Brito et al. (2025).

Flow cytometry assessment was performed using a CytoFlex (Beckman Coulter, Inc.) and CytExpert version 2.4.0.28 software (Beckman Coulter, Inc.). The following fluorescent dyes were utilized: MitoTracker Green (λab = 525 nm, detector 525/40 BP), Propidium Iodide (PI; λab = 690 nm; detector 690/50 BP), CellROX Green, Sytox Red Dead Cell stain, SYBR-14 (λab = 525 nm; detector 525/40 BP)

and Acridine Orange (λab = 650-690 nm – red fluorescence corresponding to various levels of DNA fragmentation or λab = 525 nm – green fluorescence for sperm without detectable DNA fragmentation; detectors: red-690/50 BP or green- 525/40). For each sample, 10000 individual sperm were measured. Gating of the sperm population to exclude debris was based on forward scatter (FSC) versus side scatter (SSC) plots.

Plasma membrane integrity of spermatozoa was evaluated using the LIVE/DEAD™ Sperm Viability Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. L7011), following the manufacturer's protocol with slight modifications. Aliquots of 1000 µl of diluted semen samples (final concentration: 5×10^6 spermatozoa/ml in phosphate-buffered saline, PBS) were transferred into cytometric tubes. Subsequently, 5 µl of SYBR-14 working solution prepared by diluting the stock solution 1:49 in distilled water was added to each tube. Samples were gently mixed and incubated in the dark at 37°C for 10 minutes. Following incubation, 5 µl of propidium iodide (PI) was added 2 minutes before flow cytometric analysis.

Spermatozoa were classified into three subpopulations based on dual SYBR-14 and PI fluorescence: dead (SYBR-14-/PI+), moribund (SYBR-14+/PI+) and LIVE: viable with intact membranes (SYBR-14+/PI-). Gating of the sperm population was performed using forward scatter (FSC) and side scatter (SSC) parameters to exclude non-sperm events and debris. Only events falling within the FSC/SSC gate and exhibiting SYBR-14 or PI fluorescence were identified as spermatozoa and included in the analysis.

Chromatin structure was evaluated using the Sperm Chromatin Structure Assay (SCSA). Prior to analysis, semen samples were diluted to a concentration of 1×10^6 sperm/ml using DPBS. To induce partial chromatin denaturation, 1.0 M hydrochloric acid (HCl) was added to lower the sample's pH to approximately 1.3 for 30 seconds. Subsequently, Acridine Orange- AO (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. A3568) was added to the sample and, after 3 minutes of incubation at 4°C, fluorescence measurements were performed using flow cytometry, detecting green fluorescence (indicating intact chromatin) and red fluorescence (indicating damaged chromatin). Spermatozoa were analyzed to determine the DFI calculated as the ratio of red fluorescence to the total fluorescence, defined as the sum of red and green fluorescence intensities.

Mitochondrial activity was assessed with MitoTracker Green (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. M7514) and PI- aqueous solution, 2 mg/ml (Sigma Aldrich, Germany, Cat. No. P4170). One milliliter of semen (5×10^6 sperm cells) was mixed with 2 µl of MitoTracker solution (1 mM MitoTracker dissolved in DMSO) and incubated at 37°C for 30 minutes in the dark. Subsequently, PI was added and after 2 minutes the samples were analyzed. MitoTracker passively diffuses across the plasma membrane, accumulates in active mitochondria, and exhibits green fluorescence. PI is a conventional dead cell stain (dead cells – red fluorescence). Double staining enabled the classification of spermatozoa into three groups: MT−/PI+: cells with damaged plasma membranes, MT+/PI+: cells with compromised plasma membranes but still exhibiting mitochondrial activity (simultaneous green and red fluorescence), MT+/PI−: mitochondrially active cells (green fluorescence).

Reactive Oxygen Species (ROS) were evaluated with fluorescent dyes: CellROX Green (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. C10444;) and SYTOX™ Red Dead Cell Stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. S34859). One milliliter of semen at a concentration of 2×10^6 was supplemented with 5 µl of CellROX (CellROX™ Green) reagent and incubated for 15 minutes at 20°C in the dark. Subsequently, 1 µl of Dead Cell Reagent- SYTOX™ stain was added, the sample was gently mixed, and incubated for an additional 15 minutes at 20°C, protected from light. The samples were centrifuged (5 min, 500 × g), the supernatant was removed, and the cell pellet was resuspended in 1 ml of DPBS. The material was then subjected to flow cytometric analysis. CellROX is a non-fluorescent dye in its reduced state, which emits green fluorescence upon oxidation. SYTOX is a dead cell stain that produces red fluorescence when bound to DNA. Double staining enabled the classification of spermatozoa into three groups: CellROX+/DRD+ (sperm emitting red fluorescence – dead cells containing ROS), CellROX−/DRD− (unstained sperm – live cells without ROS and with intact plasma membranes), CellROX+/DRD−(sperm exhibiting green fluorescence – live cells containing ROS).

Statistical analysis

The obtained results were analyzed using Statistica 13 software (StatSoft Inc., Tulsa, USA). All values are expressed as mean ± SEM (standard error of mean), with statistical significance defined as $p<0.05$. The significance of differences between means was assessed using a one-way analysis of variance (ANOVA). When significant differences were observed between variables, post hoc comparisons were performed using Tukey's test. Pearson's linear correlation coefficient (r) was calculated, and its statistical significance (p-value) was assessed for assessment of the relationship between the analyzed quality parameters.

Table 2. Values of qualitative semen parameters in fresh canine ejaculates (n=10).

Sperm parameters of fresh semen		Mean (%) ± SEM
Motility	TM	95.86±0.58
	PM	53.56±2.25
DFI		4.50±0.01
LIVE		70.61±0.02
Mitochondrial Activity	MT−/PI+	4.99±0.01
	MT+/PI+	10.93±0.01
	MT+/PI−	84.08±0.02
Oxidative Stress Detection	CellROX+/DRD+	17.47±0.03
	CellROX−/DRD−	1.19±0.00
	CellROX+/DRD−	70.31±0.03
Morphology	Normal	82.40±1.63
	Head defects	2.72±0.63
	Midpiece defects	5.81±1.13
	Tail defects	9.91±1.88

TM – total motility, PM – progressive motility, DFI – DNA fragmentation index, MT−/ PI+: dead and mitochondrially inactive sperm, MT+ PI+: dead and mitochondrially active sperm, MT+/PI−: live and mitochondrially active sperm, CellROX+/DRD+: dead sperm containing ROS, CellROX−/DRD−: live sperm without ROS, CellROX+/ DRD−: live sperm containing ROS

Graphs and statistical plots for grouped and column data were generated using GraphPad Prism software (version 8.0.1, GraphPad Software, San Diego, CA, USA).

Results

The present study demonstrated that the cryopreservation process reduced sperm motility, regardless of the extender used (Table 2). No statistically significant differences in sperm motility were observed between the control (TCF) and the Asol 0.05% groups. In contrast, the use of the Asol 0.5% extender resulted in a significant reduction in TM (%).

Mitochondrial activity was significantly lower in semen samples preserved with lecithin extenders compared to those diluted in the TCF-EY extender. Asolectin notably reduced the proportion of live and mitochondrially active sperm cells. However, the concentration of asolectin did not significantly affect the relative distribution of mitochondrial activity subpopulations identified via MitoTracker staining. Moreover, at all tested concentrations, a significant reduction was observed in the population of viable spermatozoa without oxidative stress compared to the control groups (Figure 1). The tested concentrations of asolectin did not result in a significant reduction in the proportion of morphologically normal sperm compared to the control extender (Figure 2).

In fresh semen, a strong correlation was observed between the following assessment parameters: live sperm and MT+/PI− ($p = 0.001$), moribund sperm and MT+/PI+ ($p = 0.03$), dead sperm and CellROX+/DRD+ ($p = 0.006$), live sperm and CellROX+/DRD− ($p = 0.001$), and MT+/PI− and CellROX+/DRD− ($p = 0.001$).

In frozen semen, a significant correlation was found between the following parameters: dead sperm and CellROX+/DRD+ ($p = 0.029$ for TCF-EY, $p = 0.003$ for Asol 0.05%), and live sperm and CellROX+/DRD− ($p = 0.013$ for TCF-EY).

Discussion

The obtained results are consistent with those previously published on the use of soy lecithin extenders for canine semen cryopreservation. The mean TM (%) values observed in our study (22.33±9.62 for Asol 0.5% and 32.23±4.16 for Asol 0.05%) are comparable to the results reported by Hermansson et al. (2021) for the TCF extender containing 1% soy (22.0±3.3 for soy type II and 26.2±4.3 for soy type IV). Axnér and Lagerson (2016) obtained TM (%) results of 24.2±15.5 for 1% and 18.0±15.0 for 2% soy bean lecithin extenders. Compared with the aforementioned authors and ours results, higher values of total motility TM (%) were obtained by Beccaglia et al. (2009), who used 0.04% soybean lecithin (42.0±7.43) and by Sánchez--Calabuig et al. (2017) (35.9±5.5), who used the soy-based extender at an unspecified concentration. The cryopreservation procedures applied in our study did not significantly affect the DFI. In all tested groups, the index value remained below 5%, indicating no impairment in the fertilizing potential of the semen (Evenson et al. 1994, Niżański et al. 2012). In contrast to our findings, Kim et al. (2010) reported an increase

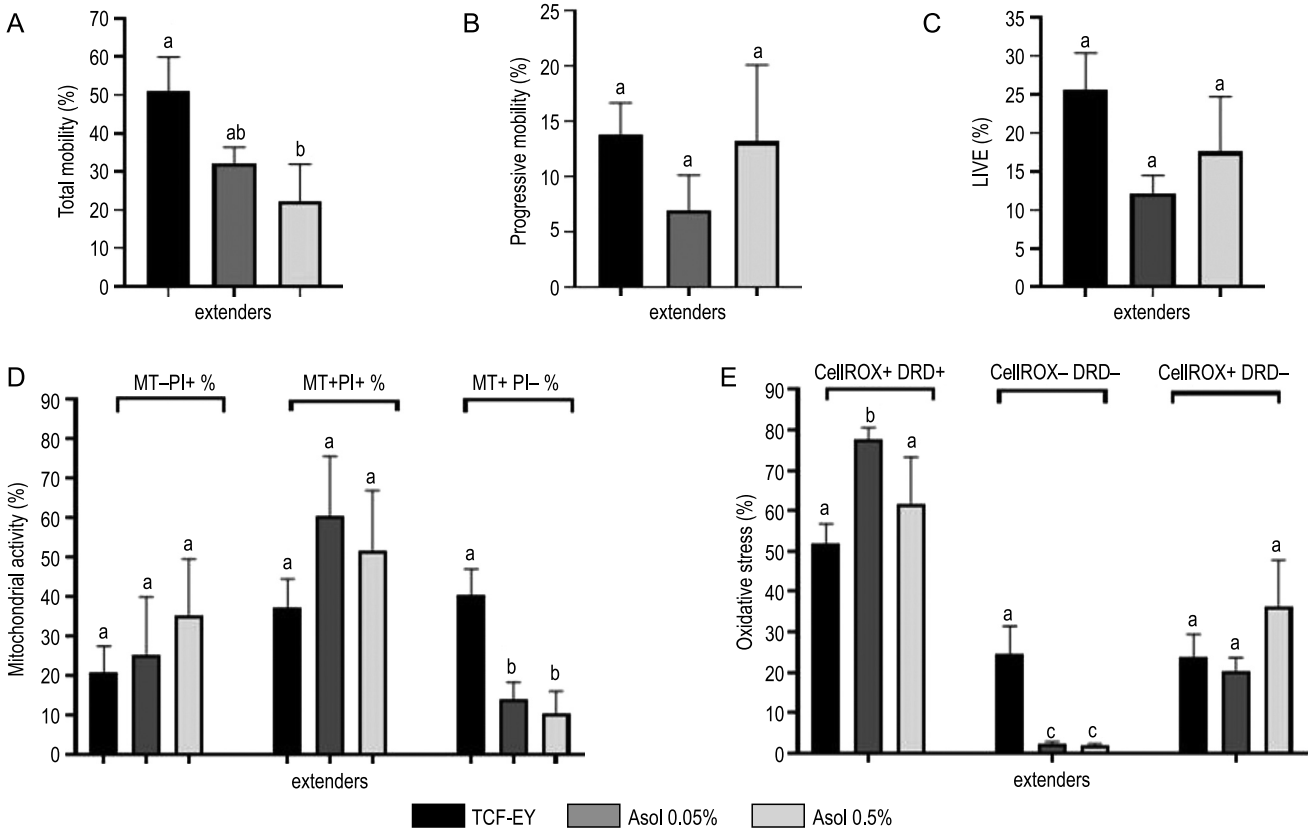

Fig. 1. A-E. Parameters and values used for the assessment of quality of dog thawed semen. Different colors represent the respective extenders evaluated. Values marked with different letters (a, b, c) differ significantly at $p<0.05$. MT−/ PI+: dead and mitochondrially inactive sperm, MT+ PI+: dead and mitochondrially active sperm, MT+/PI−: live and mitochondrially active sperm, CellROX+/ DRD+: dead sperm containing ROS, CellROX−/DRD−: live sperm without ROS, CellROX+/ DRD−: live sperm containing ROS

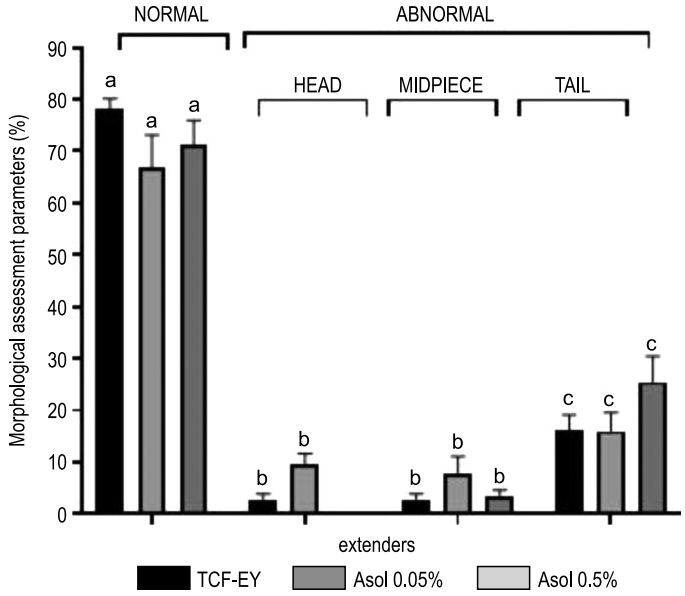

Fig. 2. Values of sperm morphology assessment of thawed dog semen. Different colors represent the respective extenders evaluated. Values marked with different letters (a, b, c) differ significantly at $p<0.05$.

in DFI in frozen dog semen to approximately 6.47%. Interestingly, other authors reported DFI values above 10% for 1% soybean extender (Hermansson et al. 2021).

The mechanism of free radical detection using the CellROX dye requires detailed discussion to ensure proper interpretation of the presented results. This method detects the presence of reactive oxygen species

(ROS), such as the hydroxyl radical (•OH) and the superoxide anion (O_2^-) (Palacin-Martinez et al. 2024). Both forms of oxygen are biological by-products of cellular respiration. Additionally, hydroxyl radicals are among the most destructive ROS, particularly harmful to proteins, lipids and DNA (Foutouhi et al. 2022).

In our study, the population of cells in which ROS were not detected was marginal, both in fresh and cryopreserved semen. The population indicating the presence of ROS showed a strong correlation with mitochondrially active and viable sperm. These findings suggest that in fresh semen, a high proportion of ROS does not necessarily indicate oxidative stress leading to cell damage, but rather reflects increased mitochondrial activity and intense metabolic processes aimed at producing energy for viable and motile sperm cells. Our results are consistent with the conclusions presented by Plaza Davila et al. (2015), who studied ROS production following experimental inhibition of mitochondrial respiratory complexes I and III in fresh stallion semen samples. They demonstrated that inhibition of these respiratory complexes led to a decrease in the CellROX positive population, confirming the affinity of this dye for mitochondrially active cells in fresh semen.

In contrast, in our study, high ROS levels in frozen-thawed semen were associated with cell death, which may be linked to the destructive effects of •OH and O_2^- According to this hypothesis, the sperm cell membranes in the Asol 0.05% group were particularly susceptible to oxidative damage caused by hydroxyl radicals, likely due to the low phospholipid content in the extender used. Consequently, the highest percentages of oxidatively stressed and dead cells were observed in these samples. This was also reflected in a significant correlation with the proportion of dead sperm parameter in fresh and frozen semen.

Investigating the effect of the soybean-based extender on mitochondrial activity is important for assessing the appropriate phospholipid content and their ability to protect the function of the mitochondrial membrane. As mentioned before, oxidative phosphorylation, which is essential for energy production, occurs within the inner mitochondrial membrane. We observed that plasma membrane integrity is closely associated with mitochondrial activity, which has been previously reported (Volpe et al. 2009). The results demonstrated that following the freezing process, the percentage of live and mitochondrially active spermatozoa significantly decreased, in favor of the population that is mitochondrially active but has damaged plasma membranes in each of the tested extenders. This phenomenon can be explained by active energy production through oxidative phosphorylation, which aims to maintain cell survival, although it is insufficient to sustain high motility (Rigau et al. 2002), which is also supported by the positive correlation between the moribund (%) parameter and the percentage of dead sperm with active mitochondria. Currently, there are few publications describing the use of MitoTracker staining to assess mitochondrial activity in dog sperm via flow cytometry. A decreasing trend was observed in the population stained with MitoTracker following the use of the soy-based extender (43.0±5.4%) compared to the commercial egg yolk-based extender CaniPRO (90.2±5.2%) (Sánchez-Calabuig et al. 2017). Our analysis did not reveal a statistically significant association between mitochondrial activity and motility, as is commonly observed in human semen (Piomboni et al. 2012, Agnihorti et al. 2016).

Moreover, the available research findings still do not resolve which type of soybean lecithin, and at what concentration, can be used to provide protection against cold shock. Dalmazzo et al. (2014) studied the concentrations and types of soybean lecithin and concluded that the use of soybean lecithin of the FP40 and 8160 types at concentrations of 0.05-0.1% is detrimental to sperm cells. In contrast, they demonstrated that Solec-F soybean lecithin at lower concentrations (0.01-0.1%) resulted in motility and mitochondrial activity comparable to those observed with an egg yolk extender. Alternatively, Hidalgo et al. (2014) demonstrated effectiveness comparable to egg yolk-based extenders when using a 1.5% solution of soybean lecithin (type not specified). Therefore, in our study, we tested asolectin – a standardized phospholipid mixture with higher purity than conventional soybean lecithin (Johns et al. 2015).

In summary, the comparison of asolectin-based extenders to the commonly used TCF-EG extender showed a statistically significant increase in dead and oxidative stress-positive cells (for both 0.05% and 0.5% asolectin extenders) and a decrease in the population of live and mitochondrially active spermatozoa (Asol 0.5%). This indicates that the concentrations of asolectin proposed by us in TCF solutions do not significantly affect viability, morphology, or plasma membrane integrity. Nevertheless, we observed a decreasing trend in all these parameters following the use of asolectin compared to the TCF-EY extender. No significant differences were observed in any of the semen quality analyses between the Asol 0.05% and Asol 0.5% groups.

An objective evaluation of soy-based extenders should be conducted based on an extended functional assessment. This approach could significantly improve the sanitary safety of frozen sperm and help determine their fertilizing properties. Considering the results of semen quality analyses, the egg yolk-based extender exhibits the most effective cryoprotective properties.

The substitution of egg yolk with soy-derived substances should be considered only at low concentrations

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Author Declarations

Ethics approval

Ethical approval was not required, as the semen samples were collected from dogs undergoing routine fertility examinations performed at the owners' request for diagnostic purposes. With the owners' informed consent, the material obtained during these procedures was subsequently used for scientific analysis.

Use of generative artificial intelligence

No generative artificial intelligence (AI) tools were used in the preparation of this manuscript.

Conflict of interest

The authors declare that they have no financial, personal, or institutional conflicts of interest related to this work.

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