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Evaluation of Metformin Supplemented Diluent on Cryopreservation of Canine Semen

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ABSTRACT

Background: Cryopreservation of semen in canines is getting more advanced lately, however the fertility following the insemination of the frozen thawed semen is lower than other species. The higher content of PUFA and lipid peroxidation leading to production of reactive oxygen species is one of the reasons for the same. Antioxidants play a role in improving the post thaw semen quality in canines.

Methods: Semen collected from 10 male dogs were further divided into two aliquots. The first aliquot was extended with TRIS-fructose egg yolk extender and the second one was extended with TRIS-fructose egg yolk extender supplemented with 50 µM of metformin. The extended semen was cryopreserved using conventional methods. Subsequently, both routine semen parameters and advanced semen parameters including viability, acrosomal integrity, DNA integrity and mitochondrial reactive oxygen species (ROS) were measured in the spermatozoa through the utilization of flow cytometry.

Result: Present study indicated that, post thaw motility was significantly higher in the metformin supplemented group in comparison with control while, the mitochondrial ROS was significantly lower in metformin supplemented group. Our study concludes metformin could be effectively used to reduce the ROS produced during the process of cryopreservation of the canine semen.

Key words: Canine semen cryopreservation, Flowcytometry, Metformin.

INTRODUCTION

Sperm cryopreservation aims at preserving the fertilizing ability of the spermatozoa along with its viability (Cerolini *et al*., 2001). The factors affecting the cryopreservation of canine spermatozoa includes but not limited to low resistance to cooling (Hori *et al*., 2014), sensitivity to fructose (Bucci *et al*., 2011), higher ratio of cholesterol: phospholipid ratio along with high concentrations of poly unsaturated fatty acids on the membrane which makes it susceptible to lipid peroxidation (Bencharif *et al*., 2008).

Reactive Oxygen Species (ROS) that is produced by the spermatozoa during the cryopreservation due to lipid peroxidation of the sperm membrane in response to the oxidative stress has been attributed as one of the important reasons for the damages that are associated with semen cryopreservation (Dutta *et al*., 2020). Unlike other cells, spermatozoa possess very low antioxidant enzyme activity of superoxide dismutase, glutathione peroxidase, peroxiredoxin, thioredoxin, thioredoxin reductase *etc.* which makes it less capable in fighting against the ROS that is produced during the process of cryopreservation (Kumaresan *et al*., 2020). Increased level of ROS coupled with deficiency in antioxidants results in the oxidative stress resulting in nuclear and mitochondrial DNA damage, telomere shortening, epigenetic alterations and Y chromosomal microdeletions (Bui *et al*., 2018).

Reducing the oxidative stress to the spermatozoa during the process of cryopreservation remains one of the major interventions to improve the post thaw semen quality. Recently, metformin, a drug used to treat various conditions like diabetes mellitus and cancer has also been shown to protect cells from oxidative damages by acting on AMP associated protein kinase pathways. Metformin is a molecule of the biguanide family and has the ability to decrease reactive oxygen species (Montalvo *et al*., 2013) and to activate the transcription factor Nrf2, resulting in increased expression of antioxidant genes (Onken *et al*.,

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2010). At the cytoplasmic level, metformin is able to lower the activity of mitochondrial complex I, which results in less reactive oxygen species. Metformin, molecule is reported to have been modulating the cell metabolism through the mitochondria activity and was tested in extender for the cryopreservation of mouse (Bertoldo *et al*., 2014), chicken (Nguyen *et al*., 2014), stallion (Cordova *et al*., 2014) and boar (Hurtado *et al*., 2015) spermatozoa. Although metformin has been reported to increase the fertilizing capacity of spermatozoa and improve the post thaw motility in canines (Grandhaye *et al*., 2020) due to its effect on oxidative stress reduction and the role of metformin on different sperm functional attributes is not clear. In this regard, the present study was designed to understand the cryoprotectant effects of metformin and further its effect on the sperm functional attributes.

MATERIALS AND METHODS

Preparation of 50 µM metformin solution

Metformin was procured as 500 g powder (Sigma Aldrich ®, St Louis, USA) and the final concentration used was 50 µM. A stock solution of 1 M was prepared from which a second stock of 500 µM was further prepared. Final dilution was achieved by diluting 10 ml of the second stock with 90 ml of diluent (Tris egg yolk fructose) to make the final volume of 100 mL. All dilutions were made in normal saline as indicated by (Bencharif *et al*., 2008). The final concentration of 50 µM was chosen based on the results of previous reports who claimed that 50 µM concentration of metformin exhibits activity without adverse effects on germ cells (Bertoldo *et al*., 2016; Faure *et al*., 2018).

Semen collection and processing

The study involved ten male dogs of different breeds which were referred to the Small Animal Gynaecology Unit (SAC-OP-OG) of the Madras Veterinary College Teaching Hospital, Chennai, Tamil Nadu, India for evaluation of their semen during the period of June 2020 to December 2022. Semen ejaculates were collected by digital manipulation technique in absence of oestrus bitch. The collected semen samples were divided into 2 groups *viz*. Group I, Group II with each group comprising of 10 samples. The first group was designated as control and was extended with TRIS-fructose egg yolk extender and the second group was supplemented with 50µM of metformin in TRIS-fructose egg yolk extender. The final concentration was 150 to 200 $\times 10^6$ spermatozoa per mL of diluted semen. The dilution was done at 37°C and cooled down to proceed for equilibration. The diluted semen samples were cooled to 4°C in 1 hour. The equilibration was performed in cold handling cabinet at 5°C for 30 minutes. The equilibrated semen was filled in French mini straws (0.25 mL) and the laboratory end of straws were filled with polyvinyl alcohol (PVA) powder for sealing the sealed ends. The straws were then dipped into ice chilled water bath for 2 minutes for firm sealing. Post equilibration, the samples were checked for pre-freeze motility before proceeding for racking. After racking, the straws were placed horizontally at a height of 4 cm above the liquid nitrogen level in a styrofoam box and exposed to the vapours of liquid nitrogen for 10 minutes and the straws were plunged into liquid nitrogen at -196°C till further use.

Evaluation of semen

The different gross and microscopic examination of the semen is evaluated in the study. Grossly, volume, colour and consistency has been assessed while, the microscopic analysis included progressive motility, concentration, viability, acrosomal integrity and abnormalities as described by (Patti *et al.,* 2021).

Flow cytometric evaluation of sperm functional attributes

Sperm functional attributes were assessed flow cytometrically to understand the protective effect of metformin on the spermatozoa during cryopreservation. We assessed plasma membrane integrity, acrosomal status, sperm mitochondrial reactive oxygen species (ROS) and DNA integrity in prefreeze and post thaw semen of both control and treatment groups.

Preparation of the spermatozoa

The frozen semen straws were thawed at 37°C for 30 seconds. The samples were washed twice with Sp-TALP medium by centrifugation at 2000 rpm for 5 min. The sperm pellet was then resuspended in 200 µl of Sp-TALP medium. The sperm preparation procedure for all flow cytometric assays remains same unless and otherwise mentioned. Flow cytometric analysis was performed by using Beckman Coulter (MoFloTMXDP) fitted with a bevelled tip. A total of 10,000 spermatozoa events were assessed for each sample. After gating out to exclude debris, spermatozoa were categorized as applicable to the individual tests.

Plasma membrane integrity

Plasma membrane integrity of the spermatozoa was assessed using the combination of SYBR-14 (Invitrogen, Thermo Fisher scientific, USA) and Propidium iodide (Invitrogen, Thermo Fisher scientific, USA) dyes as described by Nag *et al.* (2021). Stock solution of the SYBR14 (1mM) was diluted 50 times in dimethyl sulfoxide to prepare the working solution. For 2 million spermatozoa in 200 mL of sp TALP, 1.2 µL was added and incubated at 37°C for 10 min in the dark. This was followed by the addition of 2 µL of propidium iodide (2.4 mM) and further incubated for 2 min and analysed by flow cytometry. Excitation of the dyes were made using blue laser (488 nm). Fluorescence from SYBR14 was detected with a fluorescence channel (FL) 1 band-pass filter (525/40 nm) and PI fluorescence was measured using a FL3 band-pass filter (585/42 nm). The spermatozoa was classified as membrane intact and membrane compromised spermatozoa.

Acrosomal integrity

Acrosomal integrity of the spermatozoa was assessed by using the lectins i.e. peanut agglutinin conjugated with fluorescein isothiocyanate (FITC) as described by Nag *et al.* (2021) and Kumaresan *et al.* (2020). After the sample preparation, 1 µL of FITC-PNA (1mM) was added to the sample and incubated for 15 min at room temperature in the dark following which the exclusion i.e. propidium iodide (2.4 mM) was added (2 µL) and further incubated for 2 min. Excitation of the dyes were made using blue laser (488 nm). Fluorescence from FITC was detected with a fluorescence channel (FL) 1 band-pass filter (525/40 nm) and PI fluorescence was measured using a FL3 band-pass filter (585/42 nm). The samples were analysed and classified as 2 populations *viz*. acrosome intact and acrosome reacted populations.

Sperm chromatin structure assay

Sperm chromatin structure assay was performed as described by Nag *et al*. (2021). The spermatozoa after the final wash with TALP, will be dissolved in TNE buffer (0.01 mol/L of Tris-HCl, 0.15 mol/L of NaCl and 1 mmol/L of EDTA, pH 7.4). Detergent solution (0.08 N HCl, 0.1% Triton X-100; pH 1.2) was added to the sperm suspension and incubated for 30 second following which 600 µL of Acridine Orange (Sigma-Aldrich, Germany) staining solution (200 mmol/L of Na2HPO4; 0.1 mol/L of citric acid buffer, pH 6.0; 1 mmol/L of EDTA; 150 mmol/L of NaCl; and 6 mg/mL of AO) was added and further incubated for 3 minutes. FL1 (green fluorescence) and FL3 (red fluorescence) were measured after excitation with a blue laser (488 nm).

Sperm mitochondrial ROS

Reactive oxygen species (ROS)/ oxidative stress (OS) in sperm was assessed by using CELLROX™ Deep Red reagent fluorogenic probe (Invitrogen, Thermo Fisher scientific, USA). Briefly, 2.5 mM CELLROX™ Deep Red reagent fluorescent probe was diluted in dimethyl sulphoxide for a final concentration of 1 mM (working solution) and stored at - 20°C in dark. During use, this working solution was kept in the dark at 37°C. Two hundred microlitres of semen samples diluted in Sp TALP (25 X 10^6 sperms/ml) were added to 0.5µl of CELLROX™ and incubated for 30 min at 37°C. After incubation the solution was centrifuged for 5 min at 2000 rpm, the supernatant was removed and the pellet re-suspended in one ml of sheath fluid, filtered through a 40 µm pore size cell strainer to remove any large debris and the cells were analysed by flow cytometry to evaluate the oxidative stress. The percentage of ROS/OS was assessed using the density plot (Rangasamy *et al*., 2021).

Statistical analysis

The differences in between the groups was done using independent student t test after arcsine transformation whereas the differences within the groups was done using paired t test after arcsine transformation and the results were back transformed. The significant difference was considered when P value was <0.05.

RESULTS AND DISCUSSION

Semen collected in the study belonged to both indigenous (Kombai, Rajapalyam and Non-descript Indian breeds) and exotic breeds (Labraodor Retriever, Golden Retriever, Siberian Husky, Doberman). The details of the routine semen analysis parameters has been given in Table 1. The samples with minimum volume of 2.3 mL, concentration of 200 million per mL, progressive motility of 75% were considered for further experiments. The average volume (3.01±0.15 mL) of the canine semen in the present study is in line with (Bebas *et al.,* 2022). Sabarinathan, (2015) got an average volume of 1.7mL while Patti *et al.* (2021) observed 2.54 mL in canine semen. The variations in the volume of sperm rich fractions which might be attributed to differences in age, breed, size and frequency of semen collection compared to this study.

The average concentration observed in the present study is 280 million per mL of semen and is in congruent with previous studies (Zorinkimi *et al*., 2017). Sabarinathan, (2015) observed mean concentration of 332 million per mL of semen. The higher concentration of spermatozoa in these studies could be due to the lower volume of semen obtained in these studies and the differences in the breed from the present study.

Progressive motility observed in the present study is 82.5% and is in agreement with the previous studies (Bebas *et al*., 2022). Progressive motility observed by Cardoso *et al.* (2003) was 99.2% while, Strzezek *et al.* (2012) observed the progressive motility of 92.3%. The higher values observed in the present study might be due to the differences in the breed as well as the environmental conditions in the region of study. The hot and humid climate in the region of the present study might reduce the motility in our study in comparison with these studies.

Sperm functional attributes including viability, HOST, acrosomal integrity and morphology was assessed and significant difference was observed in both the groups, during pre-freeze as well as post thaw (Fig 1). Post-thaw sperm functional attributes has been studied between the

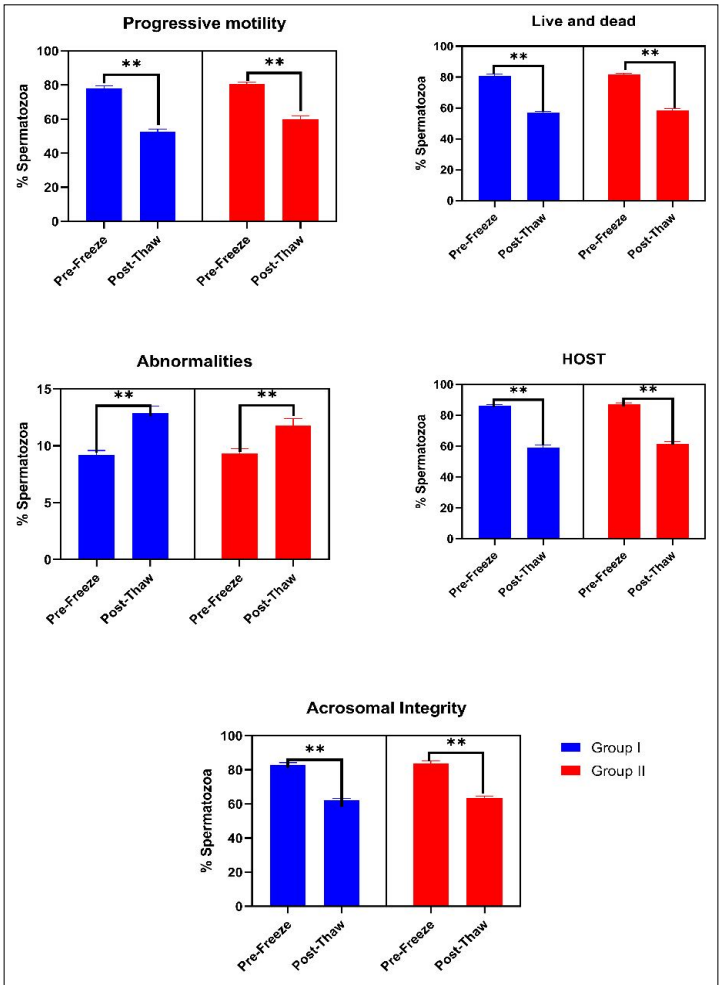
Table 1: Physical properties of sperm rich fraction of canine semen.

Parameters/Variable (n=16)	Value	Range
Volume (ml)	3.01±0.15	2.3-4.0
Colour	Greyish white to milky white	
Consistency	Medium to thick	
Concentration ($\times 10^6$ /ml)	280±15.28	200-350
Progressive motility (%)	82.73±0.04	75-90
Live percentage of spermatozoa (%)	84.5±0.01	80-88
Total sperm abnormalities (%)	7.65±0.01	6-10
Acrosomal integrity (%)	86.80±0.05	80-95
Plasma membrane integrity (%)	88.80±0.02	84-91

groups which revealed that post thaw motility was significantly higher (P<0.01) in metformin supplemented group in comparison with control group. However, there was no significant difference (P>0.05) among other attributes viz. live and dead sperm, hypoosmotic responsive sperm, acrosomal integrity and abnormalities (Fig 2).

Advanced assays viz, viability (SYBR14 and PI - Plate 1), acrosomal integrity (FITC - PNA and PI - Plate 2), sperm chromatin structure assay (Acridine orange - Plate 3) were significantly (P<0.01) different between the pre freeze and post thaw groups. But it was observed that the reactive oxygen species (Cell ROX) between prefreeze and post thaw of group I was significantly different (P<0.01) whereas the difference was not significant between the prefreeze and post thaw of group II which was supplemented with metformin (Fig 3). Flow cytometric analysis of the post thaw spermatozoa revealed that there was significantly lower (P<0.01) reactive oxygen species in metformin supplemented group in comparison with the control group (Plate 4). However, other attributes *viz*. viability, acrosomal integrity, sperm chromatin structure assay were not significantly different (P>0.05) between the groups (Fig 4).

The process of cryopreservation has been proved to render around 50% of the spermatozoa immotile and membrane compromised (Watson *et al*., 1995). Cryopreservation induces alterations in the membrane of plasma membrane during the process of cryopreservation. Higher concentrations of poly unsaturated fatty acids in the plasma membrane of canine spermatozoa have been attributed to be one of the reasons for increased production of ROS (Makker *et al*., 2009). The increased concentration of ROS in the spermatozoa is responsible for the changes in intracellular ATP and axonemal damage, decreases sperm motility, leads to the loss of intracellular enzymes

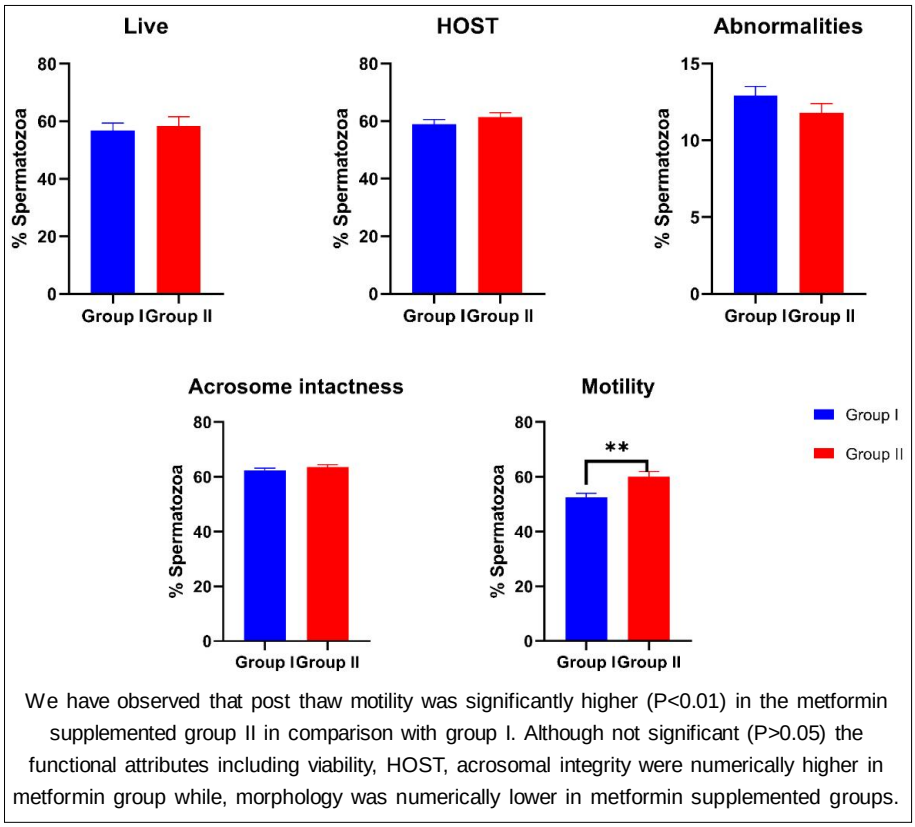


All the parameters viz. viability (Eosin and Nigrosine), HOST, Acrosomal integrity, morphology were significantly (P<0.01) different between the pre freeze and post thaw groups.

Fig 1: Mean (±SE) of prefreeze and post thaw microscopic analysis of morphological and functional characteristics of canine spermatozoa extended using TFEY and TFEY supplemented with 50 µM metformin extender.

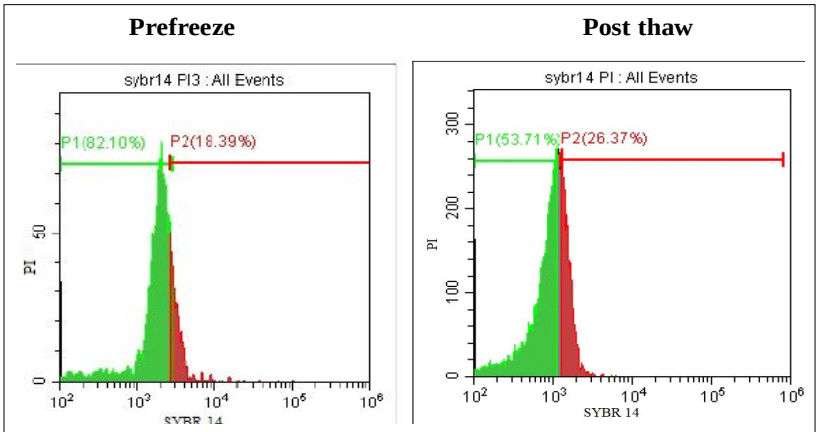
and DNA fragmentation, impairs the fertilisation ability of sperm cells and decreases pregnancy rates after IVF (Lecewicz *et al*., 2018).

Reactive oxygen species (ROS) could be neutralized by the action of antioxidant enzyme viz. catalase, superoxide dismutase and glutathione peroxidase (Zeitoun and Damegh, 2015). Reduction in the production of ROS could also reduce the damages that has been associated with cryopreservation. Metformin is a molecule of the biguanide family and has the ability to decrease reactive oxygen species (Montalvo *et al*., 2013). Bertoldo *et al.* (2014) observed a beneficial effect of metformin on the post thaw motility of the spermatozoa when the spermatozoa were pre incubated with metformin prior to cryopreservation.



We have observed that post thaw motility was significantly higher (P<0.01) in the metformin supplemented group II in comparison with group I. Although not significant (P>0.05) the functional attributes including viability, HOST, acrosomal integrity were numerically higher in metformin group while, morphology was numerically lower in metformin supplemented groups.

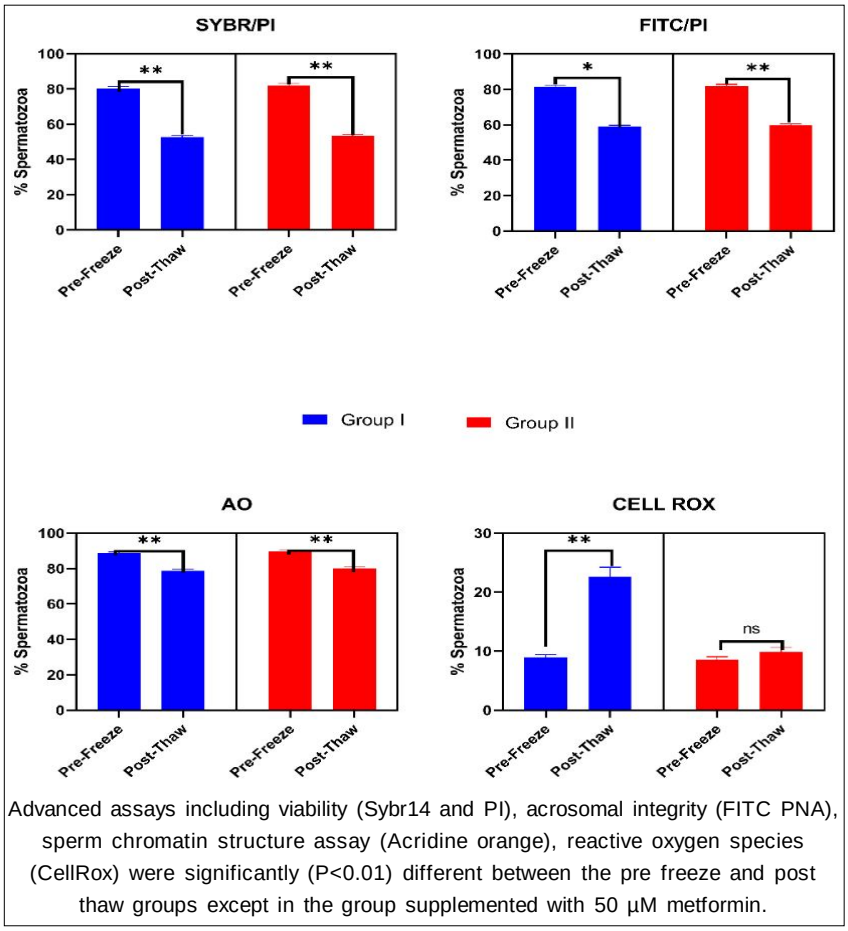
Fig 2: Mean (±SE) post thaw microscopic analysis of morphological and functional characteristics of canine spermatozoa extended using TFEY and TFEY supplemented with 50 µM metformin extender.



P1- Membrane intact sperms; P2 - Membrane compromised sperms.

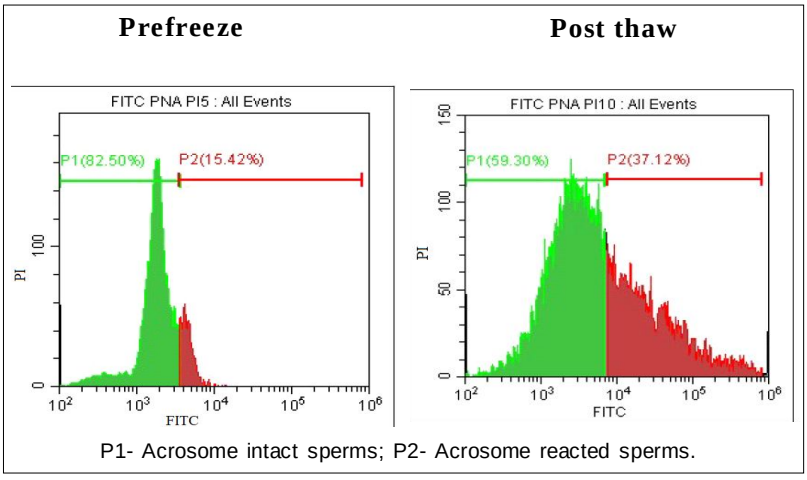
Plate 1: Plasma membrane integrity-SYBR-14/PI assay of prefreeze and post thaw extended canine semen.

Metformin, when used in extender has been reported to modulate the cell metabolism through the mitochondrial activity in mouse (Bertoldo *et al*., 2014), stallion (Cordova *et al*., 2014) and boar semen (Hurtado *et al*., 2015). Metformin is reported to activate the AMP-activated protein kinase (AMPK) pathway. AMPK is a regulator of cellular energy balance and activated AMPK switches cells from an anabolic to a catabolic state. Recent evidence suggests



Advanced assays including viability (Sybr14 and PI), acrosomal integrity (FITC PNA), sperm chromatin structure assay (Acridine orange), reactive oxygen species (CellRox) were significantly (P<0.01) different between the pre freeze and post thaw groups except in the group supplemented with 50 µM metformin.

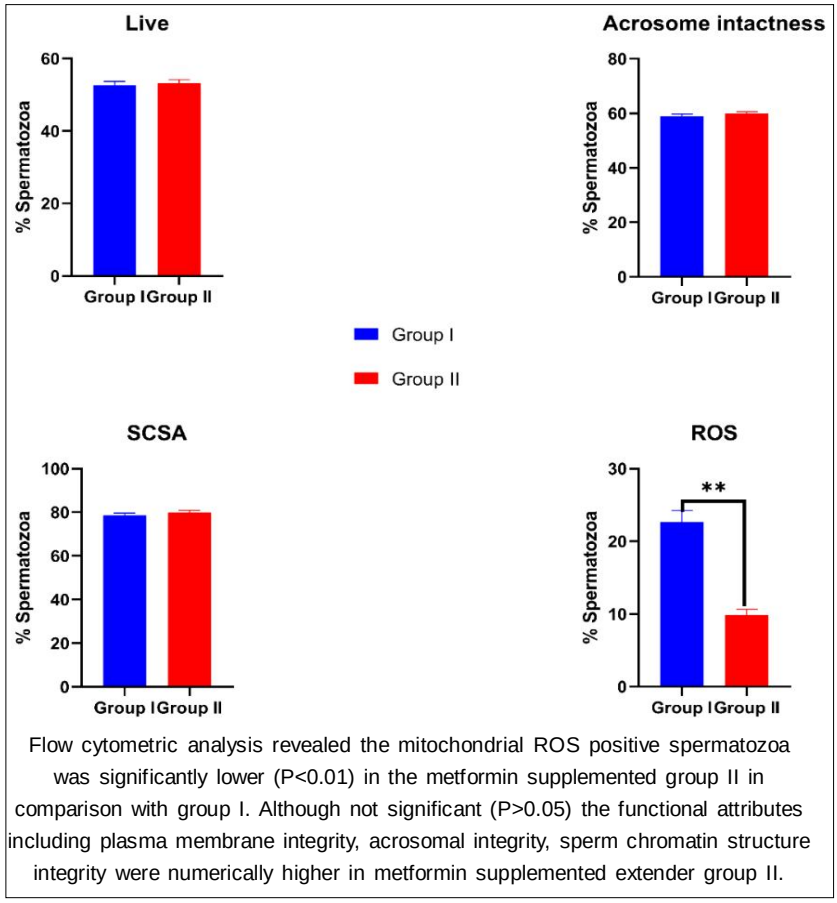
Fig 3: Mean (±SE) prefreeze and post thaw flow cytometric analysis of functional characteristics of spermatozoa extended using TFEY and TFEY supplemented with 50 µM metformin extender.



P1- Acrosome intact sperms; P2- Acrosome reacted sperms.

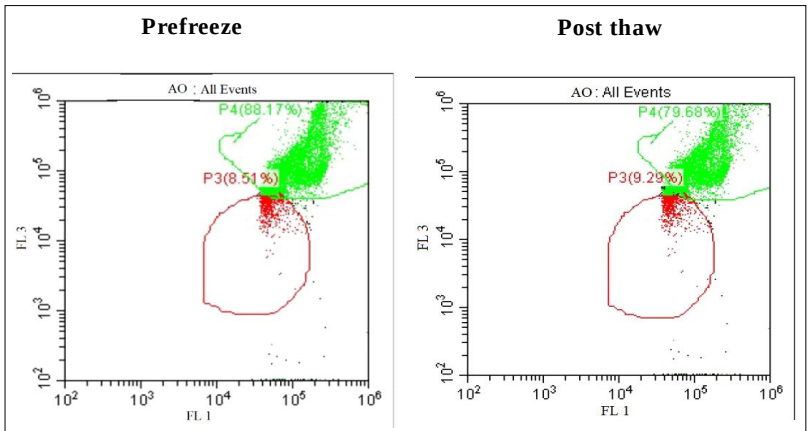
Plate 2: Acrosomal integrity-FITC-PNA/PI assay of prefreeze and post thaw extended canine semen.

that AMPK is present in spermatozoa and this protein was involved in sperm physiology, indeed absence of this protein in mice altered spermatozoa motility, quality of the spermatozoa membrane and lowered the levels of antioxidant molecules (Hidago *et al*., 2013). The reduction of mitochondrial ROS in the present study in the metformin



Flow cytometric analysis revealed the mitochondrial ROS positive spermatozoa was significantly lower (P<0.01) in the metformin supplemented group II in comparison with group I. Although not significant (P>0.05) the functional attributes including plasma membrane integrity, acrosomal integrity, sperm chromatin structure integrity were numerically higher in metformin supplemented extender group II.

Fig 4: Mean (±SE) post thaw flow cytometric analysis of functional characteristics of canine spermatozoa extending using TFEY and TFEY supplemented with 50 µM metformin.



P4- ds DNA; P3- ss DNA.

Plate 3: Sperm chromatin structure assay (acridine orange) of prefreeze and post thaw extended canine semen.

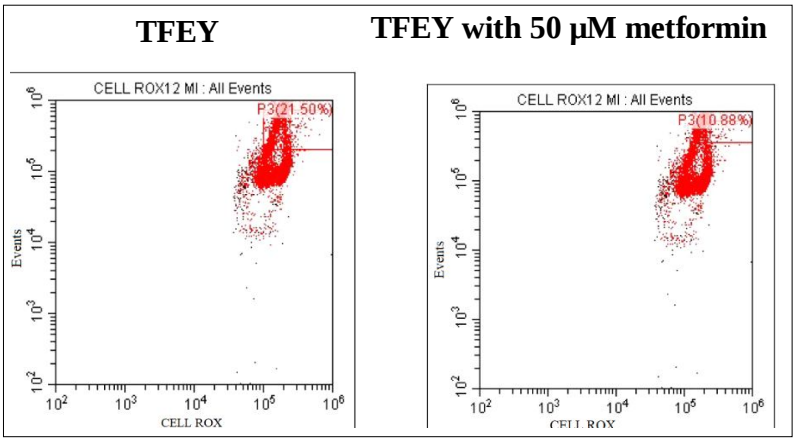


Plate 4: Reactive oxygen species/oxidative stress using CellRox® analyzed by flow cytometry of post thaw canine semen extended using TFEY (group I) and TFEY with 50 µM metformin (group II) extender.

supplemented group might be due to its action on the AMPK pathway resulting in significant increase in the post thaw motility of the spermatozoa.

CONCLUSION

The present study concludes that the addition of 50µM of metformin in the extender prior to cryopreservation significantly reduces production of mitochondrial ROS thereby, improves post thaw motility in canine spermatozoa.

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