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Research Article

Effect of Spirulina and Coenzyme Q-10 Addition to Extenders on Canine Spermiogram

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Abstract

The present study was aimed to determine the effect of addition of antioxidants to semen extender on Labrador dog semen preserved at 4˚C. The semen was evaluated for volume, colour and concentration, and extended with tris-egg yolk-citric acid containing Spirulina at 10 µg/ml (SP), Coenzyme Q-10 at 0.5 (CO) µMol/ml, a combination extender with 10 µg/ml Spirulina and 0.5 µMol/ml Coenzyme Q-10 (SC) and no antioxidant (CON). The extended semen was evaluated for acrosomal integrity, mitochondrial activity and lipid peroxidation assay at 0, 24, 48 and 72 hours. It was observed that, up to 24 hours there was no significant difference between the antioxidants' and control groups. However, at 48 and 72 hours, the addition of antioxidants showed better spermiogram compared to control group, and the combination of antioxidants showed better results when compared to individual antioxidants at 48 and 72 hours.

Keywords: Canine; Spermatozoa; Labrador Retriever; Spirulina; Coenzyme Q-10; Diaminobenzidine; Silver Nitrate; Thiobarbituric Acid Reactive Substances; Acrosome; Mitochondrial Activity; Lipid Peroxidation

Introduction

Artificial insemination (AI) is one of the most popular assisted reproductive technologies in animal breeding and has increased the reproductive potential of domestic animals in order to benefit mankind. AI has helped in increasing the number of animals, in preserving and propagating breeds and enabling breeding programmes. AI in canine breeding was first demonstrated by Lazzaro Spallanzani in 1779 with the successful and pregnancy of a spaniel dog, however canine AI has become increasingly popular only during the last decades due to intense investigation in various methods of canine semen collection, preservation, and evaluation [1]. Due to increased demand of working and pet dogs, as well as preservation of indigenous dog breeds, canine breeding has expanded to allow the preservation of semen through chilling and cryopreservation.

Compared to frozen-thawed semen, chilled-extended semen incurs lesser costs regarding shipping and processing, is easier to handle, can be deposited vaginally (as opposed to intrauterine insemination for frozen-thawed canine semen), and shows improved pregnancy rates and litter sizes [1,2]. However, in either preservation technique, the cold environment causes stress to the spermatozoa, leading to production of reactive oxygen species [3], which causes cellular and organelle damage. To counteract against the effects of chilling on spermatozoa, semen extenders are used while storing semen in refrigerated and/or frozen condition. However, semen extenders alone cannot reduce or delay the production of Reactive Oxygen Species (ROS) from damaged spermatozoa, which is why antioxidants are added to semen extenders to assist in the scavenging and neutralising of excess ROS [4].

Anti-oxidants are chemical molecules or enzymes that neutralise ROS or react with ROS to create by-products that do not cause oxidation. Endogenously present antioxidant systems in spermatozoa and semen (glutathione peroxidase, superoxide dismutase, reduced glutathione, catalase, L-carnitine, acetyl carnitine, ascorbate, urate, glutathione, albumin and taurine [5], and exogenously added antioxidants (amino acids, vitamins, enzymes, sugars and antioxidant-rich substances) have been studied by various scientists [6-16], and the results have shown a positive effect of antioxidants on both chilled-extended and frozen-thawed semen. Two commonly used antioxidants that have been commonly added to semen extenders in other animals and birds, but have not yet been utilised in canine semen extenders, are Spirulina and coenzyme Q-10.

Spirulina is a planktonic blue-green algae that comprises the primary diet of herbivorous aquatic life, and is a traditional food of certain African and Mexican communities due to its high nutritive value. It is classified as a "superfood" due to its high protein and antioxidant content. As an antioxidant, it contains both enzymatic (superoxide dismutase, glutathione peroxidase, catalase, ascorbate peroxidase) and non-enzymatic (carotenoids, ascorbic acid, tocopherols, chlorophyll derivates) antioxidant protection system [17,18]. Effect of addition of spirulina extract to semen extender as well as its oral consumption has been studied in bovines [18,19], swine [20] and in lab animals [21-23] with results showing increased progressive motility, decreased intracellular ROS production and improved post storage viability.

Coenzyme Q-10 (CoQ-10) is a lipid molecule present in mammalian cells and found primarily in the inner mitochondrial membrane. It promotes energy generation through the electron transport chain system during the production of adenosine triphosphate (ATP) by the mitochondria [24]. It is involved with several physiological processes involving energy production in the cell [25] and is considered to be the only endogenously synthesised liposoluble antioxidant molecule [26]. Its presence not only enables protection of the mitochondrial membrane against ROS stress, but also enables adequate production of ATP required in spermatozoa to promote increased metabolism and motility, which are directly linked to spermatozoa viability and overall fertility of the animal. Ensuring protection and optimal functioning of the mitochondria is essential for the fertilising capacity of the spermatozoa and spermatozoa integrity (27). CoQ-10 has been used in the extender of bovines [28], equines [29-31], caprines [32], ovines [33], poultry [34] and laboratory animals [35], with a general trend of improved spermatozoa characteristics upon the addition of CoQ-10 to the extender.

There has been meagre research reported about the addition of Spirulina extract and CoQ-10 to the canine semen extender. Keeping in view the present research regarding the addition of antioxidants in semen extender we hypothesised that the addition of Spirulina extract and CoQ-10, individually or in combination, will aid in improved spermatozoa characteristics during preservation in refrigeration temperature.

Materials and Methods

- **Outline of research:** 30 semen samples (n = 30) were collected from six dogs once a week for the required number of collections and diluted with a tris-egg yolk-citrate-fructose extender (2) containing no antioxidants (control), Spirulina at 10 µg/mL concentration, Coenzyme Q-10 at 0.25 µMol/mL and 0.5 µMol/mL, and a combination of antioxidants (spirulina at 10 µg/mL + Coenzyme Q-10 at 0.5 µMol/mL concentration). The extended semen underwent testing at 0, 24, 48 and 72 hours to evaluate the effect of the antioxidants on the semen samples.
- **Selection of animal:** 17 dogs were screened and 6 healthy intact male Labrador retrievers between 1.5 – 4.5 years old, between 25 – 35 kg body weight and semen with >70% progressive motility and >70% live spermatozoa were selected. The data of the animals used for the experiment present in the annexure.
- **Semen collection and testing:** The semen was collected from each dog using digital method with a minimum of a week sexual rest. The semen sample was assessed for acrosomal integrity, mitochondrial activity and lipid peroxidation level at 0, 24, 48 and 72 hours.
- **Semen handling:** The neat semen was checked for volume, colour, concentration and mass motility. It was then extended, and the extended semen was placed in a thermos containing water at 37°C temperature, and carried back to the laboratory for further testing. At the laboratory, the extended semen was placed sealed in a glass container containing room temperature water for 15 minutes, after which it was transferred to the door of the refrigerator for 30 – 45 minutes, or until the water in the glass container reached 4°C. The sealed tubes of extended semen were then wrapped in moist cotton, and placed in a thermos lined with moist cotton which was maintained at 4°C (1). The thermos was closed and kept in the door of the refrigerator until further processing. At the time of removal, the tubes of extended semen were unwrapped from the moist cotton, placed in a glass container with room temperature and allowed to come to room temperature over 15 minutes. After 15 minutes, the sample underwent testing.

- **Silver nitrate staining:** Silver Nitrate was taken at 1 mg/mL (modified Badr., *et al*., 2021-18) in 100 mL distilled water and mixed until dissolved. One drop (~25 µL) of diluted semen sample was taken on one end of a clean glass slide, a smear was prepared and allowed to air dry. After the smear was dry, it was allowed to fix in 70% ethanol for 2 minutes, followed by fixation in 95% ethanol for 2 minutes, after which the smear was again allowed to air dry. The smear was then stained with silver nitrate stain, and incubated at 65˚C for 3 hours (modified Badr., *et al*., 2021-18). The slide was removed from the incubator and was rinsed several times with distilled water. The slide is allowed to air dry and observed under a phase contrast microscope at 1000x magnification. 100 spermatozoa were counted per slide (modified Badr., *et al*., 2021-18) and the percent of acrosome intact spermatozoa was calculated by the following formula:

- **Diaminobenzidine staining:** 25 µL of diluted semen was added to 50 µL of freshly prepared stain (36) present in the labelled amber microcentrifuge tube at 1:2 dilution ratio while taking care to not expose the stain to light. This was incubated in a thermos containing 37˚C temperature water for 1 hour. After incubation, a smear was prepared and allowed to air dry in the dark. Once dried, the smear was fixed in 10% formalin for 10 minutes, and removed. At this point, the smear could be exposed to light without any adverse effects towards the staining reaction. The smear was observed under a phase contrast microscope at 1000x magnification. The mitochondrial staining was classified as per Hrudka's classification into class 1 (100% mitochondrial staining), class 2 (Greater than 50% mitochondrial staining), class 3 (Lesser than 50% mitochondrial staining) and class 4 (0% mitochondrial staining). 100 spermatozoa were counted per slide, classified into one of four classes, and the number of each class was multiplied with their relative score (class 1 = 1; class 2 = 0.50; class 3 = 0.25; class 4 = 0). These were added to obtain the total score, which was applied in the formula below to arrive at the index (I). The percentage of DAB class I, II, III and IV spermatozoa was calculated separately.
- **Thiobarbituric reactive substances assay:** The reagents in the TBARS assay kit were prepared according to the instructions given along with the kit (Cayman Chemicals, Ann Arbor, MI, USA). The procedure followed was as per the kit specifications and modified from Ogata., *et al*., 2015 and Badr., *et al*., 2021. 100 µL of the diluted semen sample was taken in a 15 mL capped centrifuge tube, and 100 µL of SDS solution was added to it and mixed well. To this, 4 mL of the colour reagent (10% (v/v) acetic acid, 5% (v/v) sodium hydroxide and 0.53% (w/v) TBA in HPLC-grade water) was added and mixed. The mixture was capped, kept upright and placed in a boiling water bath for 60 minutes. After 60 minutes, the centrifuge tubes were immediately cooled in an ice bath for 10 minutes. The mixture was centrifuged at 1400xg at 4˚C for 10 minutes in a refrigerated centrifuge. After stabilisation at room temperature, the supernatant from each vial was loaded into the glass cuvette of the spectrophotometer, and the optical density (O.D.) value was determined using an UV Spectrophotometer (Evolution 201/220 UV Visible Spectophotometer; Thermo Fisher Scientific India Pvt. Ltd, Mumbai). The OD values were noted down, and the concentration was calculated using the formula obtained from the standard curve, which was prepared from the MDA standard provided with the kit. From the OD value, the concentration was derived using the slope of the standard curve y = 0.0033x, where y was the concentration, and x was the OD value. The MDA concentration was derived from applying the OD value to the formula.

Statistical analysis

The data was analysed by completely randomised block design using WASP 2.0 (Web Agri Stat Pack), ICAR, Goa and by using Student t-test (Microsoft Excel; Microsoft Office 2021).

Results and Discussion

Acrosomal integrity of spermatozoa: The mean percentage of intact acrosomes observed in spermatozoa extended in four different extenders at 0, 24, 48 and 72 hours is given below

Hour	CON	SP	CO	SC
0 HR	87.00 ± 05.59^{a}	79.83 ± 9.44^{a}	80.67 ± 12.11^{a}	86.67 ± 08.00^{a}
24 HR	84.17 ± 09.95^{a}	81.83 ± 8.13^{a}	79.93 ± 12.80^{a}	82.50 ± 10.93^{a}
48 HR	60.50 ± 14.49^{b}	73.83 ± 7.83^{a}	73.33 ± 06.59^{a}	82.50 ± 10.93^{a}
72 HR	53.67 ± 12.68^{b}	71.00 ± 9.98^{a}	70.33 ± 06.98^{a}	76.67 ± 10.44^{a}

Table 1: Comparison of acrosomal integrity in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

Mitochondrial activity: The mean percentage of DAB class I spermatozoa in different extenders at 0, 24, 48 and 72 hours is given below.

The mean percentage of DAB class II spermatozoa in different extenders at different testing times is given below.

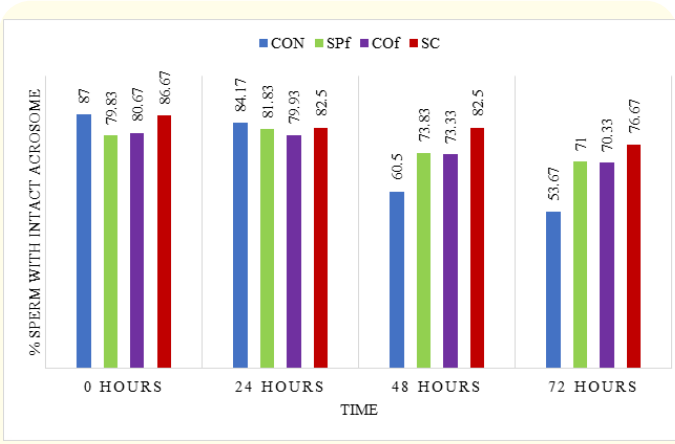

Figure 1: Acrosomal Integrity of spermatozoa in Four Extenders.

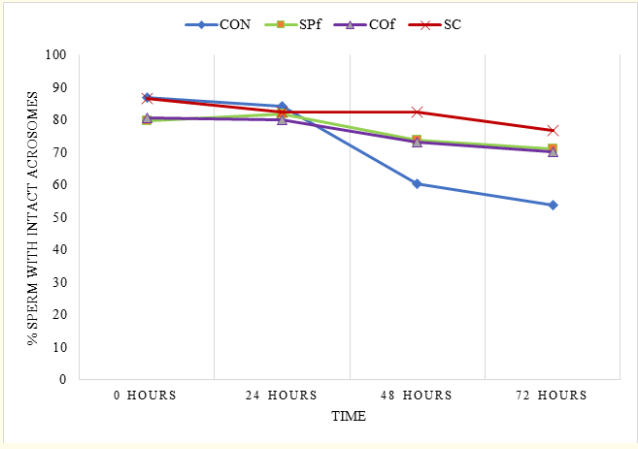

Figure 2: Acrosomal Integrity of spermatozoa in Four Extenders.

HOUR	CON	SP	CO	SC
0 HR	40.00 ± 29.35[a]	57.00 ± 23.65[a]	48.00 ± 28.71[a]	71.00 ± 24.67[a]
24 HR	40.36 ± 21.13[a]	53.54 ± 21.63[a]	49.40 ± 26.68[a]	68.34 ± 19.50[a]
48 HR	26.58 ± 07.04[b]	41.48 ± 16.22[b]	42.59 ± 26.96[b]	67.07 ± 21.71[a]
72 HR	19.87 ± 08.19[b]	28.33 ± 16.22[b]	33.83 ± 21.97[b]	62.90 ± 18.03[a]

Table 2: Comparison of DAB class I spermatozoa in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

HOUR	CON	SP	CO	SC
0 HR	37.00 ± 19.98[a]	27.00 ± 10.13[a]	17.00 ± 13.26[a]	25.00 ± 22.27[a]
24 HR	22.23 ± 18.73[a]	26.01 ± 11.42[a]	19.66 ± 15.15[a]	18.97 ± 10.86[a]
48 HR	31.20 ± 09.46[a]	36.09 ± 06.32[a]	34.10 ± 17.92[a]	16.25 ± 18.16[a]
72 HR	15.58 ± 04.78[b]	38.12 ± 06.73[a]	23.55 ± 15.38[b]	17.42 ± 17.42[b]

Table 3: Comparison of DAB class II spermatozoa in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

There were no references to DAB class II spermatozoa observed for 72 hours in chilled canine semen in extenders containing antioxidants in the scanned literature. There was no significant difference between the mean percentage of DAB class II spermatozoa at 0, 24 and 48 hours between the four extender groups. The mean percentage of DAB class II spermatozoa was significantly higher in the SP group compared to the other three groups. There was no significant difference between the CON, CO and SC groups with respect to DAB class II spermatozoa.

The mean percentage of DAB class III spermatozoa in the CON, SP, CO and SC groups at different testing times is given below.

HOUR	CON	SP	CO	SC
0 HR	22.00 ± 21.87[a]	15.00 ± 17.14[a]	33.00 ± 22.65[a]	3.00 ± 04.44[a]
24 HR	34.55 ± 15.92[a]	18.07 ± 17.20[a]	29.61 ± 15.81[a]	11.47 ± 12.60[a]
48 HR	24.05 ± 05.09[a]	17.64 ± 14.11[a]	20.69 ± 16.70[a]	15.01 ± 07.78[a]
72 HR	46.84 ± 16.81[a]	29.27 ± 20.99[ab]	37.02 ± 20.14[ab]	16.73 ± 10.60[b]

Table 4: Comparison of DAB class III spermatozoa in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

The mean percentage of DAB class IV spermatozoa at 0 hours in the CON, SP, CO and SC groups at 0, 24, 48 amd 72 hours is given below.

HOUR	CON	SP	CO	SC
0 HR	02.00 ± 02.11[a]	2.00 ± 3.52[a]	3.00 ± 6.07[a]	1.00 ± 1.88[a]
24 HR	02.87 ± 03.93[a]	2.37 ± 3.10[a]	1.33 ± 1.51[a]	1.22 ± 1.99[a]
48 HR	18.17 ± 11.09[a]	4.78 ± 3.76[b]	2.61 ± 1.50[b]	1.66 ± 1.10[b]
72 HR	17.71 ± 09.54[a]	4.28 ± 1.72[b]	5.60 ± 4.12[b]	2.94 ± 2.37[b]

Table 5: Comparison of DAB class IV spermatozoa in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

There were no references to DAB classified spermatozoa observed for 72 hours in chilled canine semen in extenders containing antioxidants in the scanned literature.

Mitochondrial activity index: The mean MAI of spermatozoa at different testing times for spermatozoa in different extenders is given below. There were no references to mitochondrial activity index observed for 72 hours in the scanned literature.

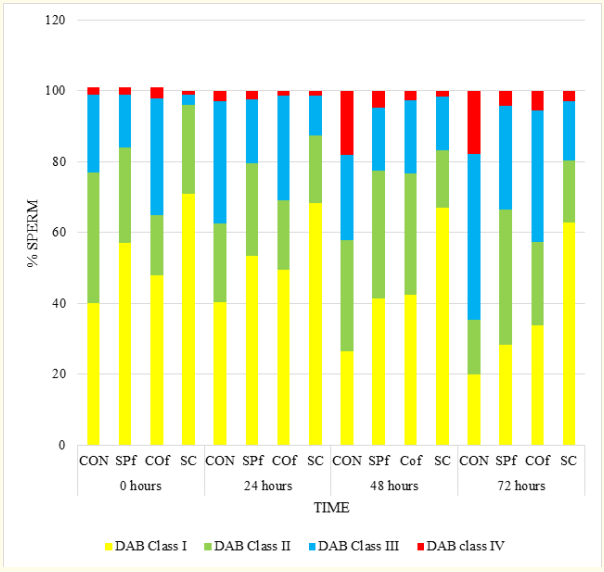

Figure 3: Mitochondrial Activity Classification of spermatozoa in Four Extenders (Hrudka 1987).

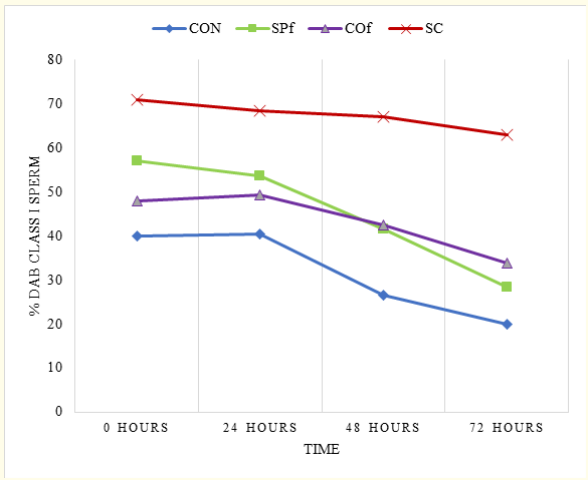

Figure 4: DAB Class I Spermatozoa in Four Extenders.

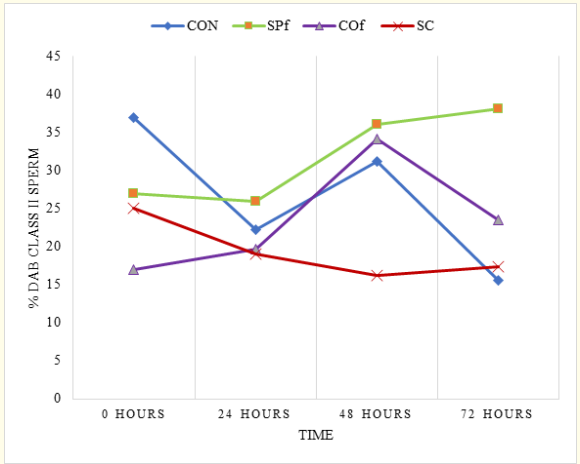

Figure 5: DAB Class II Spermatozoa in Four Extenders.

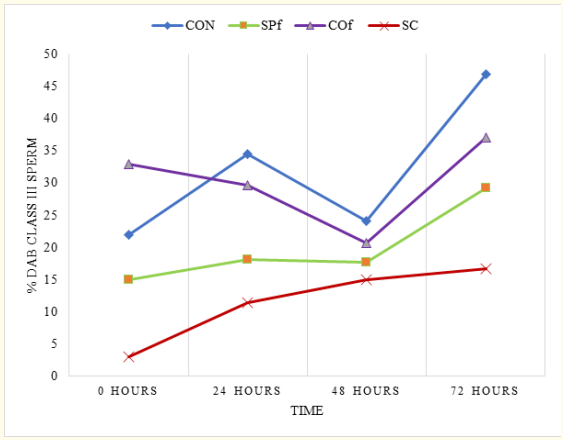

Figure 6: DAB Class III Spermatozoa in Four Extenders.

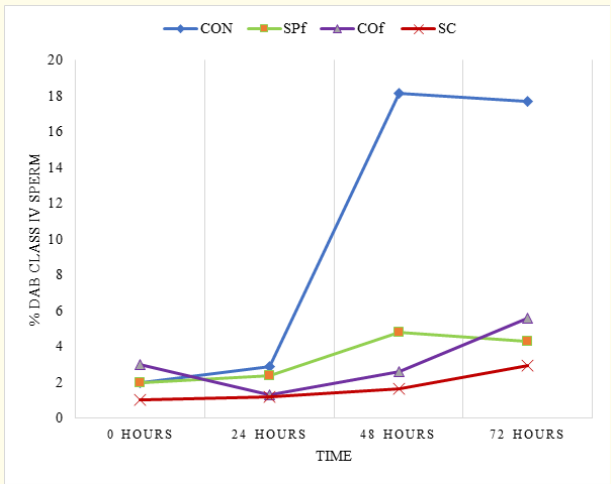

Figure 7: DAB Class IV Spermatozoa in Four Extenders.

Figure 8: Mitochondrial Activity Index of Spermatozoa in Four Extenders.

HOUR	CON	SP	CO	SC
0 HR	63.75 ± 19.67[a]	73.72 ± 17.41[a]	64.31 ± 21.44[a]	84.50 ± 13.13[a]
24 HR	60.17 ± 13.56[a]	71.20 ± 16.09[a]	66.50 ± 17.26[a]	80.67 ± 12.71[a]
48 HR	48.17 ± 08.21[b]	64.00 ± 13.02[a]	64.83 ± 17.17[a]	79.00 ± 12.07[a]
72 HR	39.33 ± 03.98[c]	54.67 ± 13.63[b]	54.83 ± 16.65[b]	76.00 ± 12.49[a]

Table 6: Comparison of mitochondrial activity index in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

There was no significant change seen in between the mean MAI of the four extenders till 24 hours. The highest mean MAI was seen in the SC group (80.67 ± 12.71%) and the lowest was in the CON group (60.17 ± 13.56%), suggesting that while statistically there was no difference, there was numerical improvement seen due to the addition of antioxidants to the extender on the MAI of spermatozoa.

Thiobarbituric acid reactive substances concentration

The mean values of TBARS-C of spermatozoa in different extenders at different testing times are given below.

HOUR	CON	SP	CO	SC
0 HR	117.93 ± 20.47[a]	123.18 ± 64.51[a]	125.91 ± 61.29[a]	124.04 ± 49.85[a]
24 HR	130.35 ± 36.16[a]	110.20 ± 13.25[a]	113.28 ± 15.85[a]	119.44 ± 18.40[a]
48 HR	152.98 ± 61.18[a]	96.01 ± 19.32[b]	92.73 ± 39.88[b]	86.26 ± 24.19[b]
72 HR	127.63 ± 45.58[a]	80.15 ± 35.26[ab]	80.25 ± 47.16[ab]	58.54 ± 27.19[b]

Table 7: Comparison of TBARS-C in four extenders.

[Different superscripts within the same row denote significant differences ($p < 0.01$ and $p < 0.05$)].

There was no significant difference in the TBARS-C between the four extenders at 0 hours and 24 hours, indicating that there was no significant production of ROS till 24 hours. At 48 hours, the TBARS-C was significantly higher in the CON group than the other groups, signifying that the action of antioxidants had begun reducing the ROS level within the sample. However, there was no significant difference between the TBARS-C levels amongst the extenders containing antioxidants at 48 hours. At 72 hours, it was observed that the TBARS-C levels were significantly lower in the SC group compared to the CON group.

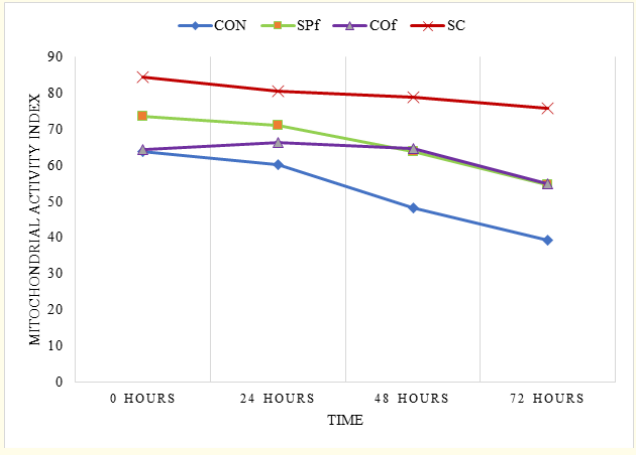

Figure 9: Comparative Mitochondrial Activity Index of Four Extenders.

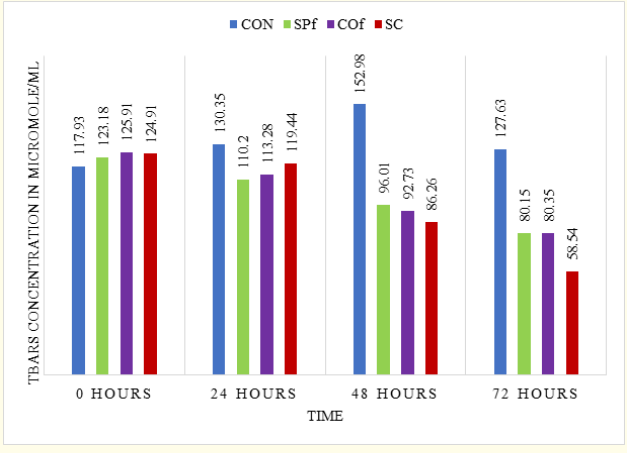

Figure 10: TBARS Concentration of Four Extenders.

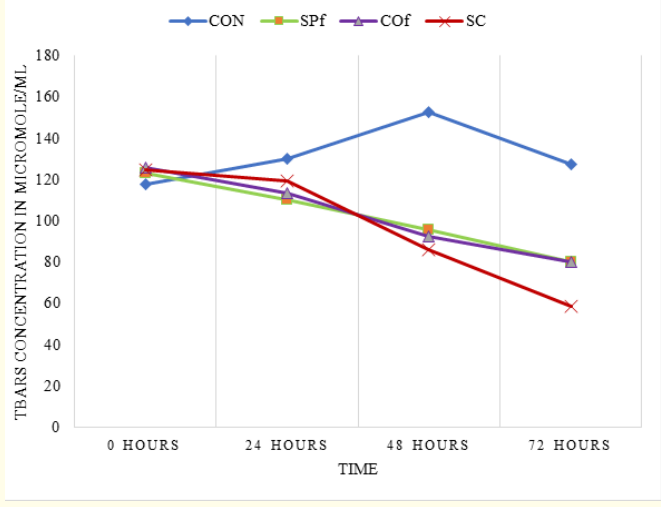

Figure 11: Comparative TBARS Concentration of Four Extenders.

The results for the TBARS-C values of the groups of the canines were higher, however the research findings of the buffalo semen were lower [18], semen preservation technique (chilled v cryo-preserved) and antioxidant concentrations (Spirulina at 10 µg/ml, Coenzyme Q-10 at 0.5 µMol/ml and a combination of the two v Spirulina at 0, 1, 5, 10 and 20 µg/ml). Aitken., *et* al (2017) The presence of ROS is essential for the capacitation process through the activation of various enzymatic pathways, excessive ROS can promote over-capacitation and a state of oxidative stress in the spermatozoa, leading to apoptosis [4]. This phenomenon to occur in the in the female reproductive tract at the time of insemination as an important step following fertilisation; however, it is important to prevent this from happening while the semen is undergoing stress due to cold storage. The addition of Spirulina, Coenzyme Q-10 or both may ensure that the spermatozoa is maintained in a relatively stress-free state while awaiting insemination, so that it can be as fertile as possible after undergoing cold stress.

The improved effect in the SC group can be attributed to the presence of Coenzyme Q-10 in the extender, which supports and protects the mitochondrial helix [24]. Spirulina on its own did not show any beneficial effect on the DAB class I spermatozoa, but seemed to enhance the activity of the Coenzyme Q-10 when in combination, as per the results. As mentioned previously, the fluctuation in DAB class II spermatozoa over time and between extenders could be due to an increase in number of spermatozoa with mitochondrial activity close to 50%, increasing the ambiguity while classifying the spermatozoa. The SP and CO groups were found to have mean percentages of DAB class III at an intermediate level when compared to the CON and the SC groups. This underlines the significance of the presence of Coenzyme Q-10 in protecting and preventing the deterioration of the mitochondria, and its synergistic effect with Spirulina.

At 48 hours, it was observed that the mean MAI of the CON group was significantly lower than the mean MAI of the SP, CO and SC groups. There was no significant difference between the antioxidants' added groups. This suggested that while there was improvement in the mean MAI of the spermatozoa by the addition of antioxidants, there was no significant difference between the antioxidants themselves at 48 hours. At 72 hours, it was observed that the mean MAI of the SC group was significantly higher than all other groups. It was also observed that the mean MAI of the CO and SP groups was lower than the SC group but still significantly higher than the CON group.

Combining Spirulina with Coenzyme Q-10 may have enhanced the activity of Coenzyme Q-10, at least for spermatozoa showing greater than 50% mitochondrial activity, which constitutes the population of fertile spermatozoa which are most likely to fertilise the ovum. This implied that the addition of a combination of Spirulina at 10 µg/ml and Coenzyme Q-10 at 0.5 µMol/ml did improve the MAI and may have helped retain the overall fertility of extended semen when compared to addition of either antioxidant alone and to no antioxidant at all.

The results obtained in the present experiment were greater than the findings reported by the other author. Kaur., *et al.* (2020) The mitochondrial activity in the semen sample may vary due to modifications in procedure, difference in extender components (difference in base extender as well as additives utilising egg yolk plasma, coconut water and low-density lipoproteins), however egg yolk plasma could be a better substitute for egg yolk [14].

Few comparisons could be made by other authors who have performed the same test as there was a difference in testing procedure at various stages. For example, the amount of semen sample added to the test varied amongst authors, with the addition of semen sample reported as 1 ml [37], 500 µL [18,38], 0.1 mL [10] and 400 µL [13]. The time interval for incubation in boiling water bath was varied between authors, with the time period reported as 15 minutes by Buege and Aust (1978-36), 20 minutes by Blumer., *et al.* (2012-38), Dalmazzo., *et al.* (2018-13) and Kaur., *et al.* (2020-14) and 1 hour by Ogata., *et al.* (2015-10). There was a difference in centrifugation temperature and speed as well, with reports of 1000xg by Beuge and Aust (1978-37), 16100 x g and 15˚C by Blumer., *et al.* (2012-38), 1400xg by Ogata., *et al.* (2015-10), 18000xg and 15˚C by Dalmazzo., *et al.* (2018-13), 2500xg by Kaur., *et al.* (2020-14) and 800xg by Badr., *et al.* (2021-18). The absorbance wavelength was also varied, and was reported as 535 nm by Beuge and Aust (1978-37), 532 nm by Blumer., *et al.* (2012-38), Dalmazzo., *et al.* (2018-13) and Kaur., *et al.* (2020-14), 540 nm by the TBARS Assay Kit Catalogue and 485 excitation/535 emissions by Ogata., *et al.* (2015-10). The other hinderance in comparison was the units used by different authors for the concentration of TBARS. Blumer., *et al.* (2012) used ng TBARS/ml, Ogata., *et al.* (2015) used µM MDA/20 x 10^6 spermatozoa, Dalmazzo., *et al.* (2018) used ng TBARS/10^6 spermatozoa, Kaur., *et al.* (2020) used µM/10^6 sperms and Badr., *et al.* (2021) used nmol/10^9.The absence of any method of converting one unit to the other did not allow to compare the present findings with the authors mentioned above.

Conclusion

The present antioxidants used showed beneficial effect on TBARS-C in combination, while their effect diminished in indi-

vidual extenders. While the addition of antioxidants showed an improvement in reducing the TBARS-C over time, the combination of antioxidants reduced the TBARS-C to nearly half of what was observed at 0 hours. This shows that a combination of Coenzyme Q-10 and Spirulina can allow preservation of the canine semen sample while neutralising excessive ROS production from the spermatozoa due to cold stress and is better than utilising Coenzyme Q-10 or Spirulina alone.

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Conflict of Interest

Authors have no conflict of interest.

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