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Effects of Linolenic Acid supplementation on Seminal Attributes of Labrador during *in-vitro* Storage at 4°C

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ABSTRACT

The purpose of this study was to evaluate the probable effects of the linolenic acid addition in different levels to the Tris based egg yolk plasma (T-EYP) extender in Labrador dog semen on spermatozoa quality during storage of semen at 4°C for 0, 24,48 and 72 hours. The collected semen from all dogs was mixed together and diluted with T-EYP. The diluted pooled semen was divided into 5 treatments (T). T1 was a control group without any linolenic acid addition. For T2 to T5 groups 0.5 ng/ml , 1.5 ng/ml, 2.5 ng/ml and 3.5 ng/ml linolenic acid were added respectively. Treatments were evaluated for sperm motility, sperm viability, morphological defect, acrosome integrity and membrane integrity after 0, 24, 48 and 72 hours of incubation at 4°C. The evaluations of spermatozoa immediately after semen collection, were revealed no significant differences among values of treatment groups, whereas after incubating the treatments for different spans of time, the sperm progressive motility and viability rates for groups supplemented with linolenic acid were significantly ($P<0.05$) higher than that of the control group. Motility, viability and acrosome integrity were higher in 2.5ng/ml linolenic acid concentration. According to the results of this study we conclude that, the most excellent level of linolenic acid for supplementation to the extended semen of dog in order to improve the sperm motility and viability plus to reduce the morphological defect rates of the spermatozoa up to 72 hours storage time at 4°C is 2.5 ng/ml.
Key words: Chilled Semen, Linolenic Acid, Sperm Attributes, Labrador Dog Semen

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INTRODUCTION

The artificial insemination (AI) has become the important breeding tool for genetic improvement throughout the world. Till date only on the basis of phenotypic traits *viz* body weight, coat color, etc. the selection of the males has been made but these phenotypic traits are not promising, thus don't yield the quality results. So, the semen has to be evaluated for its quality traits as it determines the Breeding Value of the males. AI with chilled semen has become very

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popular among the breeders (Smith, 2006). AI with preserved semen collected from study dogs of documented high quality provides the breeder with a circle for dispersal of the desired germpool (Rota *et al*., 1998). Moreover, during cryopreservation sperm membrane is more prone to lipid peroxidation by free radicals such as O_2 and H_2O_2 which leads to structural damage of sperm membrane (Mavi *et al.,* 2019a; 2020a; 2020b; Kaur *et al*., 2020; Bisla *et al*., 2020; 2021a; Kumar *et al*., 2021; 2022). The major detrimental changes due to oxidative stress mainly occur at the plasma membrane and nucleus of the spermatozoa (Mavi *et al.,* 2017; 2019b; Ngou *et al*., 2020; Bisla *et al*., 2021b; Rautela *et al*., 2022). Dietary supplementation of fatty acids had previously been reported to improve semen quality in different species (Gholami *et al*., 2010). Fatty acids have also been added directly to the semen extender with variable effects in different species. Linolenic acid, a major long chain omega-3 fatty acid, is the main antioxidant in biological membranes. Linolenic acid present in the plasma membrane provides energy, modulate the structure and composition of lipid rafts. Preliminary studies had shown successful such as use of linolenic acid in cryopreservation of bovine semen sperm (Abavisani *et al*. 2013), Cattle (Kaka *et al*., 2015) and Goat (Hundal *et al.,* 2020). Therefore, the purpose of this study was to determine the effect of Linolenic acid supplementation on canine sperm quality.

MATERIALS AND METHODS

Semen ejaculates from five healthy Labrador dogs aged between 2-3 years were collected by hand massage/manipulation method at weekly interval. Each ejaculate was divided into five equal fractions and diluted to final concentration of $200x10^6$ sperm/mL (Cheema *et al*., 2021) using Tris based egg yolk phosphate (EYP) extender supplemented with different doses of linolenic acid (Sigma-Aldrich, St. Louis, USA), *viz*. 0.0 (control, T1), 0.5 (T2), 1.5 (T3), 2.5 (T4), and 3.5 (T5) ng/ml to optimize the most effective linolenic acid concentration for its chilling effect. As fatty acids are insoluble in water, ethanol (0.05%) was added as a solvent for preparation of linolenic acid concentrations.

Ejaculates having sperm motility >70% subjectively assessed under phase contrast microscope equipped with a warm stage (37°C) at 400x magnification were used throughout the study. Samples in tubes were shifted to a container containing warm water (37°C) and placed in a cooling cabinet at 4°C. Semen was analyzed for motility, viability, membrane integrity, acrosome integrity and morphological defects after every 24h till 72h of storage.

Statistical analysis

Data of various sperm attributes was analyzed by one way ANOVA using SPSS21 program (Student version for windows, SPSS Inc.233 South Wacker Drive, 11th floor Chicago, IL 60606-6412) to find out difference among the treatments. Normality of the data was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using the Levene test.

RESULTS AND DISCUSSION

Sperm motility, viability, membrane integrity, acrosome integrity and morphological defects of semen enriched with different concentrations of linolenic acid are shown in table 1, 2 and table 3. The percentage of total motility for 0.5, 1.5, 2.5, and 3.5 ng/ml linolenic acid was not significant at the start of sampling, but motility was quite comparable to that of the control group using linolenic acid ($P>0.05$). Till 72 hrs, non-significant difference was found in T3 and T4 for the motility but in viability non-significant difference was found in T3 and T4 till 48 hrs. S significant difference can be seen at 72hrs in all the treatment groups. Linolenic acid levels did not significantly improve membrane integrity, acrosome integrity and morphological defects at the start of the experiment compared to those of the control group. Linolenic acid supplementation at the dose of 2.5 ng/ml showed significant difference, with maximum activity at membrane integrity (70.09±4.13) and acrosome integrity (69.19±4.01) hours after storage of semen at 4 °C. Ejaz *et al*., 2017 reported in buffalo bulls in Tris-citric acid extender also revealed a higher ($P<0.05$) percentage of total motility in linolenic acid supplemented group at 5.0 ng/ml potential of post-thaw spermatozoa.

Morphological defect rates of spermatozoa in the groups supplemented with different levels of Linolenic acid were significantly ($P < 0.05$) lower than the control group. Semen obtained from dogs supplemented with Linolenic acid characterized better resistance to storage at 4°C. This was reflected by better motility and viability as well as lower morphological defects of spermatozoa after semen storage for 72 hours.

CONCLUSIONS

Addition of antioxidants such as Linolenic acid to the preservation media could improve longevity and quality of sperm in chilled dog semen. The appropriate level of

Table 1: Effect of Linolenic acid to EYP Extender of Dog Semen on Motility and Viability

Experimental period	Motility					Viability				
	T 1 (Control group)	T2 (0.5ng/ml)	T 3 (1.5ng/ml)	T4 (2.5ng/m)	T5 (3.5ng/ml)	T 1 (Control group)	T2 (0.5ng/ml)	T 3 (1.5ng/ml)	T4 (2.5ng/m)	T5 (3.5ng/ml)
0 hr	79.33^{a} ± 3.33	80.82^{a} ± 3.14	82.48^{a} ± 3.32	83.44^{a} ± 4.02	79.33^{a} ± 3.33	80.20^{a} ± 5.89	79.17^{a} ± 3.86	80.39^{a} ± 2.56	85.61^{a} ±1.56	80.33^{a} ± 3.33
24 hrs	68.33^{b} ± 3.33	73.03^{b} ± 2.24	79.50^{a} ± 4.08	80.14^{a} ± 3.29	70.33^{b} ± 3.33	65.67^{b} ± 2.85	68.59^{b} ± 4.81	78.25^{a} ± 4.36	81.48^{a} ±0.83	68.33^{b}± 3.33
48 hrs	59.00^{c} ± 5.00	65.90^{b} ± 5.85	71.67^{a} ± 4.65	75.32^{a} ± 3.52	63.00^{b} ± 5.00	51.67^{c} ± 0.67	66.68^{b} ± 3.93	75.86^{a} ± 3.15	77.03^{a} ±1.40	62.00^{b}± 5.00
72 hrs	45.33^{c} ± 6.01	55.23^{b} ± 4.50	69.90^{a} ± 5.85	72.09^{a} ± 4.13	53.33^{b} ± 6.01	42.00^{d} ± 1.00	53.83^{c} ±2.67	63.20^{b} ± 4.56	78.21^{a} ±1.39	50.33^{c} ± 6.01

Mean ± SE values with different superscripts in same row differ significantly (P<0.05)

Table 2: Effect of Linolenic acid to EYP Extender of Dog Semen on HOST and Acrosome Integrity

Semen attributes	HOST					Acrosome Integrity				
	T 1 (Control group)	T2 (0.5ng/ml)	T 3 (1.5ng/ml)	T4 (2.5ng/m)	T5 (3.5ng/ml)	T 1 (Control group)	T2 (0.5ng/ml)	T 3 (1.5ng/ml)	T4 (2.5ng/m)	T5 (3.5ng/ml)
0 hr	77.33^{a} ± 3.33	76.33^{a} ± 1.67	78.00^{a} ± 2.89	80.44^{a} ± 4.02	77.33^{a} ± 1.67	79.33^{a} ± 3.33	79.37^{a} ± 2.43	81.04^{a} ± 2.83	82.23^{a} ± 2.08	80.67^{a} ± 3.33
24 hrs	69.33^{b} ± 3.33	71.67^{b} ± 1.67	76.33^{a} ± 4.41	78.14^{a} ± 3.29	70.00^{b} ± 2.88	68.33^{b} ± 3.33	74.73^{b} ± 0.84	74.60^{a} ± 4.55	78.04^{a} ± 2.83	75.00^{b}± 2.88
48 hrs	59.29^{d} ± 5.00	63.33^{c} ± 1.67	70.67^{b} ± 4.41	77.32^{a} ± 3.52	61.67^{c} ± 1.67	52.21^{d} ± 5.00	61.03^{c} ± 1.50	68.10^{b} ± 4.01	74.60^{a} ± 4.55	60.33^{c} ± 1.67
72 hrs	48.33^{d} ± 6.01	56.58^{c} ± 1.67	61.67^{b} ± 3.33	70.09^{a} ± 4.13	54.33^{c} ± 1.67	47.33^{d} ± 6.01	54.27^{c} ± 2.85	60.08^{b} ± 3.47	69.19^{a} ± 4.01	51.33^{c} ± 1.67

Mean ± SE values with different superscripts in same row differ significantly (P < 0.05)

Table 3: Effect of Linolenic acid to EYP Extender of Dog Semen on sperm morphological defects (%)

Experimental period	T1 (control)	T2 (0.5ng/ml)	T 3 (1.5ng/ml)	T4 (2.5ng/m)	T5 (3.5ng/ml)
0 hr	14.86^{a} ± 2.85	14.12^{a} ±2.41	13.50^{a} ±1.28	15.22^{a} ±1.39	13.60^{a} ±3.05
24 hrs	21.63^{a} ±2.53	16.10^{b} ±2.31	11.26^{c} ±1.96	11.39^{c} ±2.20	18.29^{ab} ±3.17
48 hrs	34.55^{a} ±3.17	17.73^{c} ±2.86	16.93^{c} ±2.15	12.13^{c} ±1.81	23.19^{b} ±2.54
72 hrs	57.60^{a} ± 4.31	30.86^{b} ±3.66	20.47^{c} ± 4.55	19.20^{c} ± 2.47	32.26^{b} ±3.93

Mean ± SE values with different superscripts in same row differ significantly (P < 0.05)

Linolenic acid advised for supplementation to the extended semen of dog in order to improve the sperm motility and viability as well as reduce the morphological defect rates of the spermatozoa up to 72 hours during storage at 4°C was 2.5ng/ml.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest with this manuscript.

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