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Computer assisted semen analysis of deccani ram semen presevability at 5ºC

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ABSTRACT

To investigate the preservability of Deccani ram semen in three different extenders - SC (Sodium Citrate), T (Tris), C (Coconut milk) egg yolk extenders after dilution and chilling (5ºC) at 1, 24 and 48 hr. of storage. Eight Deccani adult rams (aged 2-4 years) were selected and ejaculate from each ram was divided into 3 aliquots and diluted with SC, T and C extenders (final concentration - 400 million spermatozoa/0.2 ml semen) and stored at 5ºC. Semen evaluation was done by computer assisted semen analyser (CASA). Analysis of Variance (ANOVA) was used to test the significance difference between extenders. **S:** Sodium citrate (SC) and Tris (T) - Egg yolk extender was significantly ($P<0.05$) better as compared to Coconut milk extender (C). No significant difference ($P>0.05$) was observed between SC and T. Sperm kinematics assessment by CASA revealed Sodium citrate-based egg-yolk extender (SC) as better extender for preservation of Deccani Ram semen upto 48 hr. of storage as compared to T and C.

Key words: Computer assisted semen analysis, Liquid preservation, Ram semen, Sperm kinematics

INTRODUCTION

Reproductive efficiency of the livestock can be improved by selection of breeding sires with high fertilizing capacity. Artificial insemination (AI) proves to be the most accurate method of assessment of fertilizing capacity, but it is time consuming and at a time, only limited number of animals can be tested. Conventional semen analysis of sperm motility using light microscopy resulted in a 30-60% (Cancel *et al.,* 2000) variability caused by the evaluator's skills (Tretipskul *et al.,* 2010). Computer-assisted sperm analysis information on motility characteristics and morphometric dimensions (Rijsselaere *et al.,* 2005) and offers additional insights into sperm kinetics (Freour *et al.,* 2010). Freour *et al.,* (2012) reported that the use of CASA allowed the identification of different age-related decreases in velocity parameters of young men with proven fertility. Motility determined by CASA has been correlated with both in vitro and *in vivo* fertility in addition to heterospermic performance (Utt, 2016). Kasimanickam *et al.* (2006) also concluded a significant positive correlation between progressive motility and a heterospermic Competitive Index in vivo. Hence, the use of highly sophisticated instrument like CASA is needed for assessment of sperm quality in terms of motility, swimming pattern, head behavior, etc. in understanding possible sperm functions and semen quality (Rai *et al.,* 2017).

In India, Deccani sheep breed was known for its draught resilience nature. Animal husbandry department focused on Deccani breed improvement under Andhra Pradesh Drought Adaptation Initiative (APDAI) project through AI. Use of fresh, cooled and frozen semen is in practice for AI. Paulenz *et al.* (2002) reported that Tris based extender preserved better sperm viability than sodium citrate and milk based extender when stored upto 30 hr. (5ºC) while Albiaty *et al.,* (2016) concluded that tris and sodium citrate extenders were preferable as compared to milk Extender. Detailed research is required for determination of best extender for liquid preservation (5ºC) of Deccani ram semen. Therefore, the present study aimed at investigation of preservability of three semen extenders (Sodium Citrate, Tris and Coconut milk based egg yolk extenders) upto 48 hr. of storage (5ºC) by Computer assisted semen analysis.

MATERIALS AND METHODS

The study was carried out at the Livestock Farm Complex (LFC), C.V.Sc, P.V. Narsimha Rao Telangana Veterinary University, Rajendranagar (Longitude: 78.4018° E, Latitude: 17.3203° N) during January to May, 2015. Semen was collected twice a week from Eight Deccani rams (aged 2-4 years) (Fig. 1 and 2). Experimental animals were fed with concentrate feed (300 gm/day/head) in addition to adlibitum green fodders. Selected Rams were screened for brucellois and vaccinated as per schedule. A total of 144 semen ejaculates (18 ejaculates/ram) were collected with the aid of Artificial vagina (42-45ºC) during the experimental study. Semen was collected in a graduated glass test tube in a thermos flask containing warm water maintained at 37ºC. Immediately after collection, macroscopic parameters like semen volume, colour and consistency were recorded and then, the semen was diluted (1:2) with one of the following extenders maintained at 37ºC, namely-

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Fig-1: Deccani Ram species for semen collection

Fig-2: Semen collection from Deccani ram species

(i) **Extender SC (pH-6.8; 325 mOsm/kg)**: Sodium citrate-based egg-yolk extender was prepared from 2.9% trisodium citrate aqueous solution and egg yolk (20% v/v).

(ii) **Extender T (pH-6.8; 325 mOsm/kg)**: Tris-based egg-yolk extender containing Tris (3.028 g), fructose (1.25 g); citric acid (1.675 g), distilled water (100 ml) and egg yolk (20% v/v).

(iii)**Extender C (pH-7.4; 325 mOsm/kg)**: Coconut milk extender contains 15ml of coconut water (heated to 95ºC for 10 min and filtered), sodium citrate dihydrate (2.2 g), egg yolk (7% v/v).

Antibiotics added were streptomycin (1000 µg/ml) and pencillin (1000 IU/ml).

Semen samples were sent to laboratory for concentration and mass activity (0-5 score) estimation. Then, diluted to a final concentration of 400 million spermatozoa/ 0.2 ml semen and stored at 5ºC.

Computer assisted semen analysis (CASA): After 1, 24 and 48 hr. of storage (5ºC), semen samples were transferred in an insulated ice box (internal temperature maintained at 5ºC) to LaCONES (Laboratory for Conservation of Endangered Species, Hyderabad) for CASA analysis (HTMIVOS v.10.6; Hamilton–Thorne, Beverly MA, USA) . Semen samples for motility analysis were prepared by diluting the semen with normal saline to attain a final concentration of 25×10^6 spermatozoa/ml. 5 µl of diluted semen was loaded into pre-warmed microscopic slide (37°C) and covered with coverslip. Sperm kinematics were determined by random selection of six microscopic fields (>200 spermatozoa/sample) utilising factory CASA ram settings (Table No.1) Motility and Velocity parameters measured in the study was adopted from Larsen *et al.,* (2000). Wobble percentage (WOB) was derived by the formula given by Mircu *et al.* (2008):

Wobble percentage (WOB) = (VAP/VCL) x 100

The data obtained was statistically analysed by Analysis of Variance (ANOVA; SPSS, v.16). The significance of the parameters was measured at $P<0.05$ level of significance. The comparison of the means was done by Duncan's Multiple Range test (DMRT) to test the significance between the extenders.

RESULTS AND DISCUSSION

The overall mean scrotal circumference, semen ejaculate volume, sperm concentration, colour, consistency and mass activity of Deccani ram semen were represented in Table 2. There was significant difference between the semen volume obtained per ejaculation and mass activity (0-5 score) between the animals ($P\leq0.05$). There was no significant difference between the sperm concentration of spermatozoa between the animals ($P\geq0.05$). Aguirre *et al.* (2007) also reported no variation in sperm concentration in Pelibuey ram semen where semen volume differed significantly when semen was collected twice per week compared to one collection/week. The difference might be

Table 1: Analysis set-up for CASA (HTM IVOS v.10.6) used to evaluate Deccani ram spermatozoa

Variables	Settings
Frame rate (Hz)	60
frames acquired	30
Minimum contrast	15
Minimum cell size (pixels)	4
Minimum static contrast	30
Threshold straightness (%)	80
Low VAP cut off (µm/sec)	5
Medium VAP cut off (µm/sec)	15
low VSL cut off (µm/sec)	25
Non motile head size (pixels)	10
Non motile head intensity	180
Static head intensity	0.15-0.74
Static elongation limits	0.15-0.74
Magnification	1.95
Stage configuration	Slide-coverslip

Table 2: Scrotal circumference, Semen Volume, Concentration, Colour, Consistency and Mass activity in Deccani rams

Ram No	Scrotal circumference(cm)	Semen volume (ml)(n=144)	Semen concentration (millions/ml)	Mass activity(0-5 score)
1	27.00	0.88 ± 0.24^{a} (n=18)	11640.00 ± 1953	4.25 ± 0.88^{a}
2	25.50	0.35 ± 0.27^{d}(n=18)	9458.300 ± 3584	3.41 ± 0.51^{bc}
3	26.50	0.64 ± 0.16^{bc} (n=18)	11499.00 ± 2835	3.71 ± 0.61^{bc}
4	25.00	0.50 ± 0.20^{cd} (n=18)	9275.700 ± 2618	3.64 ± 0.49^{bc}
5	25.00	0.56 ± 0.22^{c} (n=18)	11335.00 ± 3230	3.23 ± 0.43^{c}
6	25.50	0.78 ± 0.21^{ab} (n=18)	11445.00 ± 2002	3.66 ± 0.48^{bc}
7	28.50	0.85 ± 0.26^{a} (n=18)	11052.00 ± 2517	3.83 ± 0.38^{ab}
8	24.00	0.54 ± 0.17^{cd} (n=18)	11323.00 ± 1575	3.57 ± 0.53^{bc}
Overall Mean ± S.E	**25.90**	**0.63 ± 0.27**	**10825.00±2742**	**3.64±0.58**

Values are Mean ± S.E and values in parenthesis are the no. of ejaculates collected from each ram; Values with different superscripts within column differ significantly ($p<0.05$).

attributed to semen collection frequency (one collection/two weeks *Vs* two collections/week) (Aguirre *et al.*, 2007).

The semen volume obtained in the present study was in accordance to that of the volume obtained in Muzzafarnagari rams (0.68 ml) (Jayaganthan *et al.*, 2015). Higher volume of semen was reported in Zulu rams of South Africa (0.9-1.1 ml) (Chella *et al.*, 2017). Lower semen concentration was reported in Kivircik and Awassi rams (Alcay *et al.*, 2014), Sanjabi rams (Ghorbankhani *et al.*, 2015) (1200 - 4900 million sperms/ml), Muzzafarnagari rams ($2667x10^{6}$/ml) (Jayaganthan *et al.*, 2015), Zulu rams of South Africa (2300-4000 millions/ml) (Chella *et al.*, 2017) compared to the semen concentration obtained in the present study. The variation in semen concentration in the present study could be attributed to lower semen volume and genetically higher semen reservoir function in Deccani rams. Mass activity obtained in the present study was comparable to that of the mass activity of Muzzafarnagari ram semen (4.0) (Jayaganthan *et al.*, 2015). The average semen colour of Deccani rams was similar to that of Indigenous ram semen of Bangladesh (Azizunnesa *et al.*, 2014). The motility, velocity and morphology parameters assessed by CASA were presented in the Table 3 and 4. The chilled (5ºC) semen after 1 hr. of storage differed significantly ($P\leq0.05$) between extenders pertaining to total motility, progressive motility, rapid motility and BCF. The parameters like total motility, progressive motility, rapid motility, VAP, VSL and VAP, decreased significantly ($P\geq0.05$) as duration of storage increased which was inaccordance to the findings of Kasimanickam *et al.*, (2011). The variation in mean VAP, VCL might be due to concentration x days of storage interaction (Kasimanickam *et al.*, 2007). The decline in the parameters like Motility, PM, Rapid Motility, VAP, VSL, VCL and STR during storage period may be due to the depletion of sperm energy source and decrease in sperm quality since the pH was changed by the metabolic products resulting in intoxication (Jeyendran *et al.*, 1984). Cold shock occurs during semen preservation at lower temperatures poses stress on the sperm cell membrane (Thuwanut *et al.*, 2011) via production of Radical Oxygen species (ROS) which damages the sperm cell membrane resulting in lower sperm motility and survivability (Salamon and Maxwell, 1995).

The mean values of Straightness (STR, %), Linearity (LIN, %), Amplitude of Lateral Head displacement (ALH, µm), Elongation (ELG, %) and Area (μm^2) did not differ significantly ($P\geq0.05$) between the semen extenders. Kasimanickam *et al.*, (2011) reported no notable changes in ALH and STR, together with the storage period and different extenders at a point in time which was in agreement to the present findings. Robayo *et al.*, (2008) concluded that VCL) and VAP kinematic parameters presented significant positive correlations with sperm migration efficiency in ovine mucus. The VAP (average path velocity) and VCL were significantly higher ($P\leq0.05$) for Sodium citrate and Tris based extenders at 48 hrs of storage than that of Coconut milk based extender

Table 3: Semen motility parameters (Mean ± S.E) of Deccani ram semen observed with CASA after dilution with three semen diluents and stored (5ºC) at 1hr., 24 hr. and 48 hr.

EXTENDER	STORAGE	MOTILE (%)	PM (%)	RAPID (%)	Elongation(%)	Area(μm^2)
EYC	1hr	82.38 ± 1.78^{c}	78.05 ± 2.08^{c}	80.16 ± 1.46^{c}	39.80 ± 1.15	6.06 ± 0.15
	24hr.	80.38 ± 1.77	76.97 ± 1.49	78.55 ± 1.71	40.52 ± 1.09	6.09 ± 0.13
	48hr.	77.77 ± 1.52	73.61 ± 1.63	72.25 ± 2.28	40.42 ± 0.82	6.01 ± 0.16
TCFEY	1hr	89.52 ± 0.94^{b}	83.52 ± 1.10^{b}	85.30 ± 1.01^{b}	40.96 ± 0.90	6.01 ± 0.14
	24hr.	78.08 ± 1.82	74.91 ± 1.32	73.88 ± 1.77	39.51 ± 0.94	6.10 ± 0.17
	48hr.	79.44 ± 1.38	73.61 ± 1.56	72.58 ± 2.47	40.45 ± 0.87	6.10 ± 0.14
CME	1hr	91.00 ± 0.68^{a}	86.01 ± 0.88^{a}	86.84 ± 0.95^{a}	40.07 ± 1.09	6.19 ± 0.15
	24hr.	79.69 ± 1.18	76.02±1.24	77.41±1.08	40.72 ± 1.10	6.31 ± 0.17
	48hr.	76.38 ± 1.98	69.05 ± 1.67	71.63 ± 1.74	40.37 ± 1.01	6.19 ± 0.15

Values with different superscripts within columns (a, b, c – between extenders) differ significantly ($p<0.05$)

Table 4: Semen velocity parameters (Mean ± S.E) of Deccani ram semen observed with CASA after dilution with three semen diluents and stored (5°C) at 1hr., 24 hr. and 48 hr.

STORAGE		EXTENDER	VAP (µm/sec)	VSL(µm/sec)	VCL (µm/sec)	STR (%)	LIN (%)	WOB (%)	ALH (µm)	BCF (Hz)
EYC	1hr	T	123.80 ± 6.13	97.84 ± 5.62	217.32 ± 8.76^{a}	79.16 ± 2.30	45.58 ± 2.13	57.94 ± 2.41^{c}	7.13 ± 0.15	26.36 ± 3.75^{b}
	24hr.		114.50 ± 4.20	87.12 ± 3.58	197.43 ± 8.81^{a}	76.83 ± 2.37	46.19 ± 2.17	60.55 ± 2.46	7.10 ± 0.21	26.77 ± 1.35
	48hr.		113.93 ± 5.15^{a}	86.23 ± 5.02	190.77 ± 7.94^{a}	75.19 ± 2.36	45.91 ± 2.37	62.00 ± 3.38	6.91 ± 0.21	26.63 ± 0.65
TCFEY	1hr		138.36 ± 7.54	98.81 ± 5.46	221.57 ± 7.76^{a}	72.47 ± 2.67	44.72 ± 2.07	62.38 ± 2.39^{a}	6.88 ± 0.21	28.16 ± 0.75^{a}
	24hr.		120.81 ± 5.97	84.97 ± 4.32	195.94 ± 8.10^{a}	71.16 ± 2.25	44.94 ± 2.48	61.66 ± 2.49	6.82 ± 0.25	27.36 ± 0.84
	48hr.		118.30 ± 5.42^{a}	81.76 ± 5.16	190.67 ± 7.46^{a}	68.69 ± 2.39	44.13 ± 2.72	63.63 ± 2.83	6.73 ± 0.22	26.90 ± 0.83
CME	1hr		119.48 ± 5.10	88.33 ± 3.50	202.41 ± 6.67^{b}	75.11 ± 2.14	44.16 ± 1.63	59.61 ± 2.22^{b}	7.05 ± 0.16	24.88 ± 0.71^{c}
	24hr.		110.66 ± 4.25	80.25 ± 2.03	177.67 ± 6.37^{a}	74.63 ± 2.08	46.94 ± 1.84	63.80 ± 2.26	7.02 ± 0.25	24.55 ± 0.56
	48hr.		101.46 ± 4.65^{b}	73.47 ± 4.16	160.64 ± 6.46^{b}	72.72 ± 2.61	46.02 ± 2.10	63.83 ± 2.36	6.81 ± 0.20	24.34 ± 0.57

Values with different superscripts within columns (a, b, c – between extenders) differ significantly (P<0.05)

at 48hrs of storage indicating better migration efficiency of sperm extended with T and SC. STR was comparatively low for semen extended with T.

Even though, there was no significant difference between the LIN among the semen extenders, extender SC showed higher LIN, indicating the straightness of the spermatozoal movement as compared to other extenders. Immediately after 1hr of storage, WOB % was significantly (P<0.05) higher for semen extended with T and C while lower percentage was recorded for semen extended with SC. WOB would be low for a track with a wide trajectory (high ALH), but high for a circling track (Mircu *et al.,* 2008) showing the adverse effects of Tris on the variables related to the quality of sperm movement (higher WOB and VCL), with a higher proportion of circular trajectories.

Paulenz *et al.,* (2002) found that spermatozoa diluted in the TRIS based extender showed a higher sperm motility, membrane integrity and number of uncapacitated spermatozoa up to 30 hrs of storage at 5ºC. Paulenz *et al.,* (2003) inseminated sheep with liquid semen *in vivo* and stated that the better *in vitro* characteristics seen in the TRIS-based extender were not reflected in field fertility. The discrepancy found between the *in vitro* and *in vivo* studies might be due to (i) poor buffering capacity of Tris at a pH<7.5 (Yaniz *et al.,* 2011) (ii) TRIS is not physiologically inert (Yaniz *et al.,* 2012) (iii) interacts with metal ions which interferes with calcium uptake by the membrane vesicles and sperm cells (Upreti *et al.,* 1995), thus inhibiting capacitation and acrosome reaction, and (iv) the pH of a Tris-based solution is temperature-dependent. Yaniz *et al.,* (2012) stated that Tris buffer causes an increase in the inner pH of sperm cell which may also have an impact on sperm motility (Gadea, 2003; Jones *et al.,* 2000). However, Citrate buffer impedes the effects of toxic metal ions and supports mitochondrial ATP production. In the present study, higher VAP, VSL and motility % might be due to masking effects of citrate and egg yolk lipoproteins but the overall negative impacts cannot be changed which can lead to lower conception rate. Thus, indicating the fact that SC would be suitable extender for preservation of Deccani ram semen as compared to T and C.

CONCLUSION

Eventhough PM, VSL, VCL (positive indicators for fertility) were higher for both Tris and Sodium citrate (SC) extenders, higher proportion of circular trajectories, low STR and high WOB percentage (negative indicator of fertility) for Tris based egg yolk extender proved that Sodium citrate based egg yolk extender would be better for liquid preservation of Deccani ram semen as compared to Tris based egg yolk extender and Coconut milk based egg yolk extender. Thus, CASA revealed precise indepth information regarding the semen kinematics of Deccani ram semen.

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