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CASA Based Assessment of Kangayam bull semen processed with different extenders and Conception rate following AI

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ABSTRACT

Semen extenders are the essential components of cryopreservation of semen. Identification of suitable and ready to use semen extender is the need of hour for the rapid propagation of Kangayam cows through artificial insemination. Kangayam bull, aged 5 years was utilized for the study. The semen was collected twice in a week and each time two ejaculates were collected. A total of 24 ejaculates were utilized for this study. After assessing the general macroscopical examination the semen was subjected to computer aided semen analysis (CASA) for assessing the kinematic parameters in fresh diluted semen, pre freeze and post thawed such as sperm motility, progressive individual motility, VAP, VSL, ALH and LIN were analyzed using CASA. Among all the three extenders, the sperm kinematic parameters during fresh diluted, pre freeze and post thawed semen stages were higher in group III (liposome based extender) followed by group I (TEYG) and in group II (soya bean lecithin based extender). Among the three extenders used, the components of liposome based extender was better and it was able to maintain the structural integrity, plasma membrane integrity and motility in the sperms of the frozen thawed semen than the other two extenders (TEYG and soya bean lecithin based extenders). Further the fertility rate was evaluated by the *in vivo* insemination of 60 Kangayam cows after 12 hours of onset of estrum. The fertility rate observed was 45.00, 40.00 and 65.00 per cent in groups I, II and III, respectively. The overall conception rate recorded in Kangayam cow was 50.00 per cent. Based on the findings, it was concluded that liposome based extender may be used for cryopreservation of Kangayam bull semen in order to maximize the fertility rate.

***Key words*:** Kangayam, CASA, Kinematics, Conception rate, Extenders.

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INTRODUCTION

Kangayam breed of cattle is a very good draught purpose breed found in the native tract of Tiruppur, Erode, Namakkal, Coimbatore and Salem districts of Tamil Nadu. A pair of Kangayam bullocks can haul a total load of approximately 3787 kg of sugarcane over 20 kms (Kandasamy, 2001). The size of Kangayam population has been decreasing in a while. Artificial insemination helps in distributing superior germplasm to a wider parts of the country (Selvaraju *et al.,* 2008, Senthilkumar *et al.,* 2016 and Elamurugan *et al.,* 2020). Success of AI depends on the quality of semen after freezing which again rely on the composition and type of extenders used. Computer assisted semen analysis (CASA) helps in analyzing sperm concentration, sperm motion and sperm morphology and it is more precise and effective tool for fertility assessment (Kathiravan *et al*., 2011). CASA is also used in assessing the sperm quality which changed during processing, packaging and storage (Amann and Waberski, 2014). Sperm motility including average path velocity can be assessed using CASA which had clinical relevance to the fertility rate (Nagy *et al*., 2015). TRIS egg yolk glycerol extender is the universal extender commonly employed in the cryopreservation of bull semen.

Semen extenders not only increase the volume of each ejaculate but also extend the longevity of sperm cells (Selvaraju *et al.,* 2008 and Raheja *et al.,* 2018). They are basically designed for preventing change in osmolarity and pH, so that minimum sperm damage takes place. The use of egg yolk in extenders is dates back to 1939, when Phillips (1939) discovered its protective effects on cooled bovine semen. Despite the good fertility rates using extenders containing egg yolk and/or milk, these components represent a risk of contamination which can release endotoxins that reduce the fertilization capacity of sperm (Bittencourt *et al.,* 2008). To reduce the injurious effects of egg yolk, the trend is being shifting towards the substitution of tris egg yolk based extender with phospholipid/liposome-based extender (Ropke *et al.,* 2011). Accordingly, extenders free of animal protein have been tested in recent years (Vidal *et al.,* 2013). As the universal extender poses multiple constraints including microbial contamination (Shin *et al*., 1998), difficulty sperm analysis (Ansari *et al*., 2010) and had negative impact sperm plasma membrane (Ansari *et al*., 2016), the need for alternative extenders raised. Soya been lecithin (Chelucci *et al*., 2015) and Liposome based extenders (Lima-Verde *et al*., 2018) give promising results. Hence, an investigation was undertaken to compare synthetic semen extenders with universal semen extender in Kangayam bull semen. The objective of this study is to use CASA in analyzing the sperm kinematics in Kangayam bull semen diluted with the above extenders in various stages of processing.

MATERIALS AND METHODS

Source of experimental animals

A 5 year old Kangayam bull reared in Frozen Semen Bank in the Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Namakkal was selected for the study.

Semen collection

A total of 24 ejaculates of semen were collected twice a week and two collections by Artificial Vagina (AV) method. The collected ejaculate was immediately transferred to semen processing laboratory and kept at 34°C in the water bath. Immediately after collection of semen, the basic macroscopical examination was performed.

Preparation of various diluents

Tris egg yolk glycerol (TEYG) extender (Universal): The composition of 100 ml of TEYG extender is detailed below.

S. No.	Components	Quantity
Buffer		
1	Tris	2.42 gm
2	Citric acid	1.36 gm
3	Fructose	1.0 gm
4	Glycerol	7 ml
5	Distilled water (To make up to)	80 ml
Antibiotics and egg yolk		
6	Dihydrostreptomycin	100 mg
7	Penicillin G sodium	1 lac unit
8	Egg yolk	20 ml

The buffer was prepared by adding items from S. No 1 to 5, on the previous day of semen collection and autoclaved at 5 psi for 20 minutes. The extender was prepared on the day of semen collection by adding the components from S. No. 6 to 8 to the buffer. The prepared extender was kept in water bath at 34° C for processing of semen.

Soya bean lecithin based extender (BioXcell®)

The dilution ratio for the stock Bioxcell solution, IMV Tehnologies, France was used as per the recommendation

of its firm. The dilution of Bioxcell was made 1:5 using sterile triple distilled water. Prepared diluent was kept in the water bath at 37° C for 10 minutes. Then it was used for semen extension.

Liposome based extender (OptiXcell®)

The dilution ratio for the stock Optixcell solution, IMV Tehnologies, France was used as per the recommendation of its firm. The dilution of Optixcell was made 1:3 using sterile triple distilled water. Prepared diluent was kept in the water bath at 37° C for 10 minutes. Then it was used for final semen extension.

Extension and freezing of semen

The final diluted semen sample was taken for filling, sealing and printing. Then the straws were kept in the cold handling cabinet for 4 hours at 5°C for equilibration. After 4 hours of equilibration, the equilibrated semen straws were arranged on a freezing rack. The freezing rack was transferred to bio freezer and the freezing was carried out by using the preset programme available in the bio-freezer (Micro Digitcool, IMV Technologies, France). The temperature of the semen straws was reduced from 5° C to -140° C in a fast and controlled manner in 7 minutes. Then the frozen semen straws were transferred to a pre-cooled goblet and plunged into liquid nitrogen container for storage. During these above said procedures the CASA was employed to access the kinematic parameters on fresh diluted, pre-freeze and post-thaw stages (24 hours and 30 days after freezing).

Semen evaluation -CASA

Immediately after semen collection, CASA: IVOS Version 14, Hamilton Thorne was used to analyze sperm kinematics. CASA settings were fixed as described by Sundararaman *et al.,* (2008). A two µl of neat semen sample was diluted with 400 µl of Tris solution. After dilution, two µl of sample was loaded in one chamber of prewarmed Leja slide and kept in microscopic stage of CASA. Four fields were scanned automatically and sperm motility characteristics were determined with a 10X objective. Sperm kinematics such as motility (per cent), progressive motility (per cent), VAP (Average Path Velocity, µm/sec), VSL (Straight Line Velocity, µm/sec), VCL (Curvi Linear Velocity, µm/sec), ALH (Amplitude of Lateral Head displacement, µm), LIN (Linearity Index; LIN = {VSL/VCL} X 100) and the sperm concentrations were measured using CASA.

Evaluation of fertility rate

Total of 60 cows were inseminated with the frozen semen extended with all the three extenders (20 cows per extender group) at 12 hours after onset of behavioral estrus. Trans rectal ultrasound guided pregnancy diagnosis was made after 45 days of pregnancy and results were recorded and analyzed.

Statistical analysis

The collected data have been analyzed statistically by using One-Way Repeated Measures ANOVA test to compare groups at Diluted, pre freeze, post thaw 24 hours and 30th days for CASA values. Chi-square was employed to test the significance of pregnancy between groups.

RESULTS AND DISCUSSION

Sperm kinematics such as motility (per cent), progressive motility (per cent), VAP (Average Path Velocity, µm/sec), VSL (Straight Line Velocity, µm/sec), VCL (Curvi Linear Velocity, µm/sec), ALH (Amplitude of Lateral Head displacement, µm), LIN (Linearity Index; LIN = {VSL/VCL} X 100) and the sperm concentrations were measured by using CASA for neat semen, pre-freeze and post-thaw (24 hrs and 30 days post freezing) stages and results were depicted in table 1 and 2.

FRESH DILUTED SEMEN

The mean (±SE) percentage of fresh diluted sperm motility were 87.52±0.84, 87.63±1.03 and 91.27±0.41 progressive motility (PM) were 51.40±0.26, 51.42±0.17, 52.58±0.24 and linearity index (LIN) were 38.83±0.69, 39.76±0.38 and 44.22±0.23 in groups I, II and III, respectively. The sperm motility, progressive motility and LIN of group III differ significantly from groups I and II. The mean (±SE) percentages of average path velocity (VAP), straight line velocity (VSL) curvilinear velocity (VCL), beat cross frequency (BCF) and amplitude of lateral head displacement (ALH) of fresh diluted semen were analysed in this study. There was a significant difference among the 3 groups in VAP and in BCF. The mean (±SE) VSL and VCL of fresh diluted semen of group III differed significantly from groups I and II but no significant difference was noticed between groups I and II. The mean ALH of fresh diluted semen of group I and III differed significantly from each other; these groups did not differ significantly from group II.

Table 1: Estimation of sperm kinematic characteristics by casa at freshly diluted and prefreeze semen

Parameters	Freshly diluted Semen (22° C)			Prefreeze semen (5°C)		
	Group I (TEYG)	Group II (Soya bean lecithin based)	Group III (Liposome based)	Group I (TEYG)	Group II (Soya bean lecithin based)	Group III (Liposome based)
Sperm Motility (per cent)	87.52^{a}±0.84	87.63^{a}±1.03	91.27^{b}±0.41	76.83^{a}±0.67	77.43^{a}±1.03	85.45^{b}±1.34
Progressive Motility (per cent)	51.40^{a}±0.26	51.42^{a}±0.17	52.58^{b}±0.24	49.86^{a}±0.40	49.12^{a}±0.51	51.53^{b}±0.33
VAP (µm/s)	96.29^{b}±0.33	93.95^{a}±0.42	99.07^{c}±0.16	91.75^{a}±0.41	91.96^{a}±0.28	96.85^{b}±0.32
VSL (µm/s)	66.89^{a}±0.86	68.70^{a}±0.73	74.12^{b}±0.21	69.46^{a}±0.34	68.85^{a}±0.35	71.35^{b}±0.46
VCL (µm/s)	172.36^{b}±1.22	172.74^{b}±0.46	167.65^{a}±0.82	170.70^{b}±0.22	171.52^{b}±1.19	164.41^{a}±1.09
ALH (µm)	7.33^{ab}±0.10	7.16^{a}±0.08	7.42^{b}±0.04	6.87^{a}±0.11	7.10^{a}±0.10	7.61^{b}±0.17
BCF (Hz)	26.77^{b}±0.32	25.62^{a}±0.37	27.83^{c}±0.25	24.19^{a}±0.35	25.00^{a}±0.36	26.58^{b}±0.44
LIN (per cent)	38.83^{a}±0.69	39.76^{a}±0.38	44.22^{b}±0.23	40.69^{a}±0.19	40.15^{a}±0.30	43.42^{b}±0.50

Means bearing different superscripts differ significantly (P < 0.05).

Table 2: Estimation of sperm kinematic characteristics by casa at post freezing (24 hours and 30 days after storage)

	Post thaw (24 hours)			Post thaw (30 days)		
Parameters	**GROUP I (TEYG)**	**GROUP II (Soya bean lecithin based)**	**Group III (Liposome based)**	**GROUP I (TEYG)**	**GROUP II (Soya bean lecithin based)**	**Group III (Liposome based)**
Sperm Motility (per cent)	69.78^{a}±0.86	68.86^{a}±0.80	77.34^{b}±0.55	68.85^{a}±0.76	68.62^{a}±0.67	76.89^{b}±0.52
Progressive Motility (per cent)	32.49^{a}±0.23	31.80^{a}±0.30	34.97^{b}±0.28	32.91^{a}±0.35	32.25^{a}±0.20	33.73^{b}±0.26
VAP (µm/s)	64.28^{a}±0.72	63.81^{a}±0.35	70.29^{b}±0.44	63.45^{a}±0.81	63.43^{a}±0.43	69.41^{b}±0.21
VSL (µm/s)	51.66^{a}±0.24	51.09^{a}±0.13	55.28^{b}±0.43	51.85^{a}±0.18	51.44^{a}±0.26	55.31^{b}±0.35
VCL (µm/s)	125.11^{b}±0.34	124.54^{ab}±0.68	122.97^{a}±0.62	125.33^{b}±0.47	124.75^{b}±0.67	122.30^{a}±1.01
ALH (µm)	6.40^{a}±0.09	6.39^{a}±0.04	7.36^{b}±0.08	6.61^{b}±0.04	6.47^{a}±0.02	7.23^{c}±0.03
BCF (Hz)	25.07^{a}±0.24	25.21^{ab}±0.16	26.13^{b}±0.49	25.62^{a}±0.19	25.52^{a}±0.08	27.05^{b}±0.14
LIN (per cent)	41.30^{a}±0.25	41.03^{a}±0.25	44.96^{b}±0.34	41.37^{a}±0.13	41.24^{a}±0.27	45.25^{b}±0.52

Means bearing different superscripts differ significantly (P < 0.05).

The results of the present study with fresh diluted semen at 22° C was in accordance with the results of Ratnawati *et al.* (2018) who recorded motility, progressive motility, VCL, VSL, VAP, LIN, STR and ALH in Ongole crossbred, Bali and Madhura bulls. The results of the present study was less than that reported by Kumar *et al.* (2015) in Murrah bull fresh semen diluted with TEYG. They found spermatozoa motility (82.28±10.37), progressive motility (39.89±7.78), VCL (233.16±32.32), VSL (96.58±5.00), VAP (129.47±13.06), STR (75.69±6.20) and LIN (44.15±5.53) using CASA.

Pre freeze semen (5°C)

The mean (±SE) percentage of sperm motility, progressive motility, average path velocity (VAP) straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH) and linearity index (LIN) of pre-freeze semen, in groups I, II and III were studied in this investigation. The mean percentage of MOT, PM, VAP, VCL, VSL, ALH, and BCF and LIN of group III differed significantly when compared to groups I and II. But, there was no significant difference between the groups I and II. The findings of the current study were in accordance with the results of Karthikeya *et al.* (2003) in Jersey bull semen evaluation at pre freeze stage. They recorded total motility (95.9±0.5 per cent), PM (54.1±1.9) per cent, path velocity (127.1±3.4 µm/s), progressive velocity (88.4±1.9 **µm/s), ALH (10.4±0.2µm), BCF (25.7±0.8 Hz) with CASA.**

Post thaw at 24 hours and 30th day post freezing

The mean (±SE) percentage of sperm motility progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH) and linearity index (LIN) of post-thaw semen at 24 hrs. were evaluated using CASA. The mean (±SE) percentage of sperm motility, progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH) linearity index (LIN) of post-thaw semen at 30th day post-freezing in groups I, II and III were studied. There was a significant difference at 24 hours and 30th day post-freezing in group III when compared to groups I and II but no significant difference was found between groups I and II in mean values of MOT, PM, VAP, VSL, VCL and in LIN. At 24 hours post freezing, of ALH, there was no significant difference between groups I and II and group III was significantly differed from group I and II. ALH at 30th day post freezing all groups differed significantly among them. At 30th day of post freezing the BCF of group III differed significantly from groups I and II and there was no significant difference between group I and II, whereas at 24 hours of post freezing BCF of groups I and III differed significantly each other, no significant difference was found between groups I and II and II and III.

Letie *et al.* (2010) reported motility, PM, VSL, ALH, BCF, LIN, STR in TEYG diluent as 41.0±11.8 per cent, 30.8±10.4 per cent, 55.8±6.1 µm/s, 6.0±0.9 µm, 22.0±4.9 Hz, 48.6±4.8 per cent, 79.6±5.7 per cent and in Bioxcell diluent as 28.6±16.0 per cent, 22.9±2.2 per cent, 64.7±9.0 µm/s, 5.6±1.0 µm, 29.7±5.0 Hz, 53.7±5.0 per cent, 86.0±3.7 per cent respectively during post thaw evaluation. Similarly, Kumar *et al.* (2015) reported motility (45.64±7.40 per cent), progressive motility (23.96±8.95 per cent), VCL (155.31±9.96 µm/s), VSL (76.28±8.76 µm/s), VAP (90.53±12.08 µm/s), STR (84.68±2.75 per cent) and LIN (51.68±3.10 per cent) in Murrah bull semen diluted with TEYG.

Miguel-Jimenez *et al.* (2020) reported VCL, (117.9±0.20 µm/s), VSL (56.7±0.20 µm/s), VAP (77.5±0.20 µm/s) LIN (48.5±0.20 per cent), STR (71.1±0.20 per cent) and WOB (71.1±0.20 per cent) in TEYG diluent; VCL (117.0±0.30 µm/s), VSL (52.0±0.20 µm/s), VAP (72.4±0.20 µm/s), LIN (45.6±0.20 per cent), STR (69.4±0.20 per cent) and WOB (63.4±0.20 per cent) in lecithin based semen diluent; VCL (123.5±0.20 µm/s), VSL (54.8±0.20 µm/s) in liposome based diluent; VAP (72.6±0.20 µm/s), LIN (44.8±0.20 per cent), STR (72.5±0.20 per cent) and WOB (60.2±0.20 per cent) in Triladyl diluent.

In the present study, the sperm kinematic character was higher in semen extended in Liposome based extender (Optixcell) followed by TEYG and soya lecithin based semen extender (Bioxcell). Sundaraman *et al.* (2012) reported that equilibration of semen at 5° C significantly reduced the proportion of motile sperm and significant decline in most of the sperm motion character during cryopreservation. This might be due to peroxidation of fatty acids of the sperm, which destroys the structural integrity of plasma membrane leading to the loss of motility (Sundararaman and Edwin, 2012). Further the cryoprotective agent, glycerol in the extender was toxic to the spermatozoa and reduced the sperm motility (Maxwell and Watson, 1996). The progressive motility, average path velocity, beat cross frequency and straightness was altered during equilibration. Besides peroxidation and toxic effects of glycerol, phospholipid of damaged spermatozoa produced reactive oxygen species (ROS), which one was toxic to the other live spermatozoa (Sundararaman and Edwin, 2008). Among the three extenders used in this study, the components of liposome based extender (Optixcell) was found to be better and it can able to maintain the structural integrity, plasma membrane integrity and motility in the sperm of the frozen thawed semen than the other two extenders (TEYG and Bioxcell).

Fertility rate following AI with frozen thawed semen diluted in three different extenders

Pregnancy in these cows was verified by ultrasound examination at day 45 after AI and rectal examination. The conception rate (Table. 3) obtained in the present study was 45, 40 and 60 per cent in frozen semen straws extended

Table 3: Fertility rate following AI in Kangayam cow

Groups	GROUP I (TEYG)	GROUP II (Bioxcell)	Group III (Optixcell)	Overall per cent	P value
No of AI	20	20	20	100	0.246597
Pregnant	9(45)	8(40)	13(65)	50	
Non pregnant	11(55)	12(60)	7(35)	50	

*Figures in parenthesis indicates percentage, The chi-square statistic is 2.8. The result is not significant at p>0.05.

in TEYG (group I), soya bean lecithin (group II) and liposome based diluents (group III), respectively. Among the three extender groups, higher conception rate was obtained in liposome based (group III) extender followed by TEYG (group I) and lower in soya bean lecithin (group II). Statistical analysis using Fisher Exact test revealed that there was no significant difference ($p<0.05$) among 3 groups on conception rate. The liposome based diluent showed higher fertility rate compared to tris-citric egg yolk extender used in buffalo semen (Akhter *et al.* 2016). Nevertheless, similar fertility rates in mare were recorded in liposome based and egg yolk based extenders (Pillet *et al.* 2012). It has been demonstrated that in liposome based extender sperm retained higher ability to travel long distance in cervical mucus test compared to soya lecithin based tris citric egg yolk based extenders (Kumar *et al.* 2015). It revealed indirectly higher ability of the spermatozoa in liposome based extender to reach at the site of fertilization that increases the chances of conception. Moreover, the higher percentage of functional and structurally intact spermatozoa in liposome based extender compared to conventional extenders contributes in higher fertility rates. Murphy *et al.,* (2018) in HF bulls reported the conception rates of 60, 64.4 per cent following the use of liposome and soya lecithin based extenders respectively. The overall conception rate in repeat breeder cows with synchromate B system was 43.75 per cent (Selvaraju *et al.,* 2009), PGF_2 alpha group was 43.75 per cent and PGF_2 alpha + hCG group was 62.50 per cent (Selvaraju *et al.,* 2010a), synchromate B system + hCG group was 56.25 per cent (Selvaraju *et al.,* 2010b) following artificial insemination with universal diluent. Manokaran *et al.* (2023) reported that the fertility rate in Kangayam cows with single PGF_2 alpha injection group was 60 per cent, double PGF_2 alpha group was 80 per cent, CIDR+ PGF_2 alpha group was 70 per cent using tris-citric egg yolk extender. The overall conception rate in Nili Ravi buffaloes was improved with semen cryopreserved in liposome based extender (59.50 per cent) compared to semen cryopreserved in tris-citric egg yolk extender (41.50 per cent) (Ansari *et al.,* 2016). Naz *et al.* (2018), obtained an *in vivo* fertility rate of 68.18 per cent in Nili Ravi buffaloes with the semen doses cryopreserved in liposome based extender and it was 55.40 per cent in Triladyl and 45.45 per cent in tris egg yolk based extenders.

The higher conception rate observed in our study in liposome based extender (group III), might be due to the higher conception rate in group III was confirmed by sperm kinematic characters which were assessed by CASA during various stages of semen storage before and after freezing especially sperm motility, progressive motility and SLR values. In the soya bean lecithin (group II) and TEYG (group I) the conception rate was lower which was the reflection of quality of pre freeze and post thawed sperms diluted in soya bean lecithin and TEYG extenders.

CONCLUSION

Based on the evaluation of Kangayam bull semen during fresh diluted, pre freeze and post freeze (24 hrs and 30th day of storage) stages, sperm kinematic character analysis by CASA and fertility trials with Kangayam cows, it was concluded that liposome based extender found to a better semen extender among the three studied extenders for freezing of Kangayam bull semen.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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