RESEARCH ARTICLE

Evaluation of Sperm Velocity Parameters with Glutathione and Honey in Skim Milk Based Extenders by CASA on Boer Buck

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Abstract

The study aimed to evaluate the Boer buck sperm velocity (µm/sec) parameters (VCL: Curvilinear Velocity, VAP: Average Path Velocity and VSL: Straight line Velocity) with 5 mM Glutathione (G) and 1% or 2% Honey (H) in Skim milk (SM) based extenders preserved at refrigeration temperature for 0, 24, 48 and 72 hrs. A total of 72 ejaculates were collected equally from 6 mature bucks at the weekly interval by using Artificial Vagina (AV) as per the standard procedure. All the ejaculates were diluted using six Skim milk-based extenders, *viz.* SME, SMGE, SMGH(1%)E, SMGH(2%)E, SMH(1%)E and SMH(2%)E. The sperm motility was evaluated by CASA (Computer Assisted Semen Analyzer). The data obtained was statistically analyzed. The results showed that sperm velocity parameters (VCL, VAP, and VSL) differed significantly ($p < 0.05$) from the extender to extender at 24, 48, and 72 hours of refrigeration. Supplementation of optimum concentration of glutathione (5 mM) and honey (1%) maintained better sperm velocity parameters up to 72 hours of storage compared to other extenders and was successful in the preservation of buck spermatozoa at refrigeration temperature. Hence, It was concluded that Boer buck semen could be preserved effectively with SMGH(1%)E at refrigeration temperature for sperm velocity parameters up to 72 hours of storage.

Keywords: Buck, CASA, Glutathione, Honey, Skim milk, Sperm.

Ind J of Vet Sci and Biotech (2020): 10.21887/ijvsbt.15.4.6

Introduction

Egg yolk extenders have the risk of microbial contamination to the semen. Furthermore, the wide variability of egg yolk composition makes it difficult to prepare standard extenders (Karunakaran *et al.,* 2017). The conventional method for measuring the kinematic sperm characters is difficult, time-consuming, and subjective. To get over these difficulties, Computer Assisted Semen Analysis (CASA) is the equipment of choice to provide precise and accurate information on sperm motion characteristics (Anand *et al.,* 2016).

Removal of seminal plasma improved the viability and longevity of Beetal buck spermatozoa in skimmed milk-based extenders during cooling up to 72 hours (Hassan *et al.,* 2016). Glutathione is a cofactor for glutathione peroxidase (GSH), which in turn reduces hydrogen peroxide (H_2O_2) to H_2O and also lipoperoxides to alkyl alcohols (Noei *et al.,* 2015). At low temperatures, honey does not freeze and its viscosity increases with decreasing the temperature. This creates a low surface tension that eventually minimizes the formation of ice crystals inside the cytoplasm of sperm and hence reduces damage to the spermatozoa during cryopreservation (El-Sheshtawy *et al.,* 2014). Honey has potent antioxidant and antibacterial properties (Zoheir *et al.,* 2015). Honey as a natural product has been studied as a supplement with different properties like synergistic antioxidant, non-permeant cryoprotectant and energy source for the improvement of post-thaw semen quality (Yimer *et al.*, 2015). The present study aimed to evaluate the effect of Glutathione and Honey on the Boer buck sperm velocity parameters (VCL: Curvilinear Velocity, VAP: Average Path Velocity and VSL: Straight line Velocity) in skim milk-based extenders preserved at refrigeration temperature for 0, 24, 48 and 72 hours.

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How to cite this article: Arakeri, S., Tandle, M.K., Vinay, P.T., Bijurkar, R.G., Suranagi, M.D., Rao, J. and Kulkarni, S. (2020). Evaluation of Sperm Velocity Parameters with Glutathione and Honey in Skim Milk Based Extenders by CASA on Boer Buck. Ind J Vet Sci and Biotech, 15(4): 34-37.

Source of support: Nil

Conflict of interest: None.

Submitted: 19/03/2020 **Accepted:** 31/03/2020 **Published:** 30/04/2020

Materials and Methods

Six sexually mature Boer bucks maintained at the Department of Veterinary Gynaecology and Obstetrics, Veterinary

College, Bidar were included in the study. All the bucks were maintained under uniform conditions of the semi-intensive housing system. Bucks were kept in a single flock with routine deworming and vaccination as per schedule. The bucks were allowed free grazing for 5-6 hrs daily and were fed concentrate @ 250 g per day per animal and provided *ad libitum* drinking water throughout the day.

Preparation of Extenders

10 g skimmed milk powder + 100 mL double distilled water + 1 g fructose + antibiotics *viz.* penicillin-100 IU and streptomycin sulfate-100 mg/100 mL which served as a control, SME.

Five other extenders were prepared as : Skimmed milk - glutathione (5 mM) extender (SMGE), Skimmed milk - honey (1%) extender (SMH1%E), Skimmed milk - honey (2%) extender (SMH2%E), Skimmed milk - glutathione - honey (1%) extender (SMGH1%), and Skimmed milk - glutathione - honey (2%) extender (SMGH2%) were prepared. For adding honey, 1 mL of honey was mixed with 9 mL double distilled water (v/v) to prepare a 10% honey solution (El-Sheshtawy *et al*., 2014), which was then used as 1 ml and 2 ml to give 1% and 2% (v/v) concentrations, respectively, in the skimmed milk extender.

Semen Collection and Evaluation

A total of 72 ejaculates were collected, 12 ejaculates from each of 6 mature bucks at the weekly interval by using Artificial Vagina as per the standard procedure. For mass activity, a drop (0.5 µL) of neat semen was placed on a pre-warmed glass slide (37°C) without coverslip and examined under a phase-contrast microscope with low power magnification (100 X). Wave motion characteristics or swirl motion of the spermatozoa were scored from + to ++++ scale (Blokhuis, 1962). A wet semen mount was made using a small drop of semen placed directly on a microscope slide covered by a coverslip (Loskutoff and Crichton, 2001) and the individual sperm motility was scored in percentage using a phase-contrast microscope (400 X magnification) with a warm stage maintained at 37°C.

Removal of Seminal Plasma and Dilution of Sperm Pellets

The collected ejaculates were centrifuged@1500 rpm for 10 minutes, and seminal supernatant plasma was discarded. The sperm pellets washed with isosmotic phosphate-buffered saline (PBS) solution in equal volume to obtain an optimal concentration and pH for the sample. Then buck wise sperm pellet sample was diluted with six different extenders (1:60) prepared as above and stored at refrigeration temperature. The samples were brought to 37°C in a water bath at the time of evaluation.

Evaluation of Semen by Computer Assisted Semen Analysis (CASA)

All the diluted sperm pellet samples were preserved at refrigeration temperature for 0, 24, 48 and 72 hours of storage and brought to 37°C temperature in a water bath at the time of evaluation by CASA system using Biovis - CASA 2000 (Expert Vision Labs Pvt. Ltd., Mumbai, India). A drop of diluted semen was taken on a clean grease-free slide, which was covered by a coverslip, and it was focused under a phase-contrast microscope with 100 X magnification. Biovis-CASA software was turned on, a fine adjustment was made for viability and clicked on option capture which captured around 60 frames/minute and automatically analyzed for sperm concentration, motility, and sperm velocity (µm/sec) parameters, *viz*., VCL: curvilinear velocity, VAP: average path velocity and VSL: straight-line velocity.

Statistical Analysis

The data obtained were statistically analyzed by General Linear Model (GLM) and procedure using SAS - Statistics Version 9.3, SAS Inc., Cary, NC; 2010 software.

Results and Discussion

Semen Characteristics of Boer Bucks

The volume of neat semen ranged from 0.81 to 1.30 ml having a whitish-yellow color. The mass activity was ++++ scale for all semen samples, and sperm motility ranged from 91.66 ± 1.12% to 96.66 ± 1.42%, and none of the samples has shown the presence of any foreign body (Table 1). Karunakaran *et al*. (2015) recorded the semen volume of 0.75 ± 0.25 mL and sperm motility 85.40±8.20% in black Bengal bucks, whereas Kalyani *et al*. (2015) obtained lesser semen volume (0.51 ± 0.01 mL), but comparable mass activity (3.95 ± 0.028) in the same breed. Although lower semen volume (0.62 ± 0.05 to 0.73 ± 0.05 mL), mass motility (3.75 ± 0.11 to 3.83 ± 0.10) and

Table 1: Semen characteristics of Boer bucks

Buck No.	*Volume (mL) (Mean ± SE)*	*Colour*	*Mass activity (+ to ++++)*	*Sperm motility (%) (Mean ± SE)*
1	1.02 ± 0.04	Whitish - yellow	++++	94.16 ± 1.48
2	0.82 ± 0.04	Whitish - yellow	++++	92.50 ± 1.30
3	0.91 ± 0.04	Whitish-yellow	++++	93.33 ± 1.42
4	0.81 ± 0.02	Whitish-yellow	++++	91.66 ± 1.12
5	1.30 ± 0.05	Whitish-yellow	++++	96.66 ± 1.42
6	1.21 ± 0.04	Whitish-yellow	++++	95.83 ± 1.48

even sperm motility (84.14 ± 0.30%) were recorded by Mukul *et al*. (2017) in Barbari bucks.

Velocity Parameters (VCL, VAP, VSL, µm/sec)

Addition of glutathione 5 mM alone in the skimmed milk extender non-significantly reduced the curvilinear velocity (VCL) of buck sperm at all intervals of refrigeration preservation when compared with control SME. Similarly, addition of 1% honey in SME maintained relatively higher VCL till 48 and 72 hrs of storage compared to 2% level; though the higher level showed initially greater VCL value; however, statistically, the differences between extenders having 1% and 2% honey were non-significant at all periods of refrigeration preservation. Further, the addition of 1% honey in skim milk glutathione extender showed higher sperm curvilinear velocity; however, the addition of 2% honey reduced it initially and at 72 hrs of refrigeration (Table 2, Figure 1).

A very similar trend of observations was found for the other two velocity parameters, *viz.*, average path velocity (VAP), and straight-line velocity (VSL) at all intervals of refrigeration preservation in different extenders tested (Table 3 and 4).

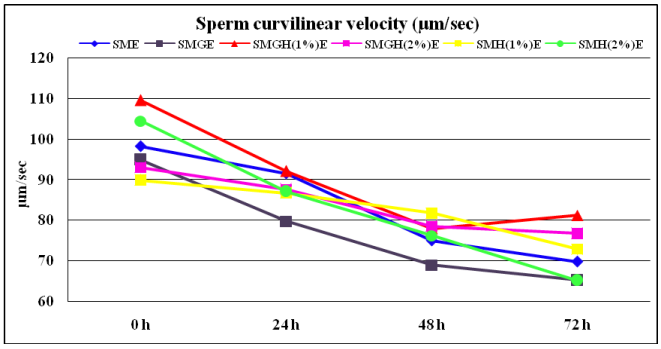

Figure 1: Sperm curvilinear velocity in sperm pellets diluted with skim milk-based extenders at 0, 24, 48 and 72 h of storage at refrigeration temperature

Table 2: Mean (±SE) sperm curvilinear velocity (VCL) in sperm pellets of buck semen diluted with skim milk-based extenders at 0, 24, 48 and 72 hours of refrigeration storage

	Sperm VCL (µm/sec) on refrigeration preservation			
Extenders	*0 h*	*24 h*	*48 h*	*72 h*
SME	98.24^{aAB} ±2.42	91.60^{a} ±3.35	75.00^{bAB} ±2.29	69.84^{bBC} ±2.30
SMGE	94.95^{aAB} ±4.12	79.80^{b} ±3.23	68.99^{bcA} ±1.09	65.24^{cBC} ±2.65
SMGH(1%)E	109.58^{aA} ± 2.48	92.16^{b} ± 2.49	78.03^{cAB} ± 2.97	81.24^{cA} ± 2.79
SMGH(2%)E	93.01^{B} ± 5.73	87.65 ± 2.84	78.54^{AB} ± 4.22	76.75^{AB} ± 3.99
SMH(1%)E	89.92^{aB} ± 3.63	86.74^{a} ±2.75	81.80^{abB} ± 2.30	72.90^{bBC} ± 1.62
SMH(2%)E	104.45^{aAB} ± 1.12	87.13^{b} ± 4.31	76.24^{bcAB} ± 3.56	65.15^{cC} ± 2.79

SME: Skim Milk Extender, SMGE: Skim Milk Glutathione Extender, SMGH(1%) E: Skim Milk Glutathione Honey (1%) Extender, SMGH(2%)E: Skim Milk Glutathione Honey (2%) Extender, SMH(1%)E: Skim Milk Honey (1%) Extender, SMH(2%)E: Skim Milk Honey (2%) Extender.

Table 3: Mean (±SE) sperm average path velocity (VAP, µm/sec) in sperm pellets diluted with skim milk-based extenders at 0, 24, 48 and 72 h of refrigeration storage

	Sperm VAP (µm/sec) on refrigeration preservation			
Extenders	*0 h*	*24 h*	*48 h*	*72 h*
SME	61.95^{aA} ± 1.86	58.75^{a} ± 2.09	51.75^{b} ± 0.89	46.90^{bAB} ± 1.76
SMGE	61.89^{aA} ± 2.78	52.33^{b} ± 2.26	51.20^{b} ± 0.76	50.46^{bAB} ± 0.61
SMGH(1%)E	71.13^{aB} ± 1.44	60.45^{b} ± 1.61	54.39^{bc} ± 1.72	53.91^{cB} ± 1.92
SMGH(2%)E	59.90^{A} ± 2.93	57.58 ± 1.75	52.90 ± 2.40	51.73^{AB} ± 2.18
SMH(1%)E	58.95^{aA} ± 2.26	57.58^{a} ± 2.10	57.82^{a} ± 2.24	46.71^{bA} ± 1.06
SMH(2%)E	66.42^{aAB} ± 0.59	56.45^{b} ± 2.45	51.91^{b} ± 1.85	44.74^{cA} ± 1.35

Means with different superscripts differ significantly at p<0.05L superscripts ^{abc} indicate the difference between time within the row, and superscripts ^{ABC} indicate the difference between extenders within the column.

Table 4: Mean (±SE) sperm straight-line velocity (VSL, µm/sec) in sperm pellets diluted with skim milk-based extenders at 0, 24, 48 and 72 h of refrigeration storage

	Sperm VSL (µm/sec) on refrigeration preservation			
Extenders	*0 h*	*24 h*	*48 h*	*72 h*
SME	56.54^{aA} ± 1.82	53.69^{ab} ± 2.01	47.69^{bc} ± 0.80	42.95^{cAB} ± 1.75
SMGE	56.88^{aA} ± 2.70	47.83^{b} ± 2.10	47.94^{b} ± 0.74	47.12^{bBC} ± 0.47
SMGH(1%)E	65.77^{aB} ± 1.48	55.49^{b} ± 1.63	50.11^{b} ± 1.67	49.65^{bB} ± 1.83
SMGH(2%)E	54.78^{A} ± 2.79	52.94 ± 1.78	48.73 ± 2.32	47.56^{BC} ± 2.04
SMH(1%)E	53.49^{aA} ± 2.19	52.45^{a} ± 2.00	53.11^{a} ± 2.26	42.55^{bAB} ± 1.10
SMH(2%)E	60.55^{aAB} ± 0.61	51.49^{b} ± 2.22	47.87^{b} ± 1.63	41.67^{cA} ± 1.28

Means with different superscripts differ significantly at p<0.05; Superscripts ^{abc} indicate the difference between time within the row, and superscripts ^{ABC} indicate the difference between extenders within the column.

The results, in general, showed that inclusion of glutathione alone@5 mM in SME either suppressed or had no beneficial effect on any of the velocity parameters studied, however the addition of honey@1% in SME improved all the velocity parameters compared to 2% level, though statistically, it was indifferent from control SME or 2% honey. Moreover, a combination of 5 mM glutathione and 1% honey in SME definitely improved all the velocity parameters initially and even maintained them at a significantly higher level over control SME and/or a combination of 5 mM glutathione and 2% honey in SME.

Kadaganchi (2017) and Nancy (2018) also recorded decreased sperm velocity values (VCL, VAP, and VSL) with an increase in storage time from 0 to 72 hrs at refrigeration temperature in SME without and with the inclusion of fructose, glutathione, honey, almond oil or olive oil. Further, there was a beneficial effect of the addition of olive oil (0.25%) in SM extender at 0-hour and in TEY at 48 hours of semen storage. Similarly, almond oil (0.25%) reflected beneficial effect in TEY dilutor at 24 hours of semen storage, whereas sperm VAP was higher in TEY extender at 72 hours of semen storage without the addition of almond or olive oil (Nancy, 2018). Špaleková and Makarevich (2012) found a significant increase in sperm VAP and VSL after 24 hours of cooling-storage with glutathione at 1.5 mmol.L^{-1} concentration.

Conclusion

Addition of 1% honey in skim milk glutathione (5 mM) extender showed higher sperm VCL (µm/sec), sperm VSL (µm/sec) and sperm VAP (µm/sec); however, the addition of 2% honey in skim milk glutathione extender reduced the same at 72 h of refrigeration. Honey at 1% level in SME maintained sperm velocity at a higher magnitude compared to 2% honey with increased storage time, while glutathione alone did not reflect any advantage in skimmed milk extender for buck sperm velocity parameters.

Acknowledgment

The author wishes to thank Dr. M. K. Tandle (Dean, DI PG'S, KVAFSU, Bidar, Karnataka) and Dr. Dileep Kumar D. Dean, veterinary college, Bidar, Karnataka for their advice, arbitration and review of research for publication.

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