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CAMEL REPRODUCTION

Effect of different proteolytic enzymes on liquefaction of semen of dromedary camel

Mohd. Matin Ansari[1]*, Sumant Vyas[1], Rajesh K Sawal[1], Sanjay K. Ravi[2], Niteen V. Patil[1], Mustafa. Hasan Jan[3]

[1]Indian Council of Agricultural Research-National Research Centre on Camel, Bikaner, India, [2]ICAR-National Research Centre on Equines sub-campus, Bikaner, India, [3]ICAR-Central Institute for Research on Buffaloes, Hisar, India

ABSTRACT

In most of domestic animals, semen biology is well studied, but it is not so well studied in camelids particularly in dromedary camels. In camelids, the ejaculated semen is highly viscous, so before its evaluation and processing for preservation, it is necessary to be liquefied. Our study was designed to evaluate the effect of three different enzymes (0.1% Collagenase, 0.5 X Accutase, 0.1% Trypsin-EDTA - Ethylene-diamine-tetra-acetic acid) on seminal viscosity at different time intervals along with its effects on seminal parameters. Semen was collected from the six adult healthy male camels using female camel as dummy. Bovine artificial vagina was used for the semen collection and ejaculate was kept at 37°C for further processing after its collection. All three enzymes were diluted in 1x phosphate buffered saline (PBS) and the semen samples were kept at 37°C. The samples were evaluated at different time intervals 0, 5, 10, 15, 30, 60, 120 and 240 min (T0–T240) for viscosity and it was evaluated by pipette method. Immediately after collection, the viscosity was very high, and no sperm mass motility was seen. However, after enzyme treatment viscosity was showed declining trend in all treatment groups compared to control. In trypsin treated group maximum motility was reached in 15 minutes. Live-dead percent was observed to be similar among treatment groups. Observations on liquefaction of semen revealed that use of enzymes decreasing the viscosity but with different rates.

Keywords: Computer assisted semen analyzer; Dromedary camel; Liquefying agents; Sperm physiology

INTRODUCTION

Genetic progress is very slow in camel due to monotocous female having long gestation period of around 380-390 days (Vyas and Sahani, 2000). Use of assisted reproductive technology applications like artificial insemination (AI) and *in-vitro* fertilization (IVF) may overcome these problems (Skidmore et al., 2013). The use of artificial insemination is limited in camels due to difficulty in collection and handling of semen for its subsequent processing and analysis. For AI and IVF to be successful, sperms must be progressively motile. However, in the ejaculates of camelids, spermatozoa have only oscillatory or vibratory movement and no progressive or free motility is seen, due to high seminal plasma viscosity (Deen et al., 2004; 2005; Giuliano et al., 2002; Lichtenwalner et al., 1996; Vaughan et al., 2003). Therefore, in order to achieve freely motile sperm, it is essential to remove the seminal viscosity (Conde et al., 2008).

Handling of camelid semen in the laboratory is difficult due to viscous nature of semen (Tibary and Vaughan, 2006; Del Campo et al., 1994; Maxwel et al., 2008; Sansinena et al., 2007) and is difficult to assess spermatozoal motility for further semen analysis and processing. For efficient cryopreservation, the sperm-cryoprotective and nutritive compounds ought to come in contact with sperm cell membrane during equilibration, therefore liquefaction of camelid ejaculates is necessary for improving post-thawing semen parameters (Monaco et al., 2016). Previous researchers have evaluated the effect of different enzymes for liquefaction of camelid semen and reported both beneficial and detrimental effects (Bravo et al., 1999, 2000; Ghoneim et al., 2010; Giuliano et al., 2010; Maxwel et al., 2008; Shekher et al., 2012), and marked reduction of sperm motility with 10 min (Morton et al., 2008) to 45 min (Ghoneim et al., 2010) of exposure. Sperm fluid containing Bromelain, though effective in enhancing the liquefaction of human ejaculates, could not break down the camelid's highly viscous seminal plasma matrix. Therefore, the objective of our study was to elucidate comparative efficacy of Collagenase, Accutase and Trypsin-EDTA for

***Corresponding author:**
Mohd. Matin Ansari, Indian Council of Agricultural Research-National Research Centre on Camel, Bikaner, India.
E-mail: matin.ansari1@gmail.com

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reducing semen viscosity and assess their effect on seminal attributes.

MATERIALS AND METHODS

Chemicals and media

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO).

Semen collection

Initially 60 ejaculates from 6 adult healthy male camels (10 ejaculates from each camel) were collected using female camel used as a dummy. 20 ejaculates that were watery, azoospermic, voluminous or dusty were discarded keeping a final overall sample size of 40 ejaculates. Semen was collected using bovine artificial vagina (AV) (IMV France) after diverting the penis into the A.V. (Vyas et al., 1998). All animals were kept under similar management conditions of housing and feeding. After semen collection, semen was kept at 37ºC in water bath for further processing.

Enzymes used for treatment (Collagenase, Accutase and Trypsin-EDTA) were diluted in 1x phosphate buffered saline solution (PBS), where 1x PBS and raw semen were used as control. Collagenase was used as 1% in PBS (C-PBS), Accutase as 0.5 x in PBS (A-PBS), and Trypsin-EDTA as 0.1% in PBS (T-PBS). After dilution, semen samples were evaluated at different time intervals 0 to 240 (0, 5, 10, 15, 30, 60, 120, 240 min) for viscosity, motility, viability, acrosome integrity and membrane integrity. Sperm viability was assessed by Eosin–Nigrosin staining whereas viscosity was evaluated by pipette method.

Experimental design

Viscosity

Viscosity of semen was estimated as described by Kershaw-Young et al. (2013). Briefly, 50µl of semen sample was taken in 100 µl pipette, around 25 µl was released on glass slide and then the pipette was raised with speed. The length at which the semen thread breaks was measured using scale and taken as the standard of viscosity. The semen samples were kept at 37ºC in water bath and the samples were evaluated for its viscosity before (at 0 min) and after (at 5, 10, 15, 30, 60, 120, 240 min) dilution. The viscosity percentage was calculated using the following formula: (Viscosity at given time/Initial Viscosity) ×100 (Kershaw-Young et al., 2013).

Motility

Motility in camel semen was assessed under Computer Assisted Semen Analyzer (CASA) (Hamilton Thorne, USA). Ten µL of semen was taken on warm grease free glass slide and a cover-slip was placed over it. All motile sperms, whether oscillatory or progressive were considered as motile and used to generate a value for total motility as used previously (Kershaw-Young and Maxwell, 2011; Morton et al., 2008).

Live dead/acrosome integrity

Viability was assessed by using conventional method eosin-nigrosin staining technique. For this, we take semen sample and mix with eosin-nigrosin dye, after that the smear were made on clean grease free glass slide and dried. After staining the spermatozoa were classified as live if they were not stained with eosin and dead if they were stained with the dye.

Acrosome integrity was assessed using a method described for ethanol-fixed alpaca sperm (Morton et al., 2010).

All observations of live dead and acrosome integrity were taken at 5, 10, 15, 30, 60, 120, 240 minutes after dilution.

Membrane integrity

Membrane integrity was evaluated by hypo-osmotic swelling test (HOST). One ml of hypo-osmotic solution (150 mOsm, sodium-citrate-fructose solution) was added to 100 µl of semen, samples mixed properly and incubated for 45 minutes. After incubation, one drop of mixed semen was taken on a clean grease free glass slide and covered with cover-slip. The spermatozoa with coiled tail as the effect of swelling due to water influx were counted. Percent membrane integrity was calculated using following formula

Intact plasma membrane (%) = No. of coiled tail/Total No. of spermatozoa × 100.

All observations of membrane integrity were taken at 5, 10, 15, 30, 60, 120, 240 minutes after dilution.

Statistical analysis

Data were tested for normality by Shapiro-Wilk test and arc-sine transformed. Various parameters were analyzed by DMRT followed by LSD test to find difference between treatment groups at each time interval with completely randomized design (CRD). A probability value of $P<0.05$ indicated that the difference was statistically significant. Data are presented as mean ± SEM.

RESULTS

Semen quality parameters

Viscosity

Percentage semen viscosity and absolute viscosity decreased significantly in enzyme treated group with time interval. The decrease in viscosity was rapid in enzyme treated groups as compared to control groups; it was significantly lower at 10, 15, 30 and 60 minutes ($p<0.05$) (Fig. 1). Within enzyme treated groups, in trypsin treated group the viscosity was decreasing with higher rate compared with accutase and

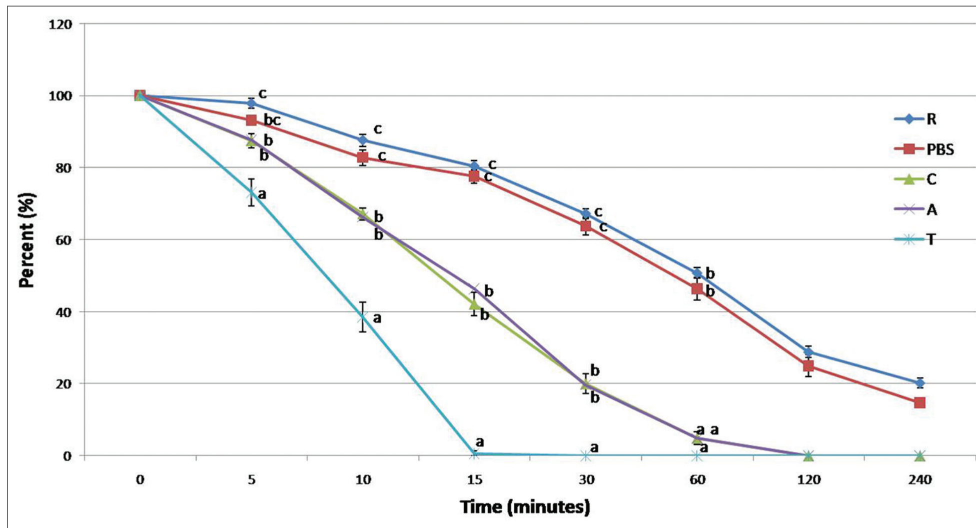

Figure 1. Viscosity percent of treatment and control groups with reference to time, R=Raw; PBS=Phosphate Buffer Saline;C=0.1% Collagenase, A=0.5 X Accutase and T=0.1% Trypsin-EDTA. Letters among treatments (a,b, c) indicate significant differences.

Table 1: Percentage of free motile sperm of treatment group and control group at different time interval

Percent samples that attained free motility (%)								
Groups	Minutes of incubation							
	0	5	10	15	30	60	120	240
R	0	0	0	0	0	10	30	50
PBS	0	0	0	0	0	20	40	50
PBS-C	0	0	0	40	80	100	100	100
PBS-A	0	0	0	0	70	100	100	100
PBS-T	0	0	30	100	100	100	100	100

R=Raw; PBS=Phosphate Buffer Saline; C=0.1% Collagenase; A=0.5 X Accutase and T=0.1% Trypsin-EDTA.

collagenase treated groups at 5, 10 and 15 min ($p<0.05$). The viscosity almost disappeared at an interval of 15 min in trypsin-EDTA and at 120 min interval in accutase and collagenase treated groups (Fig. 1). Within untreated and PBS treated semen, the viscosity remained appreciable and significantly elevated even after 240 min (Fig. 1).

Motility

No free motility was seen, only vibratory motion of spermatozoa was observed at 0 min in all samples irrespective of group (Table 1). Before any treatment most of sperms were trapped in viscous gel and showing vibratory/oscillatory motion. Thereafter, the spermatozoa started to attain free motility due to liquefaction gel fraction either spontaneously (in R and PBS groups) or due to addition of enzymes (PBS-C, PBS-A and PBS-T groups). Fifty percent of semen samples achieved free motility at 240 min in R and PBS, while 100% of semen samples achieved free motility at 60 min in PBS-C and PBS-A and at 15 min in PBS-T group (Table 1). Free sperm motility is achieved only after seminal breakdown of gel network after incubation either spontaneously or due to addition of enzymes in camelids (Table 1).

Live-dead and acrosome intact sperm

Percentage of viable sperms decreased uniformly over time in all the groups, no significant difference was recorded between groups at any time interval from 0 to 240 min ($p>0.05$) (Fig. 2). In case of acrosome integrity of sperms, at 15 min of enzyme treatment significant reduction in percent of intact acrosome was observed in collagenase treated group when compared with raw/untreated group ($p<0.05$) (Fig. 3). At the same time in trypsin and accutase treated group, there was no significant reduction in acrosome intact sperm compared with control. At 30 min post treatment the acrosome intact sperms were significantly lowest in collagenase and accutase treated group (Fig. 3).

Within enzyme treated groups, collagenase treated samples had lowest acrosome intact sperm as compared to accutase and trypin-EDTA treated groups (Fig. 3).

Membrane Integrity

The percentage of membrane intact sperms decreased in all groups over the time and there was no significant decrease up to 30 min ($p>0.05$) (Fig. 4). However, the decrease was significant in enzyme treated samples as compared to untreated and PBS treated semen at 240 min time interval ($p<0.05$). Within enzyme treated groups, trypsin-EDTA treated group had significantly lower membrane integrity at 240 min interval as compared to other groups ($p<0.05$) (Fig. 4).

DISCUSSION

Semen of camel is highly viscous because it forms coagulum immediately after copulation or after collection

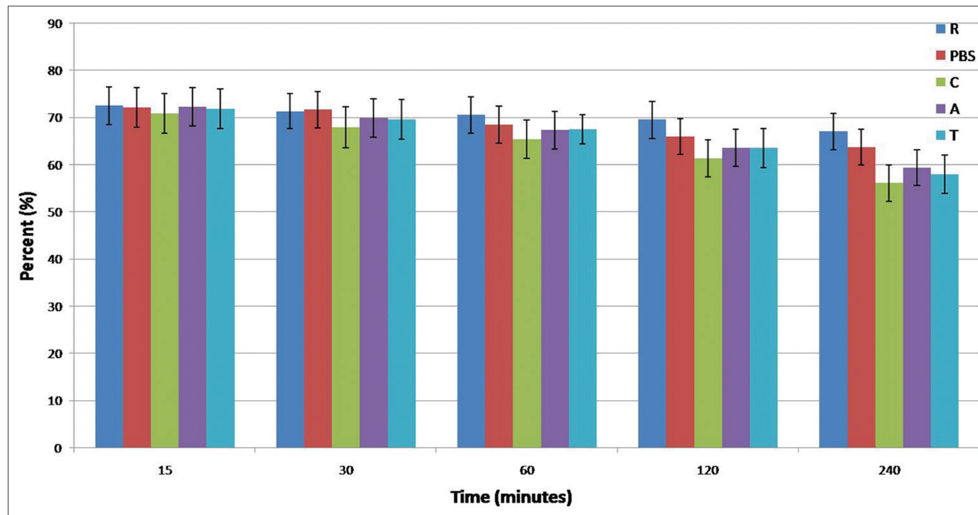

Figure 2. Live dead percent of different treatment groups at different time interval. R=Raw; PBS=Phosphate Buffer Saline;C=0.1% Collagenase, A=0.5 X Accutase and T=0.1% Trypsin-EDTA.)

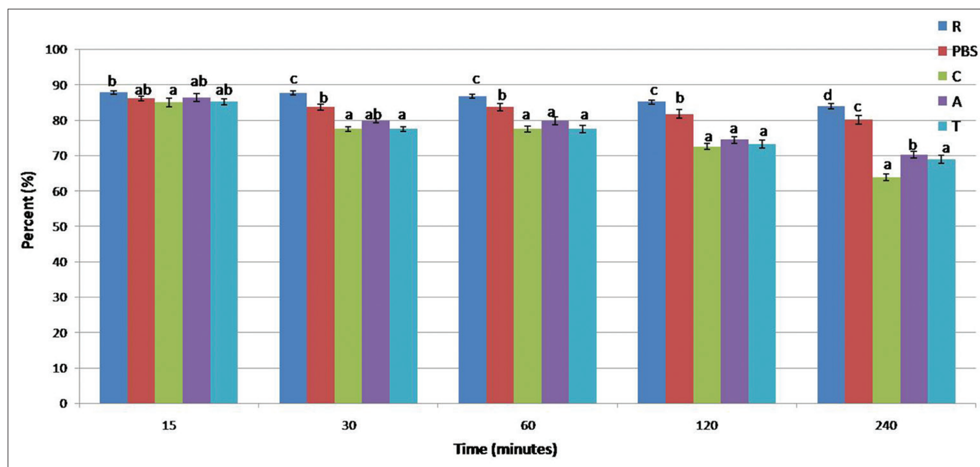

Figure 3. Percent of acrosome intact sperms of different treatment groups at different time interval. R=Raw; PBS=Phosphate Buffer Saline;C=0.1% Collagenase, A=0.5 X Accutase and T=0.1% Trypsin-EDTA.Letters among treatments (a,b, c) indicate significant differences.

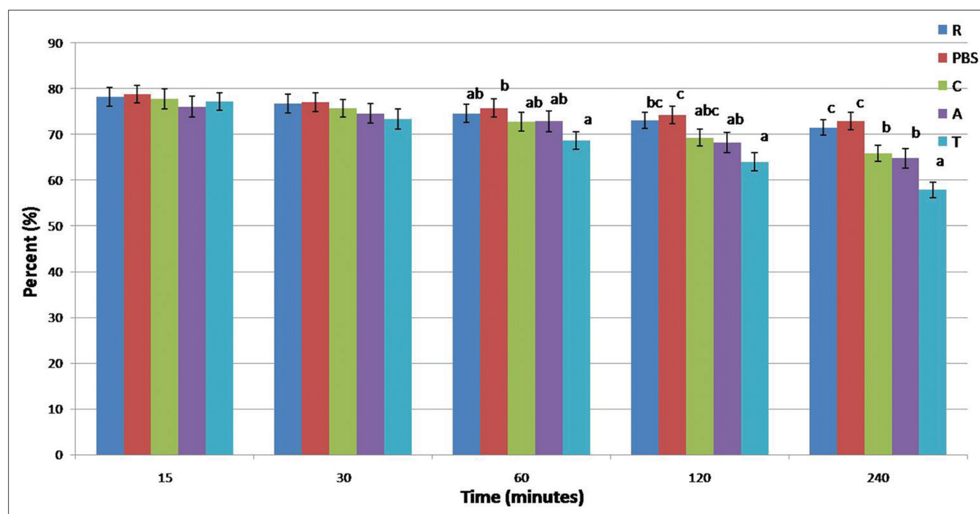

Figure 4. Membrane integrity of different treatment groups at different time interval. R=Raw; PBS=Phosphate Buffer Saline;C=0.1% Collagenase, A=0.5 X Accutase and T=0.1% Trypsin-EDTA. Letters among treatments (a,b,c) indicate significant differences.

in A.V. It becomes difficult to handle this semen for further processing and semen evaluation as mass motility, sperm concentration and sperm morphology. Our study investigated effects of liquefaction agents (enzymes) as collagenase, accutase and trypsin on camel semen viscosity and sperm quality of camels. Observations of the experiment will be helpful in developing semen evaluation protocol and preservation techniques. The high viscosity of camel semen prevents proper mixing with other liquids/ extenders before complete liquefaction (Wani et al., 2005). Similar effect was also observed in our study. Unlike cattle buffalo and other domesticated animals, camelid spermatozoa move in an oscillatory or vibratory manner in seminal plasma at the time of ejaculation resulting in very low to no mass motility as seen in fresh semen of camelids (Deen et al., 2003; Garnica et al., 1993). Accutase enzyme is frequently being used in cell culture research for the detachment of cell from stem cell mass (Bajpai et al., 2008) without affecting further culturing of the cells. In our study, accutase was used for the first time for digestion study to free the entrapped spermatozoa from the semen gel network. Accutase enzyme digestd the viscous part of camel semen and makes the sperm free from the matrix. More efficient protocol could further be designed with different concentration of this enzyme but currently only available ready to use concentration is 1x (Sigma Aldrich).

In the present study after 4 hours of incubation, 0.1% Collagenase, 0.5x Accutase and 0.1% Trypsin-EDTA all the enzymes reducing the viscosity with different rates, reduced the viscosity by 100% in all treatment groups compared to around 80% in the control group samples. In a previous study, complete liquefaction was observed at 23.89±1.49H varying from 18 to 41H without any enzyme treatment (Mal et al., 2016).

In alpaca, glycosaminoglycans were found in semen and keratin sulphate was associated with viscosity (Kershaw-Young et al., 2012). However, in our study, the seminal plasma viscosity was reduced using collagenase/serine protease, suggesting that the viscosity may be due to seminal plasma proteins or collagen.

Hyaluronidase reduced seminal plasma viscosity in llamas and alpacas (Bravo et al., 2000). It was observed that papain and proteinase K completely removed the seminal plasma viscosity in alpacas is caused by proteoglycans (Kershaw-Young et al., 2013).

Many enzymes as collagenase, trypsin, chymo-trypsin, papain and fibrinolysin have been used previously to reduce the seminal viscosity of camelid (alpacas) (Bravo et al., 1999; 2000; Giuliano et al., 2010; Morton et al., 2008) human (Cohen and Aafjes, 1982) and monkey (Hoskins and Patterson, 1967). In our study, the effect of accutase enzyme on camelid semen was tested for the first time. In Humans, studies with trypsin, chymotrypsin, and papain reduced the semen viscosity, but it also impaired acrosome integrity (Morton et al., 2008; Pattinson et al., 1990a; 1990b). However, in our study, trypsin did not affect the acrosome integrity while reducing the viscosity.

In our study, 0.1% collagenase and accutase (proteolytic and collagenolytic enzyme) did not act as spermicidal. Similar results were observed with 0.1% collagenase that improved sperm motility which was otherwise trapped in viscous gel (Shekher et al., 2012). Lower concentration of enzymes used in our study was sufficient to reduce semen viscosity and the concentration of enzyme was low enough to prevent sperm damage.

Therefore, enzyme treatment can reduce viscosity of camel semen without impairing sperm function. In addition, the fertilizing ability of trypsin-treated semen needs to be investigated. Enzyme treatment of camel semen may aid further to develop protocol for cryopreservation/refrigeration.

CONCLUSION

The seminal plasma of camel is highly viscous which traps spermatozoa and cause no mass or progressive sperm motility in raw sample mainly in freshly collected samples. However, after digestion with proteolytic enzymes, the viscosity of semen reduced and the sperms became free to move in the semen. Among all enzymes used in our study trypsin was of advantage in terms of liquefaction with reference to time.

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