2017, Scienceline Publication
World's Veterinary Journal
World Vet J, 7(3): 108-116, September 25, 2017
ISSN 2322-4568

DOI: http://dx.doi.org/10.5455/wvj.201708100

Influence of Enzymatic and Mechanical Liquefaction of Seminal Plasma on Freezability of Dromedary Camel Semen

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ABSTRACT

This study aimed to investigate the efficiency of mechanical and enzymatic elimination of semen viscosity in adult dromedary camel bulls' semen on cryopreservation potential of spermatozoa during the breeding season. Bulls showed reaction time 40.0±8.23 seconds and 251±24 seconds mating duration. Physical properties of raw semen showed volume mean value 5.28±0.66 ml, initial viability 2.5±0.6, initial raw motility 59.34±4.99%, livability 95.3±2.36%, first and second abnormalities 4.13±0.88% and 7.01±1.254%, respectively and acrosomal integrity 5.03±1.05%. The researcher examined three different treatments for viscosity elimination; namely; Amylase Enzymatic Treatment (AET), Syringe Mechanical Treatment (SMT) and Amylase Syringe Mixed Treatment (ASMT). The results revealed that, a significant deleterious effect of the ASMT on the post-thaw motility (M_{PT}) 25.00±3.69% was observed, with sperm Recovery Rate (RR) 35.02±5.02%, contrary to a clear superiority of AET treatment on (M_{PT}) 49.00±4.87%, followed by the SMT treatment (M_{PT}) 41.67±6.72%, with significantly higher RR% (76.86±4.63% and 62.10±6.65%) respectively. The AET recorded the highest acrosomal reaction (10.17±1.11%), followed by the mixed treatment (8.33±0.14%), with the least significant effect ($P<0.05$) on the mechanically treated group (7.33±0.99%). The results also showed the same trend for first and second abnormalities. Computer assisted semen analysis showed a significant superiority for the AET on mostly all sperm kinetics (DCL, DAP, VAP, VSL), except for DSL, VCL that showed highest significant value for SMT treatment. Conversely, the study recorded the lowest significant values for LIN, STR and WOB in the SMT. These results clarified that both enzymatic and mechanical methods have a positive influence on dromedary camel semen cryopreservation.

Key words: Amylase, Cryopreservation, Dromedary, Semen syringing, Viscosity

ORGINAL ARTICLE
pii: S232245681700014-7
Received: 17 Jul 2017
Accepted: 22 Aug 2017

INTRODUCTION

Semen processing and cryopreservation are becoming a prerequisite in the application of assisted reproductive technologies in camelids, especially with the increasing of genetically prized valued camels involved in camel racing and beauty contests, although, the technology is yet being sub-optimal in camels due to several challenges. The steady development of Artificial Insemination (AI) with frozen-thawed sperm requires efforts to improve the quality of semen processing techniques. One of the major challenges restricting the development of assisted reproductive technologies (ART's) in camelids is the high viscous nature of seminal plasma (Skidmore et al., 2013; Rateb, 2016). Under natural conditions, complete liquefaction for dromedary camel semen was observed to be 23.89 ± 1.49 hours, varying in a wide range from 18 to 41 hrs (Mal et al., 2016).

Several studies were performed during the last decade for the elimination of camelids' semen viscosity, with various degrees of success. Enzymatic treatments were the most used technique. The cause of the viscous nature of camels' seminal plasma used to be a controversial issue among different studies and authors, either depending on the

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Journal homepage: www.wvj.science-line.com

Semen diluents preparation and sample processing

Unless stated otherwise, all chemicals and reagents were obtained from Sigma (Sigma-Aldrich) for preparation of Tris-lactose egg yolk extender composed of Tris buffer (3.025%), lactose (5.5%), Citric acid (1.67%), glucose (1%), and supplemented with fresh egg-yolk (20%), the diluent was then divided into two portions A and B. Portion A represented the cooling extender which was added initially to the collected ejaculate with a ratio of 1:1, while portion B was supplemented with 6% glycerol to be added after 2 hours from equilibration (at 5^0C) with a ratio 1:1prior to freezing to make the diluted semen reach a final glycerol level of 3% and a full equilibration period of 4 hours at 5^0C, and a final dilution rate 1:3 according to (El-Bahrawy, 2010).

Experimental design

Semen samples were allocated to three groups [Amylase Enzymatic Treatment (AET), Syringe Mechanical Treatment (SMT) and Amylase Syringe Mixed Treatment (ASMT)], with equal fractions for each group.

Enzymatic Procedure [Amylase Enzymatic Treatment (AET)]

After primary check in a pre-test study of the enzyme power units. A concentration of 2.5 ul/ml TERMAMYLSUPRA enzyme, [(a trademarked, amylase available from Termamyl Supra) by Novozymes (Novo Nordisk), Denmark] extracted from, *Bacillus licheniformis* was set up to be used in this experiment. Immediately after collection, Tris lactose extender (Portion A) supplemented with 2.5ul/ml amylase was added to the semen with a 1:1 ratio, according to (EL-Bahrawy, 2010).

Mechanical procedure [Syringe Mechanical Treatment (SMT)]

Soon after the semen was collected, Tris lactose diluent (Portion A) was added with a ratio 1:1, then diluted ejaculates were immediately submitted to a physical process of expulsion of the semen and extender using a 10- or 20-mL (according to the ejaculate volume) for two or three times. This process was repeated two to three times depending on viscosity and visual observation for mixing of semen with the extender.

Mechanical /Enzymatic Procedure [Amylase Syringe Mixed Treatment (ASMT)]

Immediately after collection, Tris lactose extender (Portion A**)** supplemented with 2.5 µl/ml amylase was added to the semen with a 1:1ratio, according to (EL-Bahrawy, 2010), then the diluted ejaculates were immediately submitted to a physical process of expulsion of the semen using a 10- or 20 ml (according to the ejaculate volume) for two or three times.

Semen cryopreservation

An automatic filling and sealing machine (Minitube, model MPP UNO) and a computer controlled cryo-freezer, with comfortable data input and automatic recording of the freezing curve (Minitube type: Ice Cube 14S) was used for cryopreservation before transporting the cryopreserved straws to the storage tanks for further investigations. Semen straws were subjected to slow thawing temperatures of 40^0C for 40 seconds in water bath.

Samples assessment schedule

Immediately after collection, viscosity, viability, motility, abnormalities, livability and acrosomal integrity were initially assessed in raw semen, 10 minutes after initial dilution and performing the treatments (either mechanical, enzymatic or mixed treatments) the initial motility (M_I) was assessed, the pre-freezing motility (M_{PF}) was assessed after 4 hours of equilibration at 5^0C and prior to the cryopreservation of samples, and finally post-thawing motility (M_{PT}), recovery rate post thawing (RR%), abnormalities and acrosomal integrity were examined post-thawing.

Semen characteristics assessment

Semen volume was recorded using graduated collecting glass tubes for semen collection. A phase-contrast microscope (Carl ZEISS, AX10_Lab.A1, Germany) with a warm stage adjusted at 37°C was used for the assessment of sperm motility in five different fields at 400X magnification. Both mass motility (viability) in raw semen on a scale from 1 to 3 and motility of freely moving sperm were assessed to the nearest 5%. Sperm livability (live and dead sperm, %) and abnormalities (1^{st} and 2^{nd} abnormalities) were examined using eosin-nigrosin differential staining technique. Acrosomal reaction was examined following the procedure reported by (Johnson et al., 1976).

Semen viscosity was examined according to the (Bravo et al., 2000a) method, using a scale of 1 to 3, where 1 represented low viscosity, 2 for intermediate viscosity and 3 represented the highest viscous samples.

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Computer-assisted sperm analysis

Sperm kinetics were measured by a computer-assisted semen analysis (CASA) system [Sperm Vision Lite, a registered trademark of Minitube, USA] attached to a Zeiss warm stage microscope. For samples' evaluation, a 3 µl aliquot of the sperm sample was placed in a Lica disposable capillary counting chamber; three fields were analyzed. Kinematic parameters were recorded representing; distance curved line (DCL, µm), distance average path (DAP, µm), distance straight line (DSL , µm), sperm velocity curved line speed (VCL, µm /sec.), velocity average path (VAP µm/sec.), velocity straight line (VSL, µm/sec.), sperm linearity movement (LIN=VSL/VCL), sperm straightness movement (STR=VSL/VAP), sperm balanced movement, Wobble (WOB=VAP/VCL).

Statistical analysis

The obtained data results were statistically analyzed using one-way analysis of variance using SAS® (1999) software program. ANOVA procedure of SAS was used. Mean differences were tested by Duncan's Multiple Range tests (Duncan, 1955) when significant P value was obtained.

RESULTS

Fifty ejaculates were collected from male camels during this study. The males had a very good body condition with an average body weight of 622±40.12 kg and body condition score exceeding 2.5±0.5 (Faye et al., 2001).

Reaction time was of a mean value 40.0± 8.23 seconds, with an approximate mating duration of 251±24 seconds, ejaculate volume 5.28±0.66 ml, initial viability 2.5±0.6, initial raw motility 59.34±4.99%, livability 95.3±2.36%. First and second abnormalities were 4.13±0.88% and 7.01±1.254 %, respectively, while acrosomal integrity was 5.03±1.05%. Results shown in figure [1(A) (B)] revealed that, soon after the treatments, the initial motility (M_I) showed that the 3rd group the ASMT exhibited significantly ($P<0.05$) the highest recorded (M_I) reaching 70.00±4.26% compared to the AET (group 1) and SMT (group 2) being 56.00±4.00% and 50.00±3.25%, respectively, the same trend was recorded for the pre-freezing motility (M_{PF}) after 4 hour equilibration period, as it showed the superiority of ASMT treatment prior to cryo-preservation followed by the AET and finally the SMT. Contrarily to the (M_I) and (M_{PF}) values, a significant deleterious effect of the ASMT was observed in post-thaw motility (M_{PT}) 25.00±3.69%, with sperm RR 35.02±5.02%. Superiority of AET on (M_{PT}) 49.00±4.87% followed by SMT 41.67±6.72% was observed, with significantly higher RR, 76.86±4.63% for the AET group and 62.10±6.65% for the SMT group.

Although the highest post thaw motility (M_{PT}) was recorded for AET, but this was accompanied with the highest reacted acrosome (10.17±1.11%), followed by the ASMT group (8.33±0.14%), with the least significant effect for the mechanical treated group (7.33±0.99%) at $p<0.05$. Almost the same trend referred to the treatments effect was observed regarding the first and second abnormalities [Figure 1A and 1B)].

Results illustrated in Figures [2A , 2B, 2C] showed a significant superiority for the AET group on mostly all sperm kinetics. However, the SMT showed the highest distance straight line (DSL, µm), also recorded high sperm track speed, velocity curved line (VCL, µm /sec.), as compared with other treatments. Velocity average path (VAP, µm/sec.) and velocity straight line (VSL, µm/sec.) were significantly higher in AET group [Figure 2A, 2B and 2C)]. Moreover, the lowest values for linearity of sperm movement (LIN=VSL/VCL), straightness (STR =VSL/VAP) and sperm balance movement (wobble) percentage (WOB=VAP/VCL) were recorded in SMT.

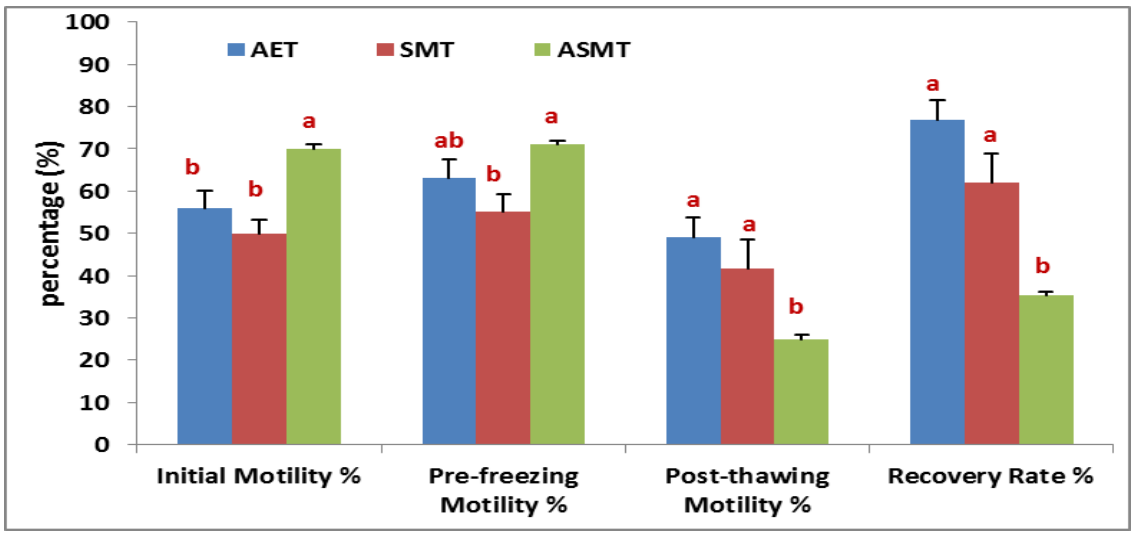

Figure 1. (A)

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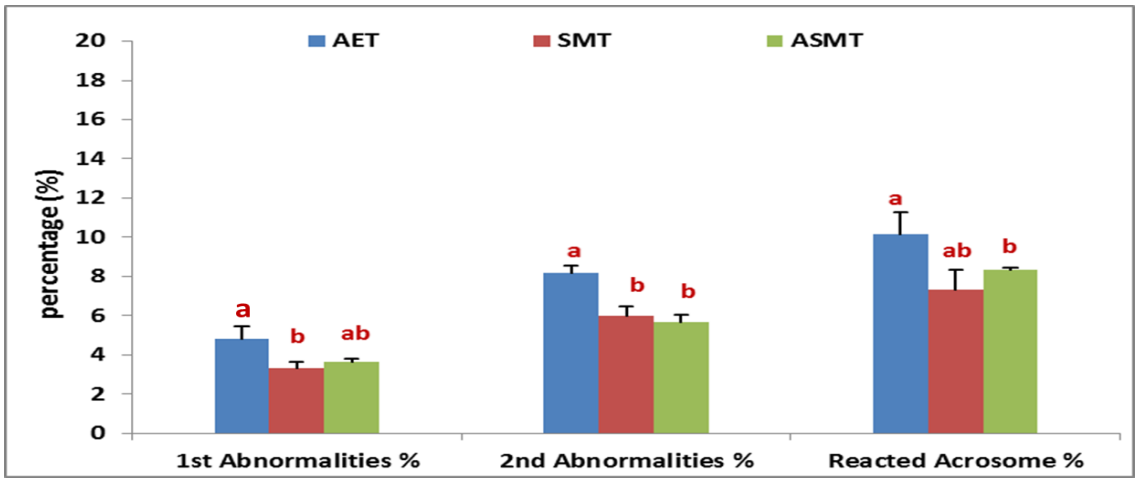

Figure 1. (B)

Figure 1. **(A) and (B):** Effect of Amylase Enzymatic Treatment (AET), Syringe Mechanical Treatment (SMT) and Amylase Syringe Mixed Treatment (ASMT) on sperm physical properties.

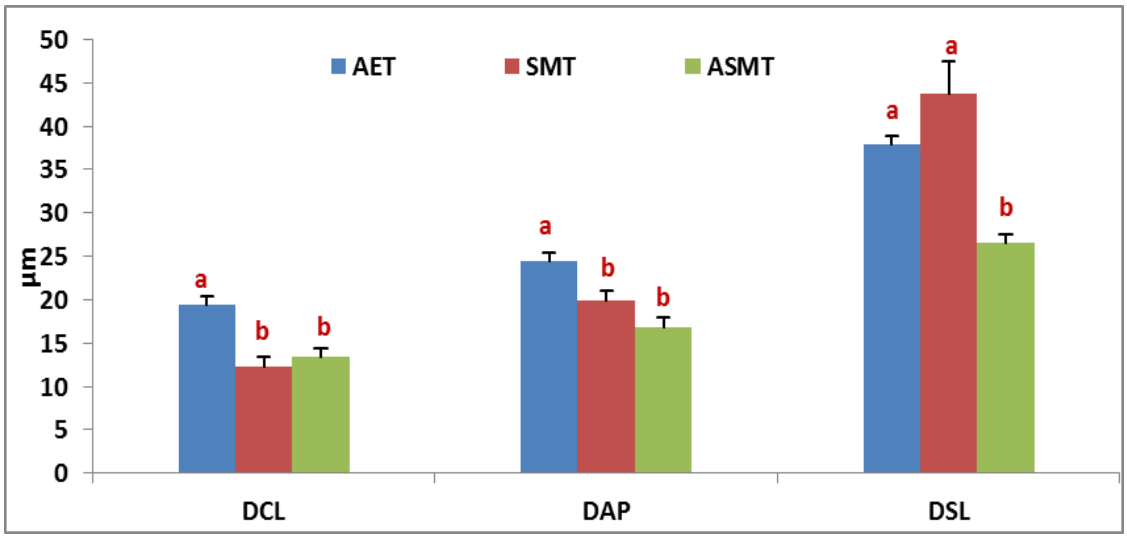

Figure 2. (A)

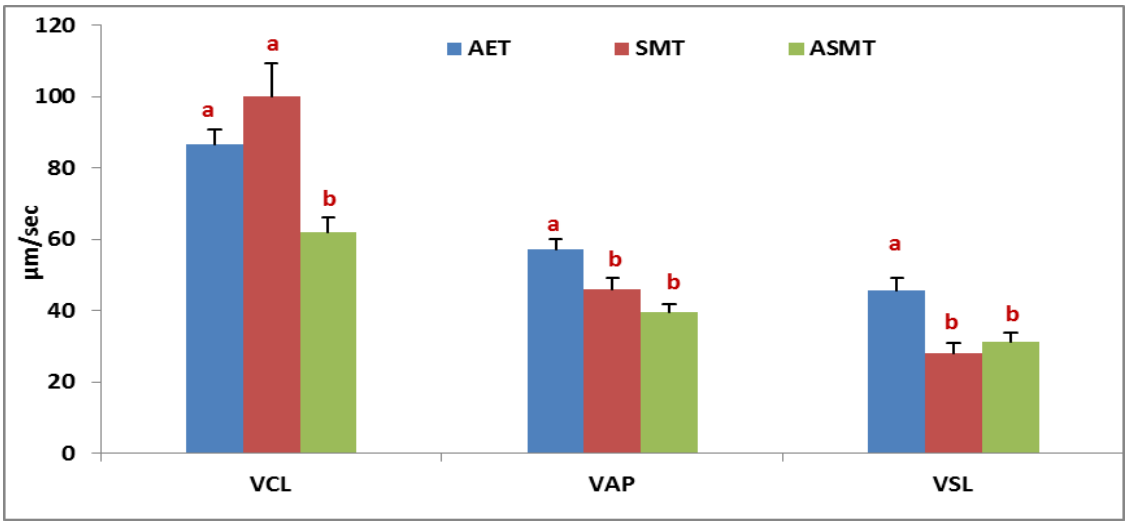

Figure 2. (B)

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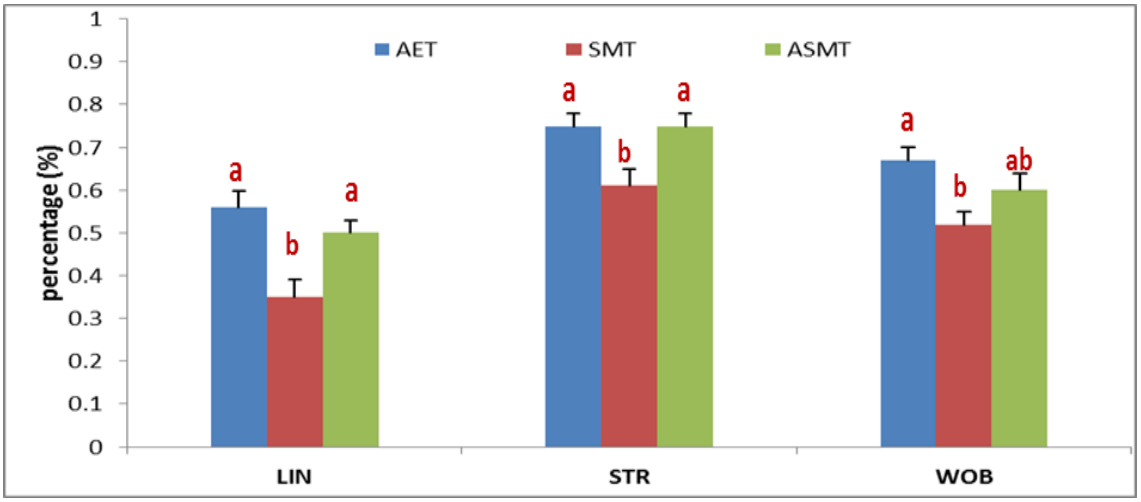

Figure 2. (C)

Figure 2. Effect of amylase enzymatic treatment (AET), syringe mechanical treatment (SMT) and amylase syringe mixed treatment (ASMT) on: Distance curved line (DCL, µm), distance average path (DAP, µm), distance straight line (DSL, µm) **(A)**; Velocity curved line speed (VCL, µm /sec), velocity average path (VAP µm/sec), velocity straight line (VSL, µm/sec) **(B);** Linearity movement (LIN =VSL/VCL), straightness movement (STR =VSL/VAP), balanced movement, wobble (WOB= VAP/VCL) **(C)**.

DISCUSSION

There is a general agreement that the difficulty of manipulating hyper viscous semen samples has been a reason to identify several methods being proposed to decrease viscosity for either initial evaluation or further processing steps of ARTs application. In mostly all species, semen rheological properties radically change immediately after collection. In this regard, viscous semen liquefaction is of fundamental importance for the application of a wide range of assisted reproductive technologies (ARTs) in different species, especially camelids (Crichton et al., 2015; Mal et al., 2016; Malo et al., 2017).

The aim of this investigation was to evaluate the sole enzymatic effect or the sole mechanical effect or a mixed in between treatment serving to reduce viscosity in dromedary camel ejaculates to attain the ability for further semen processing. Up to date, there has been a controversy regarding the reason of semen viscosity in camels.

Although camels have no seminal vesicles, but still mucin-like glycoproteins are generally known to be secreted from the bulbourethral glands (More´, 1991**).** As reported by Owen and Katz (2005**)** and later by Dissanayake et al. (2010)**,** the liquefaction factors responsible for clot lysis are derived from the prostate (plasminogen activator, a-amylase and prostate-specific antigen PSA). (Behr et al., 2009) recommended the usage α-amylase and collagenase without any effect on the quality parameters of spermatozoa, reporting that such enzymatic treatment allows better use of semen ejaculates in application sex sorting using flow cytometry technique. On the same basis, (Rateb, 2016)**,** based his study of using high-power low-frequency ultrasound efficiency in eliminating camel semen viscosity since it causes particle size reduction and viscosity alteration in different substances, this was attributed to modification in the microstructure and functional properties of carbohydrates as well as hydrolysis and cleavage of di- and polysaccharides (Kardos and Luche, 2001; Kbbani et al., 2011; Kunaver et al., 2012). Also, (Mendeluk et al., 2000) assumed that proteins are the main components responsible for the rheological behavior of the semen in its normal form. Hence, the rheological properties of hyper viscous semen samples indicated the existence of a highly organized network in which disulfide bonds and oligosaccharide chains complexes to the peptide core. Consequently, this may play a key role affecting the spermatozoa physiology and the sperm motility (Mendeluk et al., 2000; Skidmore et al., 2013).

As reported in this study, a superiority in using amylase as an enzymatic treatment for viscosity elimination, not only is succeeding in improving the motility and the kinetic characteristics of the sperm, but also the remarked softness of the rich fraction sperm clot, that allows easy packing in the French straws. The superiority of amylase for routine work examination of viscous semen specimens was reported earlier by (Dougherty et al., 1978)**,** as they noted that, the lowest level of amylase did not significantly alter semen parameters, with an 80% sufficient rate to liquefy viscous samples, but with careful regard to the level of the enzyme and the interval between addition and analysis must be controlled carefully. Based on the present data, the results elucidate that a high value of detached acrosome and abnormalities were observed when using amylase as compared to other treatments, but with no observation of sperm agglutination as reported lately by (Monaco et al., 2016) using proteolytic enzymes.

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Despite the fact that the results of enzymatic treatments surpass those obtained by mechanical methods, but still have acceptable post thaw motility compared to enzymatic treatment. Moreover, the SMT showed the highest distance straight line (DSL, µm), also recorded high sperm track speed, velocity curved line (VCL, µm/sec.), this is an indicative value for the ability of the mechanical effect to eliminate viscosity. Reduction of the seminal plasma viscosity may be attributed to the changes in the molecular behavior of semen protein fibrils (Amelar, 1962), making them less viscous and more liquefied without an observed reduction in sperm motility or morphological disorders. (Henkel and Schill, 2003) did not recommend the force elimination of viscous seminal fluid through expulsion of the semen ejaculates through a narrow-gauge needle as it caused sperm immotility. The aforementioned were totally contrary to the present data in this study showing an acceptable post-thaw motility after mechanical treatment, this finding was similar to those reported by (Kussler et al., 2014) who recently recommended the physical process of expulsion of semen through a syringe, but with a great attention to the idea that; this methodology may increase sperm DNA fragmentation. The mechanical stress (passage of diluted ejaculate through the syringe) may play a significant role in sperm DNA breaking. Unfortunately, the effect of the treatments on sperm DNA fragmentation in this study was not investigated. Taking into consideration that, although different studies had recorded high post thaw motility for eliminated viscosity with different methods, thus, still the pregnancy rates attempts depending on camelids semen processing show a very limited success (Aller et al., 2003; Deen et al., 2003; Vaughan et al., 2003; Miragaya et al., 2006; Crichton et al., 2016), as compared with fresh semen insemination, to the best of our knowledge the best of pregnancy rates using frozen semen was reported by Bravo et al. (2000b) when using collagenase for viscosity elimination or even for cryopreserved semen in different species. Literature surveys did not clarify any information regarding the reason of low pregnancy rates using frozen semen. Since the stages of embryonic development that depend on paternal genomes begin after the four-to eight-cell stage, the impact of sperm DNA fragmentation in the embryo is usually not observed in *in-vitro* embryo transfer if carried out preferably up to day three. Worth to mention that, the blastocyst stage development rate and implantation till pregnancy may be affected (Borini et al., 2006), in addition to, expected pregnancy losses (Tarozzi et al., 2007; Rougier et al., 2013). Probably the significant deleterious effect on post thaw motility when mixing between enzymatic and mechanical treatments (ASMT) in the current work was associated with the high stress that may lead to a significant DNA breaking. Lack of consistency in information should be addressed by more investigations concerning DNA fragmentation in cryo-preserved camel semen.

CONCLUSION

From this study, it could be concluded that the viscosity of dromedary camel seminal plasma could be successfully reduced by the use of either mechanical or enzymatic effect, as both may be considered a reliable alternative with different rates of success, with care to the concentration and power of the enzyme and handling and semen ejaculate manipulation during mechanical treatment.

Acknowledgments

The author is thankful to the director of the Tharb camel hospital Dr. Ashraf El-Sharabasy for his full support. Deepest thanks are due to Dr. Mostafa Osama for assisting the technical work, as well as all team members of the camel reproduction laboratory in the Tharb camel hospital. Special thanks are due to Prof Dr. Mohamed Moawad Khalifa, Dr. Sherif Rateb and Mrs. Arig Rakha for assisting statistical analysis and editing of the manuscript.

Competing interests

The author declares that he has no conflict of interest with respect to the research, authorship, and/or publication of this article, the author declares that he has no competing interests.

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To cite this paper: El-Bahrawy KA (2017). Influence of Enzymatic and Mechanical Liquefaction of Seminal Plasma on Freezability of Dromedary Camel Semen. *World Vet. J.* 7(3): 108-116. DOI: http://dx.doi.org/10.5455/wvj.201708100
Journal homepage: www.wvj.science-line.com