Effect of Different Mucolytic Agents on Viscosity and Physical Characteristics of Dromedary Camel Semen

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ABSTRACT

Elimination of viscosity in camel semen is necessary to improve assessment of its raw physical characteristics and to provide a homogeneous dilution. Five different mucolytic agents were tested for removal of viscosity; namely: α-amylase (25%), α-chymotrypsin (0.5%), trypsin (25%), sodium hydroxide (0.1 N) and bromhexine hydrochloride (0.2%). Thirty semen ejaculates were analyzed during the rutting season. The raw semen samples used in this study had an average of 13.4 ± 1.7 ml ejaculate volume, 296.3 ± 41.7 x 10^6/ml sperm concentration, 64.2 ± 4.3 % mass motility, 23.0 ± 2.3 % individual motility, 96.6 ± 0.57% intact acrosomes, 5.2 ± 0.4 % primary abnormalities and 8.1 ± 1.7 % secondary abnormalities. After collection, and raw semen analysis each ejaculate was divided into six equal portions and were diluted in a Tris-Lactose extender at 1:3. Each of the five portions was subjected separately to one of the five selected mucolytic agents at a concentration of 50 µl/ml of extender, and the remaining portion was diluted in an extender free from the mucolytic agents (control).

Seminal viscosity, sperm motility and acrosomal integrity were assessed post-dilution after incubation for 15 min at 37°C and after 240 min of equilibration at 5°C. Amylase superiorly eliminated seminal viscosity in all samples and improved individual sperm motility (46.0 ± 9.12) post-dilution compared to control (27.5± 5.57) and to samples treated with the other mucolytic agents (31.2 ± 5.42 to 37.0 ± 6.55%). However, amylase had a significant ($p< 0.05$) deleterious effect on sperm acrosomal integrity after 240 min of equilibration (11.8± 1.95% detached acrosomes). Although α-chymotrypsin partially liquefied about 60% of samples post-equilibration, it significantly ($p< 0.05$) improved individual sperm motility (46.0 ± 6.35%) and significantly ($p< 0.05$) minimized acrosomal detachment after 240 min of equilibration (7.4± 0.67%). In conclusion, α-amylase totally eliminated viscosity in camel semen, and significantly improved sperm forward motility as compared with untreated semen. However, these mucolytic agents may have deleterious effects on acrosomal integrity after equilibration.

Key Words: camel semen; mucolytic; viscosity; sperm; intact acrosome.

INTRODUCTION

The interest in applying modern reproductive technologies in new and old world camelids has increased dramatically over the last decade. This is due in part to their unique reproductive aspects and to utilize their productive potential for commercial application (Faye, 2008). Because of the features of reproduction in camelids, establishing and developing techniques for freezing and storing semen from these species have obvious advantages for prolongation of the female breeding season, cross-breeding to improve productivity (Al-Eknah, 2000), as well as production and transfer of camelid embryos by IVM and IVF (Khatir *et al.*, 2004). The mucoid nature of camelid semen is considered one of the major problems that delay the application of artificial insemination and semen processing (Skidmore, 2003). Ejaculate viscosity makes it difficult to handle semen, to determine sperm concentration and motility as well as to dilute semen in extender (Adler et al., 1997; El-Zanaty *et al.*, 2004) thus causing technical problems during filling straws. Zavos *et al.* (2006) recommended that viscous semen specimens should be liquefied prior to performance of routine semen analysis to avoid inaccurate assessment of semen characteristics. Freshly ejaculated camelid sperms mostly exhibit poor mass motility and low progressive motility comparable to that of domestic ruminants which exhibit wave and swirl movement. The high viscosity in camelid semen results in oscillatory movement of sperms (most sperms move back and forth) and only 5 to 10% of sperm actively progress forward (Agarwal *et al.*, 1995; Bravo *et al.*, 2000). This is due to entrapment of spermatozoa in mucus and sperm can develop motility only after its liquefaction. Deen *et al.* (2003) noted that when camel semen is in gel form, 35.9% of samples exhibit

no individual sperm motility and only 35.4% exhibit low fair grade of individual sperm motility. In the present study, we conducted collection and dilution of dromedary camel semen in extender containing selected five different mucolytic agents. First of all amylase is known as an enzyme with a unique mode that catalyzes the endohydrolysis of alpha- 1,4-glucosidic bonds liberating a consistent blend of lower molecular weight. Secondly, bromhexine disrupts the structure of acid mucopolysaccharides producing less viscous mucus. Moreover, trypsin and chymotrypsin hydrolyze peptide bonds at the carboxy side of a hydrophobic amino acid where peptides are broken into smaller peptides, and finally NaOH for its alkaline effect. The selected mucolytic agents were used to assess the possibility of reducing the viscosity of semen. Subsequent liquefaction, sperm forward motility and acrosomal integrity were assessed after incubation for 15 min at 37°C post-dilution and after four hours of equilibration at 5°C.

MATERIALS AND METHODS

Animals and location:

The experiment was carried out in a semi-arid area, 34 km North West of Alexandria, Egypt. Semen was collected from six dromedary camel bulls at 10 years of age and 550 kg average body weight during the (2006/2007) breeding season. Bulls were fed daily at 9 am on a pelleted concentrate feed mixture (14% crude protein) supplemented with barley as a source of energy. Berseem hay was also offered *ad lib.* as roughage and the animals were allowed to drink twice daily, with 3 - 4 hours free grazing period twice a week.

Semen collection and initial evaluation

Semen samples were collected twice a week at 8 pm in a clean collection area adjacent to the AI laboratory. El-Hassanein Camel Dummy Technique was used for semen collection (El-Hassanein, 2003). Each bull was left to seek the entrance to the fixed artificial vagina (AV) by himself with a little help to direct the penis into the AV. Five ejaculates were collected from each bull all over the experimental period.

Mucolytic agents and extender preparation

Five different mucolytic agents; namely, α-amylase (Biochemika, Fluka), α-chymotrypsin (Product of Amoun Pharmaceutical. Co., Egypt), trypsin (Sigma Chemical Co., USA), sodium hydroxide (NaOH) and bromhexine hydrochloride (Bisolvon, Chemical industries development under license of Boehringer Ingelheim, Germany) were used to study their effect on camel semen viscosity and sperm physical characteristics. Solutions of α-amylase (25%), α-chymotrypsin (0.5%), trypsin (25%), sodium hydroxide (0.1 N) and bromhexine hydrochloride (0.2%) were freshly prepared, each of which was added to a Tris-Lactose glycerolated extender at 50 µl/ml of extender before collection. Tris-Lactose extender was composed of a mixture of equal parts of Tris-buffer base (0.25 mol) and a sugar base (lactose, 11%). The extender was supplemented with 3% glycerol concentration and 20 % egg yolk as described by El-Bahrawy *et al.* (2006) in a one-step freezing method. After evaluation of the raw semen characteristics, each ejaculate was divided into six portions for dilution in one of the five different mucolytic agent portions and one portion was diluted in an extender free from the mucolytic agents and served as control.

Viscosity and semen assessments

Seminal viscosity, sperm motility and acrosomal integrity were assessed 15 min post-dilution with the mucolytic-treated extender, where samples were kept in a water bath at 37°C. Samples were then transferred to a minitübe cooling cabinet adjusted at 5°C for 240 min of equilibration before reassessing semen characteristics. Several samples of diluted semen were examined for assessment of viscosity according to the method described by Bravo *et al.* (2000). Briefly, 50 µl of a semen sample were pipetted using a micropipette, 25 µl were placed on a glass slide, and then pulled upward to compare the length of the thread. The procedure was repeated after 15 and 240 min as the thread was pulled slowly until it broke. A phase-contrast microscope (Leica) with a warm stage adjusted at 37°C was used for assessment of sperm motility in five different fields at 400x magnification. Both mass motility in diluted seminal plasma trapping sperms (due to viscosity) and individual motility of freely moving sperms were assessed to the nearest 5%. Semen volume was recorded using a graduated collecting glass tube that was used for semen collection. Concentration of semen samples diluted in sodium citrate (2.9%) containing 0.02% glutaraldehyde, were counted using a haemocytometer counting slide.

Acrosomal integrity and abnormalities were assessed in a sample of 10µl semen added to 200µl of 0.2% glutaraldehyde using a phase-contrast microscope at 800x as described by Johnson *et al.* (1976).

Statistical analysis

Analysis of variance was detected using GLM procedure by SPSS (SPSS version 11.5 for windows; SPSS Inc., Chicago, IL, USA). The differences between means were detected using Duncan's Multiple Range Test according to Snedocer and Cochran (1967). Results were quoted as arithmetic mean ± standard error of mean (SEM) and significance was attributed at $p < 0.05$.

RESULTS

All the collected ejaculates were milky and highly viscous. The average ejaculate volume was 13.4 ± 1.7 ml and mean concentration was 296.3 ± 41.7 x 10^6 sperm/ml. The average mass motility in delivered semen was 64.2 ± 4.3 % and individual

forward motility of spermatozoa was 23.0 ± 2.3 %. The percentage of intact acrosomes in ejaculates was 96.6 ± 0.57% , while spermatozoa having primary and secondary abnormalities averaged 5.1 ± 0.4 and 8.1 ± 1.7 %, respectively.

Data on semen consistency and sperm forward progressive motility and acrosomal integrity after dilution in extender with mucolytic agents versus control samples are summarized in Table (1). Fifteen min after dilution and incubation at 37°C, α-amylase superiorly eliminated viscosity in all semen samples (100%) as compared with control (30.0 %) and the other mucolytic agents (42.5, 60.0, 50.0 and 45.0 % for α-chymotrypsin, trypsin, NaOH and bromhexine hydrochloride, respectively). On the other hand, equilibration for 240 min relatively improved liquefaction in control (47.0 %) and in semen samples diluted with α- chymotrypsin, trypsin, NaOH and bromhexine hydrochloride (60.0, 77.0, 55.0 and 52.5 %, respectively).

As the consistency in camel semen samples were reduced post-dilution in the extenders, spermatozoa were rendered free from entrapment and developed forward motility. Dilution in extender free from mucolytic agents (control) slightly improved sperm individual forward motility (27.5± 5.57%) compared to the raw semen motility values (23.0± 2.3%). However, dilution in extenders containing mucolytic agents apparently ($p< 0.05$) improved sperm individual forward motilities post-dilution (46.0 ± 9.12, 34.0 ± 6.32, 36.5±6.15, 37.0 ± 6.55 and 31.2± 5.42 % for α-amylase, α-chymotrypsin, trypsin, NaOH and bromhexine hydrochloride, respectively). On the other hand, equilibration of diluted camel semen for 240 min improved ($p< 0.05$) forward motility in control samples (up to 37.0 ± 7.89 %) and in semen samples treated with α-chymotrypsin (46.0 ± 6.35 %) and bromhexine hydrochloride (40.5 ± 6.16 %) compared to values recorded at 15 min post-dilution. On the contrary, semen samples treated with α-amylase revealed a marked reduction in sperm individual forward motility post-equilibration (41.5± 10.62 %). Sperm acrosomal integrity in camel semen was markedly ($p< 0.05$) affected by the dilution process in all treatment groups. The least acrosomal detachment was recorded in semen samples diluted with trypsin (4.8 ± 0.86 %) and NaOH (5.2 ±0.48 %)

. However, dilution in extenders containing bromhexine hydrochloride increased acrosomal detachment up to 8.8 ± 1.2 % post-dilution. Similar increases in acrosomal detachment were recorded in semen samples diluted with α-amylase, trypsin, NaOH and bromhexine hydrochloride after equilibration at 5°C for 240 min (11.8 ± 1.95, 9.8 ±0.58, 8.4 ± 1.12 and 8.6 ± 0.81 %, respectively).

DISCUSSION

The structure of male camel seminal plasma is known for its high viscosity due to the mucopolysaccharide material secreted by the accessory sex glands in the male camel (Mosaferi *et al.*, 2005). Mucopolysaccharides are long chains of sugar molecules that form a sort of fibrinous network separated by spaces too narrow to allow free movement of the enmeshed spermatozoa (Deen *et al.*, 2004). Several methods for elimination of viscosity in ejaculates have been previously reported. Skidmore (2005) reported that if dromedary camel semen was allowed standing for 15-20 min it would partially liquefy and become easier to mix with the extender, and motility can be more readily assessed. On the other hand, Garnica *et al.* (1993) and Fuentes (1990) tested the value of incubation of semen samples at 37°C for 8 and up to 22 hours for viscosity elimination. Recently, Wani *et al.* (2008) achieved best liquefaction and progressive sperm motility by 60-90 min incubation at 37°C of dromedary semen immediately added after collection to Tris-lactose-egg yolk extender. The addition of different mucolytic agents for elimination of seminal viscosity in different species (monkeys, guinea pigs and humans) was reported by many authors (Freund, 1958; Hoskins and Patterson, 1967; Gozalez-Estrella *et al.*, 1994). Several workers have endeavored to reduce the viscosity of camelid semen using enzymes (Bravo *et al.*,1999; Ccallo *et al.*,1999; Bravo *et al.*, 2000; Deen *et al.*, 2003) and mechanical agitation (Niasari *et al.*, 2005) to allow easier handling, evaluation and extension of semen. Deen *et al.* (2003) studied the effect of α-chymotrypsin and caffeine on spermatozoal motility and found that the addition of caffeine, but not of α-chymotrypsin, improved motility of the individual sperms. In the present study, dilution of dromedary semen in a Tris-lactose extender supplemented with α-amylase, α-chymotrypsin, trypsin, sodium hydroxide or bromhexine hydrochloride obviously eliminated the viscous consistency post-dilution. However, the best liquefaction (100% of samples) was achieved in extender supplemented with α-amylase. The present results also revealed a marked improvement in

Table 1: Effect of different mucolytic agents (mean ± SE) on camel seminal viscosity, sperm forward motility and acrosomal integrity after dilution, incubation for 15 min at 37 °C, and after equilibration at 5°C for 240 min.

Parameters (%)	Control	Mucolytic agents				
		α-amylase	α-chymo-trypsin	Trypsin	NaOH	Bromhexine hydrochloride
EV_{15}	30.0	100	42.5	60.0	50.0	45.0
EV_{240}	47.0	100	60.0	77.0	55.0	52.5
M_{15}	27.5± 5.57^{d}	46.0±9.12^{a}	34.0± 6.32^{bc}	36.5± 6.15^{b}	37.0± 6.55^{b}	31.2± 5.42^{c}
M_{240}	37.0± 7.89^{c}	41.5± 10.62^{b}	39.5± 46.0± 6.35^{a}	39.0± 6.76^{bc}	7.06^{bc}	40.5± 6.16^{b}
DA_{15}	6.2± 1.42^{bc}	6.2± 1.65^{bc}	6.2± 0.73^{bc}	4.8± 0.86^{c}	5.2± 0.48^{c}	8.8± 1.2^{a}
DA_{240}	5.2± 0.58^{d}	11.8± 1.95^{a}	7.4± 0.67^{cd}	9.8± 0.58^{abc}	8.4± 1.12^{bc}	8.6±0.81^{bc}

- EV_{15} %: Percentage of samples in which viscosity is completely eliminated after 15 min of incubation at 37 °C
- EV_{240}%: Percentage of samples in which viscosity is completely eliminated after 240 min of equilibration at 5°C.
- M_{15} %: Percentage of forward motility (mean ± SE) after 15 min of incubation at 37 °C.
- M_{240} %: Percentage of forward motility (mean ± SE) after 240 min of equilibration at 5°C.
- DA_{15} %: Percentage of detached acrosomes (mean ± SE) after 15 min of incubation at 37°C.
- DA_{240} %: Percentage of detached acrosomes (mean ± SE) after 240 min of equilibration at 5°C.
- Means within the same row with different superscript letters are significantly different (P<0.05).

liquefaction of diluted semen samples treated with α-chymotrypsin, trypsin, sodium hydroxide or bromhexine hydrochloride after equilibration at 5°C. The proper liquefaction of equilibrated camel semen ensures the achievement of proper mixing of semen with the extender before filling as well as improving the packaging process itself.

In routine AI work, quality standards for acceptance or rejection of semen for AI are set in terms of sperm concentration, viability and morphology. Bravo *et al.* (2000) noted that the addition of enzymes for viscosity elimination had little or no effect on both motility and acrosomal integrity. However, the present study revealed a marked improvement in individual sperm motility after dilution of semen in extenders containing the tested mucolytic agents. Dilution in an extender with α-amylase superiorly improved sperm forward motility up to 46% compared to the corresponding raw seminal value (23%).

However, acrosomal integrity post-dilution was negatively affected by different mucolytic additives. Acrosomal detachment in treated samples increased up to 4.8-8.8% post-dilution and was elevated by the subsequent equilibration period, especially in samples diluted with α-amylase (11.8%) and trypsin (9.8%). Disadvantages of treating semen with some mucolytic additives to eliminate viscosity may include damage to sperm acrosome. However, Medan *et al.* (2008) reported higher conception rates by using a catalase-supplemented extended cooled semen for artificial insemination rather than using enzyme-free cooled extenders. This was attributed to motility improvement after using the enzyme. However, results of adequate elimination of the viscous nature of camel semen treated with α-amylase inspired us to conduct further investigations to attenuate the negative effect of α-amylase on acrosomal integrity post-dilution, while benefiting from its positive mucolytic effect (unpublished data).

In conclusion, our study indicated that some mucolytic agents may totally eliminate viscosity in camel semen with obvious improvement of sperm forward motility as compared with untreated semen. However, these mucolytic agents may have a deleterious effect on acrosomal integrity after equilibration

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الملخص العربي

تأثير بعض مذيبات المخاط على اللزوجة والخواص الطبيعية للسائل المنوى للجمل أحادى السنام

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استهدف البحث إستخدام بعض المواد للتخلص من اللزوجة الشديدة للسائل المنوى للجمل أحادى السنام للحصول على سائل مخفف متجانس يسهل دراسة خواصه واستخداماته. تم اختبار خمسة عوامل إنزيمية وكيميائية مختلفة وهى الألفا-أميليز (٢٥%)، الألفا-كيموتريسين (٠,٥ %)، التربسين (٢٥%)، أيدروكسيد الصوديوم (٠,١ %) و أيدروكلوريد البروموهيكسين (٠,٢ %). أجريت الدراسة على ثلاثين قذفة من السائل المنوى فى موسم التزاوج rutting season . تم تقسيم كل قذفة إلى ستة أجزاء وتخفيفها فى مخفف الترس – لاكتوز Tris-Lactose بنسبة ١:٣ لاستخدامها فى إختبار تأثير إضافة المواد المؤثرة على اللزوجة عند تركيز ٥٠ ميكرون/مل من المخفف المستخدم مع المقارنة بعينة كنترول خالية من المواد المذيبة للمخاط، وتم اجراء فحص بعد ١٥ دقيقة من التحضين على درجة حرارة ٣٧°م وبعد ٢٤٠ دقيقة (٤ ساعات) من التوازن الحرارى عند درجة ٥°م.

أظهرت النتائج أن خواص السائل المنوى الخام المستخدم فى الدراسة كمتوسطات كانت كالتالى: حجم القذفة ١٣,٤ ± ١,٧ مل، تركيز الحيوانات المنوية ٢٩٦,٣ ± ٤١,٧ × ١٠^٦/ مل، الحركة التكتلية ٦٤,٢ ± ٤,٣%، الحركة التقدمية ٢٣,٠ ± ٣,٢%، الأكروسوم السليم ٩٦,٦ ± ٠,٥٧ %، الشواذ الأولية ٥,١ ± ٠,٤، الشواذ الثانوية ٨,١ ± ١,٧ %.

وبينت النتائج بوجه عام أن الألفاأميليز أظهر تفوقا فى القضاء على اللزوجة فى كل العينات وتحسين الحركة التقدمية للحيوانات المنوية (٤٦,٠ ± ٩,١٢%) وذلك بعد التخفيف مقارنة بجرعات الكنترول الغير معاملة (٢٧,٥ ± ٥,٥٧%) بينما كانت الحركة التقدمية لباقى المعاملات المذيبة للمخاط تتراوح ما بين (٣١,٢ ± ٥,٤٢ الى ٣٧,٠ ± ٦,٥٥%). مع الأخذ فى الإعتبار الأثر المعنوى السلبى للأميليز على سلامة الاكروسوم بعد الإتزان الحرارى لمدة ٤ ساعات (١١,٨ ± ١,٩٥%). فى حين أن إنزيم الألفا كيموتريسين قد أحدث إذابة فى ٦٠% من العينات قبل الإتزان الحرارى، محدثا تحسنا معنويا بإحتمالية (٠,٠٥) للحركة التقدمية ٤٦,٠ ± ٦,٣٥% وكذلك خفض الاكوسوم المشوه الى ٧,٤ ± ٠,٦ % بعد الإتزان الحرارى لمدة ٢٤٠ دقيقة. الخلاصة، أن انزيم الألفا –أميليز قضى على لزوجة السائل المنوى للجمال. وأظهرت كل المواد المستخدمة تحسن معنوى للحركة التقدمية بعد ١٥ دقيقة من التحضين على درجة ٣٧°م، وكذلك بعد الإتزان الحرارى لمدة ٤ ساعات على درجة حرارة ٥°م مقارنة بالسائل المنوى غير المعامل، مع الأخذ فى الإعتبار الأثر السلبى للمواد المذيبة للمخاط على سلامة الأكروسوم بعد فترة الإتزان الحرارى.