

## **EFFECT OF COLLECTION FREQUENCY, EXTENDER AND THAWING TEMPERATURE ON THE MOTILITY RECOVERY OF CRYOPRESERVED DROMEDARY CAMEL SPERMATOZOA**

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### **ABSTRACT**

The primary objective of this study was to examine ejaculate characteristics and quality of camel bull semen maintained under high (3 /week) and low (2 /week) collection frequencies during the breeding season. Ejaculate characteristics included motility, volume, concentration, detached acrosome, sperm abnormalities, pH, seminal plasma osmolality and freezing point depression. Significant differences ( $P<0.01$ ) were observed for ejaculate volume being 13.5 ml versus 7.5 ml for low and high collection frequency, respectively and also pH value ( $P<0.05$ ) for low frequency of 8.7 versus 8.3 for high frequency semen collection.

The second objective was to determine the optimum thawing temperatures (slow thawing vs. rapid thawing) for cryopreserved camel semen extended in tris lactose extender supplemented with different concentrations of  $\alpha$ -amylase. Rapid thawing showed higher post-thaw motility for the control samples as well as the samples at different concentrations of the  $\alpha$ -amylase treated samples. That leads to an overall significant difference ( $P<0.01$ ) in post-thaw motility for rapid thawing being 56.33 % compared to 47.66 % for slow thawed cryopreserved samples. No significant effect was detected for neither the detached acrosome percentage nor the sperm abnormalities.

According to the present results scheduling a collection program of two semen collection times per week, using alpha-amylase supplemented tris-lactose extender with rapid thawing protocol is recommended for a desired high yield of acceptable insemination doses during the breeding season of male camels.

**Keywords:** Dromedary, Semen, Collection frequency, Cryopreservation, Thawing rate.

### **INTRODUCTION**

Efficient production of high quality semen of high genetic value is the most important aim of artificial insemination (AI) organizations. The improvement of quality and economics is limited by the number of insemination doses that can be obtained from one ejaculate and the number of appropriate quality ejaculates that can be obtained from a camel per week. For maximum reproductive efficiency, male bulls have to be managed that is to maintain normal sexual behavior and good libido. Allowing collection of high quality sperm using appropriate collection methods, intervals, and frequencies are very important aspects (Sieme *et al.*, 2004).

El-Wishy (1987) assumed that the low productivity of camels is due to the low reproductive efficiency. Studying the reproductivity and modifying semen extenders and protocols for applying advanced reproductive

technologies in family camelidea give high opportunities in improving its productivity (Al-Ekna, 2000, Bravo, *et al.*, 2000a, Aminu, *et al.*, 2003, Khatir *et al.*, 2006, Morton *et al.*, 2006, Niasari-Naslaji, *et al.*, 2006). Semen characteristics of alpacas were studied after repeated collections, (Bravo *et al.*, 1997). In sum, frequency of mating affected some semen characteristics that may be important determinants of the fertility of male alpacas.

Successful semen storage is dependent on the reversible reduction in metabolic activity and motility, which prolongs the fertilizing lifespan of sperm (Evans and Maxwell 1987). This can be achieved by storing sperm in a liquid or frozen state. Cryopreservation of sperm in camelids is inefficient, primarily owing to lack of knowledge regarding camelid sperm physiology and the viscous nature of the seminal plasma (Bravo *et al.* 2000b). Camelid sperm are generally not tolerant to freezing and thawing procedures (Morton *et al.* 2009). At present the lack of efficient methods to preserve the fertilizing lifespan of semen in liquid or frozen form prevents the widespread use of AI in camelids. (Morton *et al.*, 2009). Generally, the technical aspects of camel sperm cryopreservation have significantly improved over the last few decades. However, a standard protocol designed to optimize sperm motility recovery after thawing has not yet been established as well as, no data exists regarding characteristics of repetitively collected camel semen.

The primary objective of this investigation was to examine changes in ejaculate characteristics and semen quality over time in camels maintained under high and low collection frequencies. A secondary objective was to determine the effects of semen extender and freezing on semen quality under rapid and slow thawing conditions during the breeding season.

## **MATERIALS AND METHODS**

Three healthy adult dromedary camel males (12 years old) with live average body weight of 500 kg were used in the present study for semen collection carried out in the artificial insemination laboratory in Maryout research station, Desert Research Center (DRC), located 34 km North West of Alexandria, Egypt.

Males were trained for semen collection by 42 cm artificial vagina (with a disposable plastic inner liner to avoid the expected lethal effect of rubber on sperm viability) filled with 40°C warmed water and placed in El-Hassanien camel dummy. With this system, a male may copulate for a time comparable to that of natural mating (approximately 25 to 30 minutes), semen samples are reliable, and the male's sexual behavior is not changed (El-Hassanien, 2003). Two semen collection regimes were done for two subsequent breeding seasons (twice a week vs. three times weekly). Collecting tubes with a modified plastic water jacket were used to maintain semen samples at 37°C during the long lasting collecting duration. Semen samples were transmitted immediately to a 37°C adjusted water bath for raw semen physical characters estimation.

### **Experiment 1:**

#### **Physical semen characteristics:**

The semen ejaculates were excluded if contaminated with urine, feces, strange substances, strange colour, odour or contains no sperms (azoospermia).

**Semen volume:** was assessed by graduated collecting glass tube.

**Mass motility :** Phase-contrast microscope (Leica), with warm stage at a power of 40X and an average of 5 fields was obtained to the nearest 5%. For assessing seminal plasma

**pH :** Digital Cole-parmar pH meter was used with a micro probe indicator.

**Abnormalities and intact acrosome were determined** according to Johnson *et al.* (1976), sample of 10 $\mu$ l semen was added to 200 $\mu$ l of a solution of 0.2 % glutaraldehyde using a phase - contrast microscope at 800X.

**Concentration:** Diluted semen samples were counted with a haemocytometer counting slide taking in consideration the dilution factor.

**Osmolarity and freezing point depression (FPD):** An Osmomat 030 apparatus was used for osmolarity estimation (Osmol/kg) , from which FPD was calculated by using the equation of Osmolarity (osmol/kg) X -1.858 = (-<sup>0</sup>C) Freezing Point Depression. (FPD). All parameters were determined according to El - Bhrawi (2005).

### **Experiment 2:**

#### **Semen dilution, amylase enzyme supplementation and cryopreservation:**

A tris-lactose 3% glucerolated extender, supplemented with 20% fresh egg yolk and different concentrations of Supra-Terminal  $\infty$ -amylase enzyme with four different concentrations of 2.5, 5, 10, 15  $\mu$ l/ml. were added to the previously prepared extender to eliminate seminal plasma viscosity in-addition to a control group free of alpha-amylase (El-Baharwy, 2010). Semen extension was carried out using a split sample technique in one-step freezing method on five different concentrations of a final dilution rate of 1 semen portion to 3 portions of extender.

Diluted semen was transported to a mini-tübe cooled handle cabinet for four hours equilibration time, before packing in 0.5 ml French straws using a 133 model type mini-tübe filling and sealing machine. A -140<sup>0</sup>C nitrogen vapor biological freezer (mini-tübe) was used for doses cryopreservation before transporting to the storage tanks for further post-thawing investigations.

#### **Thawing method:**

A programmable mini-tübe thawing device was used for slow and rapid thawing. The device was programmed for the desired time and temperatures which were 37<sup>0</sup>C for 40 seconds for slow thawing, while rapid thawing needed a higher temperature of 65<sup>0</sup>C for 10 seconds

#### **Statistical analysis:**

The results of raw semen statistical analysis were conducted using SAS® (1988) software program. One way ANOVA of GLM procedure of SAS was used in each experiment. Extenders and thawing temperature data analysis were performed using SPSS software (SPSS version 11.5 for Windows; SPSS Inc., Chicago, IL, USA) computer program. Results are

quoted as arithmetic mean  $\pm$  standard error of mean (S.E.M.) and significance was attributed at ( $p < 0.01$ ).

## RESULTS AND DISCUSSION

### Experiment 1:

The optimal ejaculation frequency is dependable on age, exercise and sexual potential. There is a natural ejaculation frequency that balances the testosterone level in the body. The effects of various frequencies of semen collection on several quantitative and qualitative semen characteristics were investigated in adult camels. Motility %, volume, sperm cell concentration/ml. per ejaculate were determined for each ejaculate (Table 1).

The ejaculated volume of semen obtained with low frequency collection technique (13.5 ml.) was higher than the ejaculates volume (7.3 ml.) depending on high frequency rate ( $P < 0.01$ ). Mathematically sperm cell concentration/ml. was also higher  $454.61 \times 10^6$  vs.  $310 \times 10^6$  sperm/ml for low and high collection frequencies, respectively, with no significant differences observed between groups. However, in the present experiment there were no significant differences between low and high frequencies for semen collection on motility percentage being 48.6 and 51.7 %, respectively, or for detached acrosome being 4.6% versus 6.2%. Whereas, there were no significant differences for primary abnormalities percentage under the low and the high collection rates (5.3 versus 6.3%), respectively. The same trend was observed for secondary abnormalities being 7.4% versus 9.5%. No differences were also observed for osmolarity or for freezing point depression being 0.37 Osmol./kg and  $-0.62^\circ\text{C}$  for low collection rate and 0.36 Osmol./kg ,  $-0.6^\circ\text{C}$  for high collection rate, respectively.

**Table (1): Effect of collection frequency on semen physical characteristics of male dromedary camels:**

Parameter	Semen collection frequency per week (Mean $\pm$ SE)	
	2 collection days per week	3 collection days per week
Mass motility (%)	48.6 $\pm$ 3.3	51.7 $\pm$ 5.3
Volume (ml.)	13.5 $\pm$ 1.46 <sup>a</sup>	7.3 $\pm$ 1.01 <sup>b</sup>
Concentration $\times 10^6$ sperm/ml.	454.61 $\pm$ 65.88	310.73 $\pm$ 43.97
Detached acrosome (%)	4.6 $\pm$ 0.83	6.2 $\pm$ 8.2
First abnormalities (%)	5.3 $\pm$ 0.31	6.3 $\pm$ 0.76
Second abnormalities (%)	7.4 $\pm$ 0.41	9.5 $\pm$ 1.41
pH	8.7 $\pm$ 0.11 <sup>a</sup>	8.3 $\pm$ 0.06 <sup>b</sup>
Osmolarity (Osmol./kg)	0.370 $\pm$ 0.01	0.360 $\pm$ 0.007
Freezing pint depression ( $^\circ\text{C}$ ).	0.620 $\pm$ 0.21	0.600 $\pm$ 0.32

Different letters a, b within the same rows indicates a significant difference (  $P < 0.05$  )

The present results confirm that decreasing the frequency of semen collection in camels partly increases semen yields per male, as increasing the frequency of semen collection had negative effects on ejaculate volumes without significantly affecting sperm cell concentrations. The relative

advantages and disadvantages following high and low frequencies of semen collection have been previously demonstrated in different animal species (Bravo *et al.*, 1997, alpacas; Sieme *et al.*, 2004, stallions; Frangež *et al.*, 2005, Boars; and Aguirre *et al.*, 2007, rams).

The obtained results demonstrated a favorable effect of low semen collection frequencies on semen volume with no marked decline in other sperm characteristics. These results agree with those reported by Pickett *et al.*, (1975) for stallions. A decrease in semen volume and sperm concentration with successive ejaculations has been reported in several studies on rams (Ollero *et al.*, 1996; Kaya *et al.*, 2002). We concluded that camel males express their optimal reproductive capacity more efficiently when semen collection is undertaken at a low rather than a high frequency. The frequency of semen collection in that species also influences the number of semen doses available for insemination having a higher yield of semen volume during the breeding season. That resulted in no significant difference for ejaculate characteristics taking in consideration higher non-significant mathematical value for sperm cell concentration. However, an explanation for the absence of significant differences in sperm concentrations between ejaculates from males subjected to collection at varying frequencies may be attributed to the relatively small number of males and to the high individual variability of semen parameters between males. Generally semen collection frequency has an impact on semen quality.

#### **Experiment 2:**

**Table (2): Effect of different concentrations of  $\alpha$ -amylase on physical characteristics of camel semen under different thawing conditions:**

Thawing Treatment	Slow				Rapid			
	Semen characteristics							
	Mot. %	IA %	1 <sup>st</sup> ab.%	2 <sup>nd</sup> ab. %	Mot.%	IA %	1 <sup>st</sup> ab.%	2 <sup>nd</sup> ab.%
Control	40 <sup>b</sup> ±2.8	7.0 ± 2.1	10.3 ± 2.6	9.6 ±3.5	46.6 ± 9.2	9.6 ± 0.66	6.67 ± 0.33	7.6 ± 0.3
2.5 µl/ml	40 <sup>b</sup> ±2.8	12.3 ±1.3	10.6 ±0.3	10.6 ±1.3	53.3 ± 10.9	10.3 ± 0.67	7.0 ± 1.15	8.3 ± 0.67
5 µl/ml	55 <sup>a</sup> ±0.01	9.67 ± 2.6	9.6 ± 1.2	14.0 ± 1.7	58.3 ± 8.3	11.0 ± 0. 1	5.3 ± 0.88	6.3 ± 0.85
10 µl/ml	56.6 <sup>a</sup> ±4.4	12.6 ± 2.08	10.0 ±1.15	8.6 ±0.3	56.6 ± 4.4	10.6 ± 1.85	8.0 ±1.0	9.0 ± 2.3
15 µl/ml	55 <sup>a</sup> ±5.7	10.0 ± 3.8	8.66 ± 1.2	9.3 ±1.4	61.6 ± 4.6	10.4 ± 2.4	5.0 ± 1.57	7.0 ±1.0

a,b Means with different superscripts in the same column are significantly different at P<0.05

Mot. = Motility, IA = Intact acrosome, 1<sup>st</sup>ab. = Primary abnormalities, 2<sup>nd</sup>ab. = Secondary abnormalities

The mean values obtained from semen physical parameters under different thawing rates are presented in (Table 2). Significant increase was obtained for sperm motility under different concentrations of amylase. The

results showed that addition of 5, 10, 15  $\mu\text{l/ml}$  of alpha-amylase had a significant positive effect in increasing the motility % of semen samples post-thawed under rapid and slow protocols being higher for rapid thawing (Table 2). No significant differences were detected between 2.5  $\mu\text{l/ml}$  concentration and the amylase free extender (the control) as they both showed a significant decrease in motility percentages in the slow thawing procedure. On the other hand, rapid thawing protocol enhanced post thaw sperm motility under all treatments of alpha-amylase as well as the control group. Neither different amylase concentrations nor the thawing procedures had any significant effect on acrosomal reaction or abnormality of the post-thawed sperms, leading to no expected effect of both treatments on fertility.

The overall effect of rapid and slow thawing of the cryopreserved doses (Table 3) showed a significant effect ( $P < 0.01$ ) in enhancing the post thaw motility with 56.33 % for rapid thawing versus 47.66 % for slow thawing. The same trend was monitored for a highly significant decrease ( $P < 0.01$ ) on 1<sup>st</sup> and 2<sup>nd</sup> abnormalities with an approximate decrease of 30 % under rapid thawing conditions, while no effect was detected on acrosomal integrity.

**Table (3): Overall effect of rapid and slow thawing on cryopreserved semen dose:**

Parameter Treatment	Mot. %	IA %	1 <sup>st</sup> ab. %	2 <sup>nd</sup> ab. %
Slow thaw.	47.66 <sup>b</sup> $\pm$ 2.67	10.33 $\pm$ 1.03	9.86 <sup>a</sup> $\pm$ 0.59	10.46 <sup>a</sup> $\pm$ 0.89
Rapid thaw.	56.33 <sup>a</sup> $\pm$ 3.02	10.46 $\pm$ 0.57	6.40 <sup>b</sup> $\pm$ 0.43	7.67 <sup>b</sup> $\pm$ 0.53

a,b Means with different superscripts in the same column are significantly different at  $P < 0.01$

Mot. = Motility, IA = Intact acrosome, 1<sup>st</sup>ab. = Primary abnormalities, 2<sup>nd</sup>ab. = Secondary abnormalities

Motility is commonly believed to be one of the most important characteristics associated with the fertilizing ability of sperm. Significant differences had been observed in motility parameters between sperm that achieved a high percentage of fertilization and those that failed in the achievement of pregnancy (Donnelly *et al.*, 1998 and Paulenz, *et al.*, 2007). Thawing frozen sperm rapidly, apparently caused less membrane damage on thawing resulting in enhanced post-thaw motility and viability and reduced premature capacitation detected in primary abnormalities. This was confirmed in different animal species. Bradford & Buhr (2002) on stallion reported that the fast thaw rate significantly increased the percent of progressively motile sperm compared with the slow and moderate thaw rates. The fast thaw rate also improved sperm viability. Dong, *et al.*, (2009), on monkey also noticed that post-thaw motility was significantly lower in samples that were thawed slowly than those that were thawed rapidly. Warm water thaw exposes sperm to critically dangerous temperatures for only a brief time. The rise in temperature should be rapid enough to minimize sperm damage. A major criticism and concern for the warm water thaw is the danger of cold shock caused by mishandling of the straw following thaw. Cold shock is the permanent injury to sperm caused by a sudden decrease in semen temperature after thawing. Damage can result if the chosen thawing rate is

inappropriate, as slow thawing results in re-crystallization of microcrystals of intracellular water and resulted damage to cellular organelles (Hammerstedt *et al*, 1990). The same observations were remarked by Zhang, *et al.*, 1990, on stallion spermatozoa, while, Cumming, (1995) failed to prove these results in a study on bulls. As a generalization, the more rapid the cooling (freezing) rate, the more rapid should be the warming (thawing) rate. The ideal cooling and warming rates are also influenced by the composition of the extender. The process of thawing for insemination is potentially the most damaging to sperm due to rapid changes in volume.

## **CONCLUSION**

It can be concluded that varying the frequency of semen collection in male camels, extender constituents and thawing temperature not only increases or impairs the quantities of spermatozoa available for insemination, but also modifies the initial rate of sperm viability in post-thawed semen. At a strategic level, the practical use of adequate frequencies of semen collections, coupled with the more investigations for semen processing and insemination protocols should provide useful tools for optimizing the fertilizing potential of the best camel sires in commercial situations

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**تأثير معدل الجمع والتخفيف و حرارة الاسالة على استعادة الحركة فى سبرمات  
الجمال أحادية السنام المحفوظة بالتجميد**  
**خالد أحمد البحراوى، السيد السيد الحسانين و شريف عبدالرزاق راتب**  
**شعبة الانتاج الحيوانى - مركز بحوث الصحراء**

الهدف الاول من تلك الدراسة كان لاختبار خواص وجودة الدفقات الخاصة بالسائل المنوى لطلانق الجمال احادية السنام وذلك تحت نظامين مختلفين لجمع السائل المنوى، النظام الاول بمعدل جمع 3 مرات/اسبوعيا بينما النظام الآخر من خلال جمع السائل المنوى (2 مرة/اسبوعيا)، ودراسة أثر ذلك على خواص الطبيعية للقفذات من حيث الحركة، الحجم، التركيز، سلامة الاكروسوم، المشوهات، رقم الحموضة، أسموزية البلازما المنوية ونقطة التجمد. ظهر اثر الاختلاف في معدلات جمع السائل المنوي معنويا ( $P<0.01$ ) في حجم القذفة لتصل الي 13.5مل في حالة الجمع مرتين اسبوعيا وتنخفض الى (7.3 مل) في حالة الجمع 3 مرات أسبوعيا، وكذلك كان نفس الاثر بما يخص رقم الحموضة بحيث كان 8.7 في حالة الجمع مرتين اسبوعيا مقارنة برقم حموضة 8.3 في حالة معدل الجمع العالي في حين لم تظهر باقى الخواص فروقا معنوية.

كان الهدف الثانى من تلك الدراسة هو التعرف على معدل الاسالة الامثل (الاسالة السريعة او الاسالة البطيئة) وذلك للسائل المنوى المحفوظ بالتجميد باستخدام مخفف الترس لاكتوز والمدمع بانزيم الالفا اميليز (كمذيب للطبيعة المخاطية فى السائل المنوى) بتركيزات مختلفة. أظهرت الاسالة السريعة حركة أعلى فيما بعد الاسالة وذلك لجميع تركيزات الالفا اميليز وكذلك المجموعة الضابطة الخالية من الانزيم، وظهر الاثر الكلي لطريقة الاسالة بغض النظر عن كمية الانزيم تقوفا معنويا في ارتفاع حركة ما بعد الاسالة 56.33% مقارنة 47.66% للأسالة البطيئة. كذلك ظهر الاثر الايجابي للأسالة السريعة في الانخفاض المعنوي لنسبة المشوهات الاولى والثانوية في حين لم يتأثر سلامة الاكروسوم معنويا باثر معدل الاسالة.

وعليه فان الاعداد الجيد لبرنامج متكامل لانتاج جرعات محفوظة من السائل المنوي للجمال من خلال جمع السائل المنوى بمعدل مرتين اسبوعيا واستخدام انزيم الالفا اميليز كمادة داعمة لمخفف الترس لاكتوز ومراعاة برنامج للأسالة السريعة بعد الحفظ بالتجميد يساعد فى الوصول الى جرعات تلقحية جيدة تساهم وتطوير برامج التلقيح الاصطناعى فى الابل.

**قام بتحكيم البحث**

**كلية الزراعة – جامعة المنصورة**  
**مركز بحوث الصحراء**

**أ.د / مصطفى عبد الحليم الحرايرى**  
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