SMGroup

International Journal of Animal Science

Research Article

Studies on Semen Processing, Cryopreservation and Artificial Insemination in Dromedary Camel

Akbar SJ[1]*, Hassan SM[2] and Ahmad M[1]

[1]*Dubai (Pvt.) Camel Breeding Center, United Arab Emirates*

[2]*Animal Sciences Institute, National Agricultural Research Centre, Pakistan*

Article Information

Received date: Jan 29, 2018

Accepted date: Feb 20, 2018

Published date: Feb 23, 2018

***Corresponding author**

Akbar SJ, Dubai (Pvt.) Camel Breeding Center, Dubai, United Arab Emirates,
Tel: +971 50 657 2000;
Email: jahangirakbar@hotmail.com

Distributed under Creative Commons CC-BY 4.0

Photograph of "world's first dromedary camel calf born on January 12, 2018 with frozen semen artificial insemination".

Abstract

The aim of the present study was to evaluate the liquefaction time of semen, optimal cryoprotectant level in extender for cryopreservation of camel spermatozoa and subsequent *in-vivo* fertility for large scale application of assisted reproductive techniques. Two experiments were performed to meet the objectives of the study. In experiment I, semen samples of individual bulls (n=3) were divided for dilution in one of the three experimental extenders to evaluate the liquefaction process. In experiment II, semen samples of individual bulls (n=3) were divided for dilution in one of the three experimental extenders with variable glycerol concentrations to improve the post-thaw sperm quality and fertility. Liquefaction time, motility, live sperm, plasma membrane integrity and normal apical ridge varied significantly due to extender and bull. Sperm quality parameters were improved ($P<0.05$) with increase in glycerol concentration (7%) as cryoprotectant. Thirteen camels carried to full term pegnancy with semen frozen in extender with 7% glycerol. To date, four camels have delivered healthy calves and remaining are awaited for it. It is concluded that dilution of camel semen with Tris based extenders accelerates the process of liquefaction with improved sperm structures and functions. Supplementation of Tris based extender with 7% glycerol preserves the post-thaw quality and fertility of camel bull spermatozoa. Moreover, the fertility results of present study are very encouraging for the large scale application of assisted reproductive techniques in camelids.

OPEN ACCESS

ISSN: 2575-7806

How to cite this article Akbar SJ, Hassan SM and Ahmad M. Studies on Semen Processing, Cryopreservation and Artificial Insemination in Dromedary Camel. Int J Anim Sci. 2018; 2(2): 1018.

Introduction

Artificial Insemination (AI) is an important technique of assisted reproduction that facilitates in extensive dissemination of genetics from elite sires. While cryopreservation of spermatoza is helpful in long-term preservation of farm animal genetic resources. There is considerable interest worldwide in the application of AI in camelid breeding programs by using frozen-thawed spermatozoa. At present the lack of efficient methods to preserve the fertilizing lifespan of frozen thawed spermatozoa impedes the large scale use of AI in camelids [1,2].

Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to the osmotic and thermic shocks that occur both during the dilution, cooling–freezing and during the thawing procedures [3]. Damage occurring during the freezing–thawing procedures affect mainly cellular membranes (plasma and mitochondrial) and in the worst case, the nucleus [4]. Cryopreservation of sperm in camelids is inefficient, primarily owing to a lack of knowledge regarding camelid sperm physiology and the viscous nature of the semen [5,6]. Very few studies have addressed the subject of sperm cryopreservation and male to-male variability that limits the success in cryopreservation. Additionally, the literature reports only a single early pregnancy (1/13 animals inseminated) in dromedary camel that has resulted from AI with cryopreserved spermatozoa [7]. Therefore, the aim of the present study was to evaluate the liquefaction time, optimal cryoprotectant level in extender for cryopreservation of dromedary camel spermatozoa and subsequent in-vivo fertility for large scale application of assisted reproductive techniques.

Materials and methods

Experiment 1: Effect of extenders on liquefaction time and sperm quality parameters

Three mature dromedary camel bulls (age 7-10 years) were involved in this study. The bulls were kept in individual pens at Livestock Research Station, National Agricultural Research Centre (Islamabad, Pakistan). The bulls were offered ample amount of seasonal fodder and concentrate. For semen collection, bulls were excited using a sexually receptive she camel and allowed to mount after olfactory contact during teasing. When teased bulls mounted the female and adjusted its mating position, the penis was directed into pre-warmed Artificial Vagina (AV) maintained at 40°C. After several thrusts, semen was collected in graduated tube attached with silicone cone of AV. The sample was immediately transferred to laboratory for further experimentation.

Semen samples of individual bulls (15 ejaculates from 3 bulls; B-I, B-II and B-III) were kept in water bath and divided into four aliquots by reverse pipetting technique. Three extenders were used to dilute semen samples as described below:

TCF-I: Tris (hydroxymethyl)aminomethane (3.03% w/v), citric acid (1.65% w/v), fructose 1.7% w/v), and egg yolk (20% v/v).

TCF-II: Tris (3.03% w/v), citric acid (1.5% w/v), fructose (0.2% w/v), and egg yolk (20% v/v).

SCF: Sodium citrate (2.2% w/v), fructose (1.5% w/v) and egg yolk (20% v/v).

All the experimental extenders were supplemented with 0.1% (w/v) streptomycin sulphate and EDTA.

Each aliquot was diluted with 1:1 ratio either in TCF-I, TCF-II or SCF extenders at 33°C in water bath and time was noted as 0 min. Fourth aliquot was not diluted with any of the extender (control). Liquefaction status was evaluated after every 10 minutes of each sample and defined as disappearance of thread formation during intermittent pipetting. As the liquefaction completed, each semen sample was evaluated for motility (%), live sperm (%), plasma membrane integrity (PMI; %), normal apical ridge (NAR; %) and chromatin integrity (%) by using the procedures given below.

Experiment 2: Effect of glycerol concentrations in Tris based extenders on post-thaw quality and *in-vivo* fertility

Three mature dromedary camel bulls (age 8-15 years) were used in this study. The bulls were kept in individual pens at Dubai (Pvt) Camel Breeding Centre (Dubai, UAE). The bulls were maintained on breeding diet that included alfa alfa hay, concentrate and mineral supplement. Semen collection was carried out as described above.

In this experiment, effect of glycerol (G) was determined in three Tris based extenders. In first step, non-glycerolated extenders were prepared as given below.

Ext-I: Tris (2.43% w/v), citric acid (1.3% w/v), fructose (2% w/v), and egg yolk (20% v/v).

Ext-II: Tris (3.03% w/v), citric acid (1.65% w/v), fructose (1.7% w/v), and egg yolk (20% v/v).

Ext-III: Tris (3.03% w/v), citric acid (1.5% w/v), fructose (0.2% w/v), and egg yolk (20% v/v).

In second step, the glycerolated part were prepared by adding glycerol at concentrations of 6, 10 or 14% v/v in equally divided parts of above Tris based extenders. Finally, the extenders were designated as G-3, G-5 and G-7.

After semen collection, ejaculates were initially diluted with Ext-1, Ext-II or Ext-III and placed in water bath at 33°C. After liquefaction, each diluted sample was cooled from 33°C to 4°C over 120min and then glycerolated extender was added to make the final glycerol concentration as 3, 5 and 7% and sperm concentration 100×10^6/ml. Semen was filled in 0.5ml French semen straws and equilibrated at 4°C for 90 min. After equilibration, semen was frozen using controlled rate freezing system at slow rate (+4 to -20°C @2°/min and -20 to -60°C @3°/min). When temperature reached -60°C, semen straws were plunged into liquid nitrogen at -196°C, stored until analyzed for post-thaw semen parameters (motility, live sperm, PMI, NAR. Post-thaw analysis was carried out at least 24h after freezing. At least two semen straws per group were thawed at 33°C for 45 sec and semen was taken into a pre-heated tube in water bath and processed for analysis of semen quality parameters.

Semen parameters

Progressive motility (%): Semen motility was evaluated subjectively in samples placed under a cover slip. A 9μl drop was placed on pre-heated microscopic slide, cover slip placed and evaluated at 10x using a phase contrast microscope fitted with a 37°C warm stage. Motility was assessed at least in five different microscopic fields of slide.

The average of five fields was denoted as final motility. Rectilinear progressive motility was considered as progressive motility of semen.

Viability (%): Viable sperm were evaluated using differential staining technique with eosin-nigrosin stain. Briefly, this solution contained 10 g nigrosin and 2 g eosin in 100ml of solution of 2.9% sodium citrate. A 10µl drop was mixed with staining solution on a microscopic slide and a thin smear was prepared. Slide was air dried and observed at total 400 magnification of microscope. At least two hundred sperm were counted for viability. Sperm with complete exclusion of stain were considered as viable.

Plasma membrane integrity (%): Hypo-osmotic swelling test (HOST) was used to evaluate functional integrity of sperm. Briefly, HOS solution was prepared by dissolving tri-sodium citrate (0.75 g) and fructose (1.351g) in 100 ml of solution, prepared in distilled water to final osmolarity of 190mOsm/kg. It was performed by dissolving 50µl of semen into 500 µl of HOS solution and incubated for 30 min at 33°C. After incubation, a 5µl drop of prepared solution was observed under microscope (400 magnification) for swelling/coiling ability. A minimum two hundred sperm were counted for coiled or swollen tail of varying degrees, and considered as biochemical active plasma membrane.

Normal apical ridge (%): It was determined using formol citrate solution method. The solution was prepared by dissolving tri-sodium citrate (2.9 g) in 1% solution of formaldehyde. A 500µl of each semen sample was fixed in 50µl of formol citrate solution and incubated for 30 min. At least two hundred sperm were counted for normal, loose or damaged acrosome under microscope (1000x).

Chromatin integrity (%): Toludine blue staining was used to assess chromatin integrity. Briefly, semen smear was prepared on slide, air dried and fixed in freshly prepared 96% ethanol-acetone (1:1) at 4°C. It was air dried again and hydrolysis was performed in 0.1N HCl at 4°C for 5 minutes. Thereafter, slides were immersed in distilled water and stained with toludine staining solution (0.05% in 50% Mcllvaine citrate phosphate buffer) for 5 minutes. At least two hundred sperm were counted at 1000x under microscope using oil immersion lens. Sperm with intact chromatin present light blue and denatured integrity presented violet colour.

Artificial insemination: Twenty one dromedary females were selected based upon palpation and ultrasonographic evaluation of reproductive tract. All females possessing normal follicular development and infection-free uterus and ovarian-bursa were selected for study. They were followed for follicular growth and development using trans-rectal ultraonography (MyLab™One VET; Italy) by linear transducer with frequency of 6-8 MHz. Ovulation was induced with GnRH analogue (Buserelin; 20µg) when mature follicle reached 11-13mm. At least six semen straws of Ext-III with G-7 were thawed at 33oC for 45 sec and sperm suspension was poured in a pre-warmed tube. Thereafter, it was loaded in insemination catheter (65cm long, minitube, Gerrmany). Vulva of female camel was washed and disinfected. Insemination was performed at 30h of induction of ovulation. Ultrasonography was performed at 48 h to ascertain ovulation. Pregnancy determination was performed at 18, 30, 60 and 90th day of insemination through trans-rectal ultrasonography.

Statistical analysis

All data were presented as mean ± S.E.M and normality was determined using Shapiro-Wilk test for each parameter. The data were analyzed as 3×3 factorial analysis in both experiments for each semen parameter: 3 extenders (TCF-I, TCF-II, SC) × 3 bulls (B-I, B-II and B-III) in experiment 1; and 3 extenders (Ext-I, Ext-II and Ext-III × 3 glycerol concentrations (3, 5, 7%) in experiment 2, using PROC MIXED of SAS Enterprise Guide (Version 4.2; SAS Inst. Inc., Cary, NC, USA). The replicates were considered as covariate in analysis model. Probability level of <0.05 was considered as significant. The multiple comparisons of groups were determined using Tukey's test.

Results

Experiment 1

The data for liquefaction time and post-liquefaction motility of semen of three bulls diluted in three extenders have been presented in figure 1. Liquefaction time and motility, both varied due to extender and bull ($P<0.001$). In overall, all bulls contained longer ($P<0.05$) liquefaction time in SCF extender as compared to both Tris based extenders. The liquefaction time of B-I and B-II was recorded equal

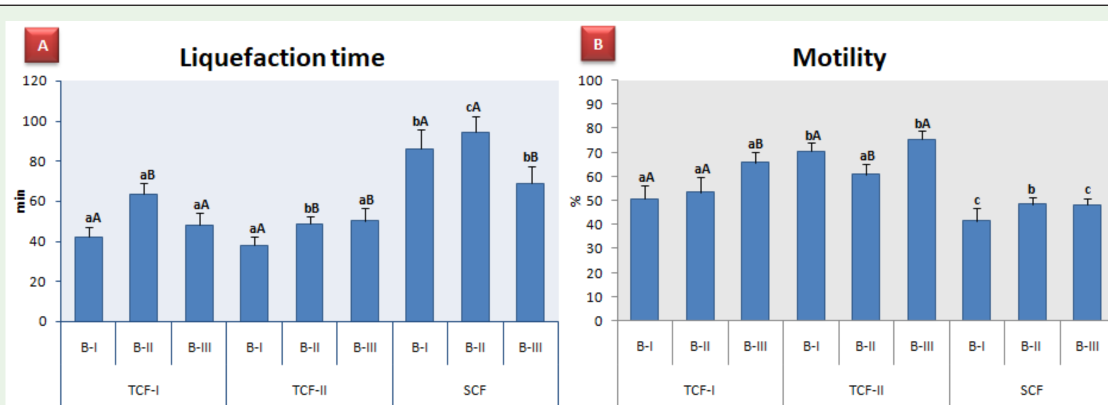

Figure 1: (Experiment 1), The liquefaction time (A; minutes) and post-liquefaction motility (B) of semen diluted in TCF-I (Tris citric acid fructose-I), TCF-II (Tris citric acid fructose-II) and SCF (sodium citrate) extenders of three bulls (B-I, B-II and B-III). The data have been presented as LS means ± S.E.M which have been analyzed as 3 × 3 factorial model for three extenders and three bulls. Probability level of <0.05 was considered for significance. Small letter superscripts (a-c) denote difference (P<0.05) among different extenders within same bull while capital letter superscripts (A-C) denote difference (P<0.05) among three bulls within same extender.

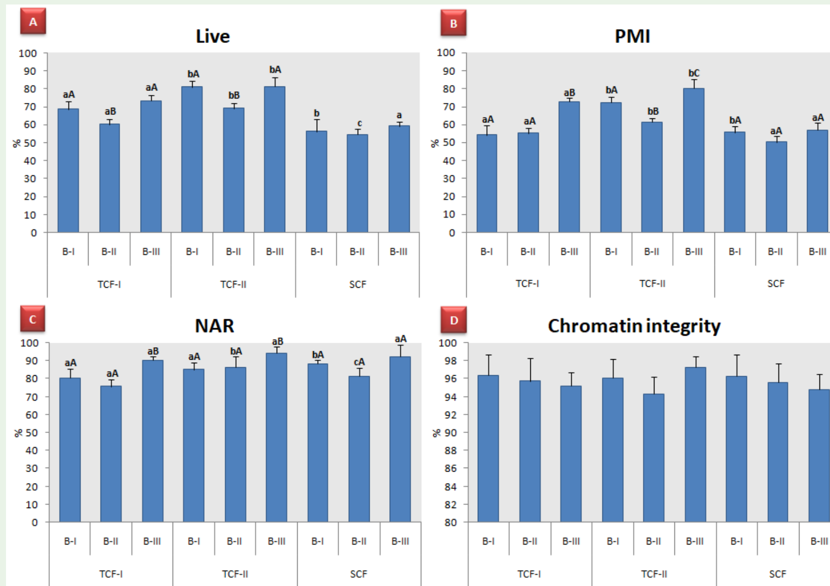

Figure 2: (Experiment 1); The data for live (A), PMI; plasma membrane integrity (B), NAR; normal apical ridge (C) and chromatin integrity (D) of semen diluted in TCF-I (Tris citric acid fructose-I), TCF-II (Tris citric acid fructose-II) and SCF (sodium citrate fructose) extenders of three bulls (B-I, B-II and B-III) after completion of liquefaction; have been presented as LSM which have been analyzed using 3 × 3 factorial model.

(*P*>0.05) in TCF-I and TCF-II extenders while semen of B-III liquefied earlier (P<0.05) in TCF-II than other both extenders. Motility was recorded higher (*P*<0.05) of B-III in both Tris extender; while B-I in TCF-II and SCF extenders.

The results for post-liquefaction live sperm, plasma membrane integrity, normal apical ridge and chromatin integrity have been presented in figure 2. All semen parameters varied (*P*<0.05) due to interaction of extender (TCF-I, TCF-II and TCF-III) and bulls (B-I, B-II and B-III) except chromatin integrity. In overall, B-III presented higher (*P*<0.05) sperm attributes in TCF-I and II extenders followed by B-I while B-II possessed relatively lower (*P*<0.05) live sperm, plasma membrane integrity and normal apical ridge. Collectively, the semen samples diluted in TCF-II possessed relatively higher (P<0.05) live sperm and functionally active plasma membrane; also depicted in lower initial liquefaction time.

Figure 3: (Experiment 2); The post-thaw data for motility (A), Live (B), PMI; plasma membrane integrity (C) and NAR; normal apical ridge (D) of semen processed in three extenders (Ext-I, Ext-II and Ext-III) containing three levels of glycerol (G-3, G-5 and G-7) in each extender; have been presented as LSM which have been analyzed using 3 × 3 factorial model. A probability level of <0.05 was considered for significant. Small letter superscripts (a-c) denote difference (P<0.05) among different extenders within same bull while capital letter superscripts (A-C) denote difference (P<0.05) among three bulls within same extender.

Figure 4: One of the dromedary camel calf born with frozen semen artificial insemination.

Experiment 2

The results for post-thaw analysis of semen samples diluted in three extenders containing three concentrations of glycerol (G-3, G-5 and G-7) have been presented in figure 3. All semen parameters differed ($P<0.05$) due to interaction of type of extender (Ext-I, Ext-II and Ext-III) and concentration of glycerol (G-3, G-5 and G-7). All semen parameters were observed highest ($P<0.05$) in Ext-III containing 7% glycerol (G-7) followed by Ext-II with G-7 and Ext-III with G-5. Motility, live sperm and PMI were recorded lowest ($P<0.05$) in all extenders containing 3% glycerol (G-3). Sperm quality parameters improved ($P<0.05$) with increase of glycerol concentration in extender.

In overall, fifteen females were found pregnant at 30th day of insemination. Six females faced embryonic mortality between 18th and 30th day which were inseminated again. All pregnant females have been confirmed for pregnancy at 60 and 90 days of insemination. Two females aborted at mid gestation. Remaining females were carried to full term. To date, four camels have delivered healthy calves (Figure 4) and remaining are awaited for it.

Discussion

It is very well documented that camel spermatozoa are densely clustered, and the heads of spermatozoa are embedded and tightly secured and some process of liquefaction of semen coagulum releases spermatozoa in batches, which develop motility from oscillatory to progressive one. Due to viscosity of the ejaculated semen, assisted reproductive technologies in camel are lacking or little progress has been achieved [8]. In the present study, both liquefaction time and motility varied due to extender and bull. Liquefaction time was significantly shorter in Tris based extenders compared with SCF extender. Similarly, higher motility, live sperm, plasma membrane integrity and normal apical ridge were recorded in Tris based extenders compared with SC extender. Regarding bull effect, the liquefaction time of B-I and B-II was similar in TCF-I and TCF-II extenders while semen of B-III liquefied earlier in TCF-II than in other two extenders. Likewise, there was bull variation in all the semen quality parameters, except the chromatin integrity which did not differ. Noteworthy, all the extended semen samples liquefied from 38 to 94 min at 33°C, whereas the samples without an added extender (control) failed to liquefy even at 180 min of incubation (33°C). Similar findings were reported in an earlier study by Wani et al [9]. It is put forward; that camel semen can be efficiently liquefied in Tris based extender for further processing.

In experiment 2, the importance of glycerol as cryoprotectant was determined. Glycerol is considered as permeable agent which reduces the crystal formation inner as well as outer of sperm cell, preventing damage to plasma membrane and enhancing viability and function [10]. In the present study, glycerol has been used from lower to bovine optimal concentration in Tris-based extenders. It has been observed that sperm parameters especially motility, viability and plasma membrane integrity was augmented due to increased level of glycerol in semen extender, which is also obvious in other species [11]. It also caused higher sperm attributes when higher concentration was used in tris extender containing lower concentration of fructose. It may be speculated that it could be due to lower level of fructose in camel semen plasma. Dromedary camel sperm could be intolerant to higher fructose concentration. Due to protection of sperm membrane structure and function with higher level of glycerol (7%) this led to increased motility and integrity of plasma membrane. These findings could lead to better development of semen extenders for enhancing post-thawed quality of dromedary camel spermatozoa.

To the best of our knowledge, this is the first study regarding successful frozen semen insemination of dromedary camels which led to live birth of camel calves at our centre. This assisted reproductive technology will lead to preservation of better genetics and later spread in dromedary camels. Previously one early pregnancy was reported by Deen et al [7]. It s worth to mention that in the present study, six of the full term pregnancies were achieved by the semen that was frozen 12-13 months before the inseminations were performed. Live births of camel calves using frozen semen insemination at our center is a way forward to develop and augment the assisted reproductive techniques and may even help to shift the previous routine procedures of embryo transfer by involving frozen semen insemination.

It is concluded that dilution of camel semen with Tris based extenders accelerates the process of liquefaction with improved sperm structures and functions. Supplementation of Tris based extender with 7% glycerol preserves the post-thaw quality and fertility of camel bull spermatozoa. Moreover, the fertility results of present study are very encouraging for the large scale application of assisted reproductive techniques in camelids.

Acknowledgments

This study was generously supported by His Highness Sheikh Hamdan Bin Rashid Al Maktoum, Finance Minister of UAE and Deputy Ruler of Dubai. The authors are thankful to Malik Muhammad Mumtaz for semen collection and assistance in laboratory work.

References

1. Skidmore JA, Morton K, Billah M. Artificial insemination in dromedary camels. Anim Reprod Sci. 2013; 136: 178-186.

2. Malo C, Crichton EG, Skidmore JA. Optimization of the cryopreservation of dromedary camel semen: Cryoprotectants and their concentration and equilibration times. Cryobiology. 2016.
3. Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci. 2000; 60: 481-492.
4. Ahmed H, Andrabi SM, Anwar M, Jahan S. Use of post-thaw semen quality parameters to predict fertility of water buffalo (Bubalus bubalis) bull during peak breeding season. Andrologia. 2017; 49.
5. Bravo PW, Alarcon V, Baca L, Cuba Y, Ordonez C, Salinas J, et al. Semen preservation and artificial insemination in domesticated South American camelids. Anim Reprod Sci. 2013; 136: 157-163.
6. Morton KM, Thomson PC, Bailey K, Evans G, Maxwell WM. Quality parameters for alpaca (Vicugna pacos) semen are affected by semen collection procedure. Reprod Domest Anim. 2010; 45: 637-643.
7. Deen A, Vyas S, Sahani MS. Semen collection, cryopreservation and artificial insemination in the dromedary camel. Anim Reprod Sci. 2003; 77: 223-233.
8. Bravo PW, Skidmore JA, Zhao XX. Reproductive aspects and storage of semen in camelidae. Anim Reprod Sci. 2000; 62: 173-193.
9. Wani NA, Billah M, Skidmore JA. Studies on liquefaction and storage of ejaculated dromedary camel (Camelus dromedarius) semen. Anim Reprod Sci. 2008; 109: 309-318.
10. Forero-Gonzalez RA, Celeghini EC, Raphael CF, Andrade AF, Bressan FF, Arruda RP. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia. 2012; 44: 154-159.
11. Rasul Z, Ahmed N, Anzar M. Antagonist effect of DMSO on the cryoprotection ability of glycerol during cryopreservation of buffalo sperm. Theriogenology. 2007; 68: 813-819.