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Effect of lactose extender with different levels of osmolality and pH on the viability of Bactrian camel (*Camelus bactrianus*) spermatozoa

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Summary

The effect of different levels of osmolality and pH of lactose extender on the viability of Bactrian camel spermatozoa was investigated. Semen was collected from 10 Bactrian camel bulls using modified bovine artificial vagina. In experiment I, the extenders consisted of 9, 10, 11, 12 and 13% lactose with respective osmolalities of 290, 333, 350, 376 and 419 mOsm/kg and the pH of 6.9. In experiment II, 10% lactose extender with different levels of pH = 5.9, 6.9, 7.5, 7.9 and 8.9 were compared. All extenders contained 20% egg yolk and antibiotics. There were three replicates (ejaculates) for each trial. After dilution, semen was evaluated for progressive forward motility (PFM), plasma membrane integrity (PMI) and live percentage of sperm, at time 0, 4, 12 and 24 hrs after incubation at 4°C. Viability of sperm was similar among 9-11% lactose concentrations at time 0 and 4. PFM was compromised (<8%) at 10 and 11% lactose extenders at time 12. Although, PFM was greatest at time 0 (P<0.05) for the pH of 6.9, it reduced to 15% at time 4 (P<0.05). At the pH of 5.9-7.9, PMI and live percentage of sperm reduced at time 12 and 24, respectively (P<0.05). In conclusion, 9-11% lactose with the pH of 6.9 may consider as a suitable extender for the short-term preservation (up to 4 hrs) of Bactrian camel semen maintained at chilled condition (4°C).

Key words: Bactrian camel semen, Lactose, Osmolality, pH, Short preservation

Introduction

Semen preservation is an effective tool to enhance the reproductive performance and to maintain the genetic potential of the male. The progress in semen preservation and related techniques in camelids has been slow in comparison with other livestock species (Bravo *et al*., 2000). Slow progress could be due partly to the lack of proper extender to maintain the viability of spermatozoa. Recently, we have characterized some attributes of Bactrian camel semen (Mosaferi *et al*., 2005), which are determinants for preservation of semen in this species. The requirements of a preservative extender have been elaborated on several occasions (Melrose, 1962; Mann, 1964; Watson, 1979; Stoss, 1983). The composition should take into account the osmolality and pH of the extender. It should also provide an energy substrate, and if intended for cooling spermatozoa, contain substances to protect spermatozoa against the adverse effects of reduced temperature. In addition, extenders commonly contain anti-microbial agents (Steel and Torrie, 1980; Anchordoguy *et al*., 1987; Watson, 1990; Salamon and Maxwell, 1995). The inclusion of sugars in semen diluents performs two main functions (Watson, 1979). Some of them like glucose, fructose, mannose and arabinose can be metabolized by spermatozoa and serve as energy substrate (White *et al*., 1954; O'Dell *et al*., 1959; Mann and Lutwak-Mann, 1981).

Some other sugars such as sucrose, lactose and raffinose, with high molecular weight and low permeability, may only act extracellularly to maintain the osmotic pressure of the extender and to preserve the membrane integrity of the spermatozoa during storage. Therefore, non-permeable sugars are considered as cryoprotectant (Nagase *et al*., 1968; Salamon and Lightfoot, 1969; England, 1993; Salamon and Maxwell, 1995; Salamon and Maxwell, 2000). Lactose has been used on several occasions for preservation of camel semen (Anouassi *et al*., 1992; Hassan *et al*., 1995; Zhao, 2000) without elaborating the appropriate pH and osmolality of this extender. This study was conducted to examine the viability of Bactrian camel semen exposed to different levels of osmolality and pH of lactose extender maintained at chilled condition.

Materials and Methods

The investigation was conducted at the Bactrian Camel Research Center, Jahadabad, Meshkinshahr, Ardabil province, Iran (latitude: 38′ 23″ N; longitude: 47′ 40″ E; altitude: 1568.5 m) during December (2001) to early May (2002). Ten male Bactrian camels, aged 4-12 years, with a sound history of fertility were used for semen collection, once a week, during rutting season. Each camel bull received 7.5 kg alfalfa hay and 2.5 kg mixed concentrate, on the daily basis, including 68% barley, 12% cotton seed meal, 17% wheat bran, 2% molasses and 1% mixed vitamins and minerals. Semen was collected and processed according to the method described previously (Mosaferi *et al*., 2005). After collection, semen was placed inside an incubator set at 37°C. For further processing, 50 ml conical flask equipped with a stainless steel clip (Gem Clips, Penguin, China) was prepared. The conical flask was floated in a separate beaker filled with 37°C water while the neck of the conical flask was maintained with the flask holder. The beaker with the conical flask was placed over the magnetic stirrer, which was set at the very low speed. The whole process was carried out inside the incubator. The semen sample was then pipetted into the conical flask. The initial stirring of the semen sample for 5 min resulted in the reduction of the viscosity, which facilitated further evaluation by providing a liquefied homogeneous specimen.

Lactose (Lactose monohydrate; Merck, Germany) was used as the basic medium to evaluate the effect of different pH and osmolality of the lactose extender on the viability of Bactrian camel semen. Lactose media with different concentrations of 9, 10, 11, 12 and 13% (w/v) were prepared in de-ionized water to produce the osmolalities of 290 (hypotonic), 333 (isotonic), 350 (slightly hypertonic), 376 (moderate hypertonic) and 419 (extreme hypertonic) mOsm/kg H_2O, respectively. Osmolality was measured using an automatic osmometer (Model 13, Roebling, France). The pH of all media was adjusted to 6.9. Ten percent lactose medium was used to make extenders with different pH. The pH of the extenders were measured by an electronic pH meter (Model CG 822, Schott-Geräte, GmbH, Germany) and adjusted to 5.9 (moderate acidic), 6.9 (slight acidic), 7.5 (slight alkaline), 7.9 (moderate alkaline) and 8.9 (extreme alkaline) using 2 M sodium hydroxide (Merck, Germany) or 2 M citric acid (Merck, Germany). All extenders contained 20% egg yolk and antibiotics (penicillin G sodium: 1000 IU/ml; streptomycin sulfate: 1000 µg/ml; Jaber Ibne Hayyan, Iran).

Additional 50 ml conical flasks with the same assembly process described before were prepared for semen dilution. The liquefied homogeneous semen, at the final dilution of 1:10, was pipetted slowly into different prepared extenders while being stirred slowly. This, in turn, resulted in substantial decrease in the viscosity of semen. The viability of spermatozoa was evaluated for progressive forward motility (PFM), plasma membrane integrity (PMI) and live percentage (livability) at the time that diluent was added to the specimen (time 0). Consequently, the diluted semen was transferred into the refrigerator (4ºC) and gradually cooled while it was inside the beaker containing 37ºC water in order to reduce cold shock. The diluted semen

reached 4ºC within 4 hrs at which time evaluation for the viability of semen was performed (time 4). The evaluation process continued at 12 (time 12) and 24 (time 24) hrs after dilution and cooling of the semen.

To determine the PFM of sperm, 10 µl of diluted semen was placed on the microscope slide (76.2 × 24.5 mm; Pearl, China) covered with a coverslip (24 × 24 mm; Menzel-Glaser, Germany) and examined under a phase contrast microscope (Nikon, Japan), at the magnification of 400. The microscope was equipped with warm plate set at 37°C. A single person assessed the percentage of the fast forward progression of 200 sperm subjectively (Comhaire and Vermeulen, 1995). The PMI was assessed using hypo-osmotic swelling (HOS) test (Kumi Diaka, 1993). Fructose solution (70 mOsm/kg H_2O) was prepared on a daily basis and kept at 4°C. Diluted semen (100 µl) was added to the pre-warmed (37°C) fructose solution in a test tube (1 ml), which was incubated for 45 min at 37°C. HOS test was conducted under a phase contrast microscope (Nikon, Japan), at the magnification of 400, to report the percentage of sperm with curled/swollen tail, out of 200 sperm counts. The total number of live and dead spermatozoa was assessed using the eosin B-fast green vital staining technique (Loskutoff, 1999). Twenty microliters of the diluted semen was combined with 10 µl of eosin B-fast green dye. A smear was made and dried immediately using a hair dryer. A total of 200 sperm was counted with phase contrast microscope (×1000) and the percentage of live spermatozoa (livability) was determined. There were three replicates (ejaculates) for each trial.

Statistical analysis

The response variables have a discrete nature with the binomial distribution therefore, all percentage data were subjected to arcsin transformation. Changes in the PFM, PMI and livability over time were analysed for the effects of treatments, time and treatment by time interaction using procedure GLM in SAS/STAT (SAS/STAT, 1993) by either univariate or multivariate analysis with repeated measures analysis included in the model. Multivariate analyses were used where variance and covariance structures over time did not conform to the analysis of variance assumptions, using sphericity test. Between groups differences at any given time, were compared using analysis of variance followed by least squares means using LSMEANS statement within GLM procedure in SAS/STAT (Steel and Torrie, 1980). Data were presented as mean ± SEM.

Results

Just after dilution (time 0), PFM of spermatozoa was similar among osmolalities of 290 (28.5%), 333 (34%) and 350 (31%) mOsm/kg (P>0.05), but significantly higher than those of 376 (13.5%) and 419 (1%; P<0.001; Fig. 1a). After 4 hrs incubation at 4°C (time 4), PFM was reduced in all extenders (P<0.01). However, it was significantly higher for isotonic (18%) and slightly hypertonic media (16%) than that of moderate hypertonic medium (3%; P<0.01). At time 12, PFM decreased in all osmotic conditions (P<0.01).

At time 0, PFM for pH of 6.9 was significantly higher than those of slight, moderate and extreme alkaline extenders (P<0.01; Fig. 2a). At this time, there was no appreciable motility of spermatozoa in moderate acidic pH extender. From time 4, the motility of spermatozoa was suppressed in all extenders (P<0.05). At this time, PFM for slight acidic and slight alkaline extenders were greater than moderate and extremely alkaline extenders (P<0.05).

At time 0, PMI of spermatozoa was greater (P<0.01) for isotonic (65.5%) and slightly hypertonic (65.5%) extenders compared to moderate (52%) and extreme (46%) hypertonic media (Fig. 1b). At time 4, PMI was still higher (P<0.01) for the isotonic (62%) than moderate (47%) and extreme hypertonic (36%) media. From time 12, PMI decreased in all media (P<0.05). At this time, isotonic medium exhibited greater PMI than hypotonic, moderate and extreme hypertonic media (P<0.05). At time 24, isotonic medium (49%) displayed greater PMI compared to hypotonic (20.5%; P<0.0001), moderate hypertonic (35%; P<0.01) and extreme hypertonic (19.5%; P<0.0001) media.

At time 0, PMI was lower for extreme

alkaline (34%) compared to moderate (53%) and slight acidic (59%), slight (58%) and moderate alkaline (48%) media (P<0.001; Fig. 2b). At time 4, pH of 8.9 (11%) exhibited a significant reduction in PMI relative to time 0 (P<0.05) from time 12, PMI reduced in all extenders relative to time 0 (P<0.05).

At time 0, the percentage of live spermatozoa (livability) was different between slightly (69.5%) and extreme (55.5%) hypertonic media (P<0.01; Fig. 1c). Incubation of spermatozoa for 4 hrs at 4°C did not reduce livability (P>0.05). It was greater (P<0.05) for slight hypertonic (65%) compared to moderate (55%) and extreme (56.5%) hypertonic media. At time 12, slightly hypertonic (61%) extender showed greater livability than hypotonic (35.5%) and extreme hypertonic (49.5%) media (P<0.05). At time 24, the sperm livability declined in all extenders compared to time 0 (P<0.01). Isotonic medium (50%) displayed greater sperm livability compared to hypotonic (25%) and extreme hypertonic (36.5%) media (P<0.05; Fig. 1c).

At time 0, livability was similar among various pHs (5.9, 78%; 6.9, 82%; 7.5, 78.5%; 7.9, 69% and 8.9, 76%; P<0.05; Fig. 2c). At time 4, livability declined at slight

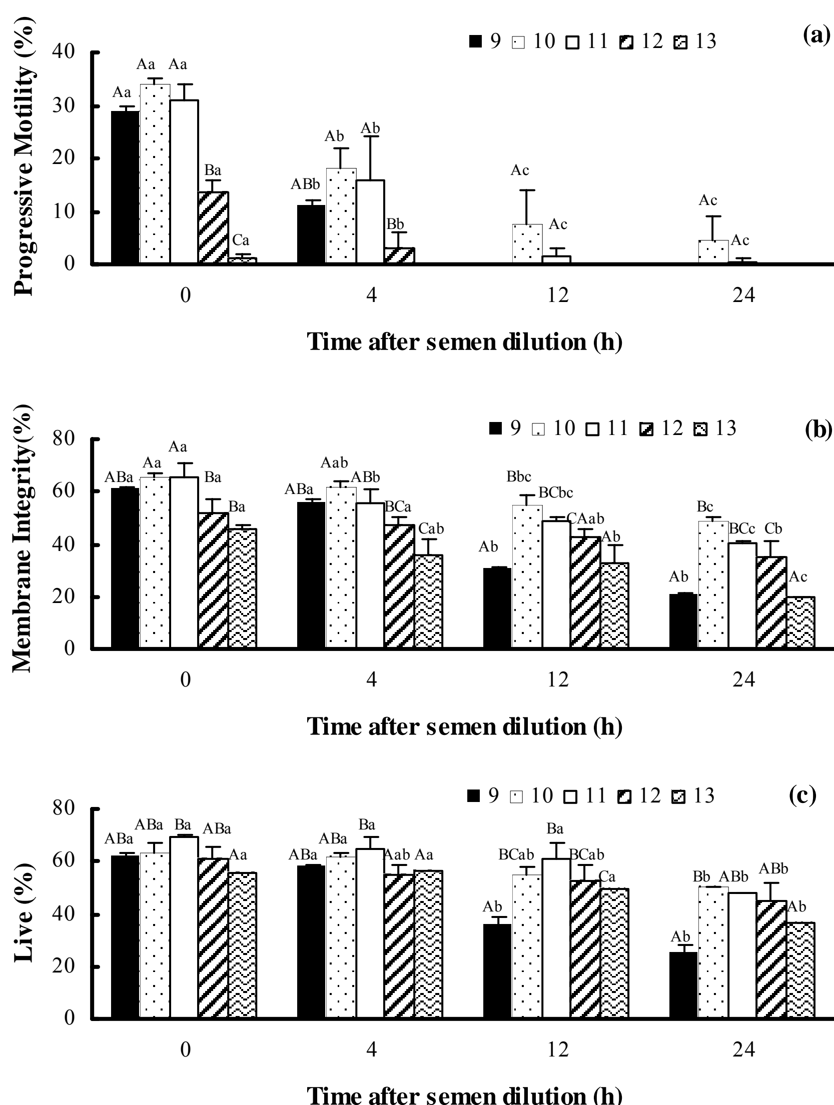

Fig. 1: The effect of lactose extender with different levels of osmolality (290, 333, 350, 376 and 419 mOsm/kg) adjusted to the pH of 6.9, on the progressive forward motility (a), plasma membrane integrity (b) and live percentage (c) of Bactrian camel spermatozoa maintained at 4°C. [abc]Values with different lower-case superscripts indicate significant difference over the time within experimental groups (P<0.05). [ABC]Values with different upper-case superscripts indicate significant difference at any particular time among experimental groups (P<0.05)

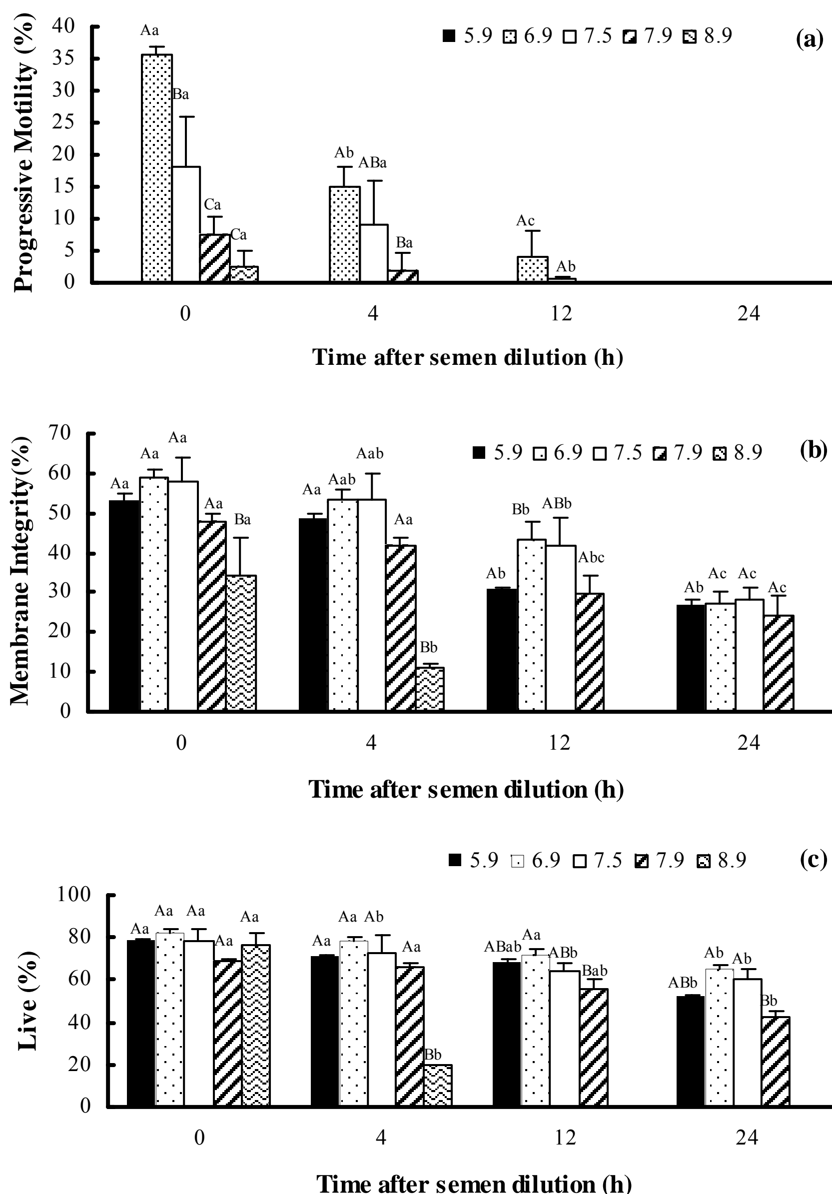

Fig. 2: The effect of pH (5.9, 6.9, 7.5, 7.9, 8.9) of 10% lactose extender on the progressive forward motility (a), plasma membrane integrity (b) and live percentage (c) of Bactrian camel spermatozoa maintained at 4°C. ^{abc}Values with different lower-case superscripts indicate significant difference over the time within pH groups (P<0.05). ^{ABC}Values with different upper-case superscripts indicate significant difference at any particular time among pH groups (P<0.05)

(73%; P<0.05) and extreme (19.5%; P<0.0001) alkaline extenders compared to time 0. At this time, extreme alkaline extender exhibited a significant reduction in livability compared to other pHs (P<0.001; Fig. 2c). At time 12, livability in moderate acidic (67.5%) and extreme alkaline (0%) media significantly reduced relative to time 0 (P<0.01). At this time, livability was greater in slight acidic (72%) than moderate alkaline (55.5%; P<0.05). At time 24, livability reduced compared to time 0 in all extenders (P<0.05). At this time, the sperm livability in moderate alkaline extender (42.5%) was significantly lower than that of slight alkaline (60%) and slight acidic (65%) extenders (P<0.05; Fig. 2c).

Discussion

This study was conducted to evaluate the effect of various levels of pH and osmolality of lactose extender on the viability of Bactrian camel semen preserved at chilled

condition (4°C).

In the first experiment of this study, the effect of different percentages of lactose (9, 10, 11, 12 and 13), with respective osmolalities of 290, 333, 350, 376 and 419 mOsm/kg on the viability of Bactrian camel spermatozoa was investigated. The pH of extenders was adjusted to 6.9. Just after semen dilution (time 0) in 9-11% lactose extenders, the PFM of spermatozoa was greater than other concentrations (28.5-34% vs 1-13.5%; $P<0.05$). This value was lower than that obtained from Green buffer extender in this species (62.4%; Mosaferi *et al*., 2005). The viability of sperm in 9-11% lactose extenders reduced significantly ($P<0.05$), at time 4 for PFM (28.5-34% vs 11-18%), at time 12 for plasma membrane integrity (61-65.5% vs 30.5-54.5%) and at time 24 for livability (62-69.5% vs 25-50%), Compared to time 0. This, in turn, indicates that lactose extender might not be a suitable choice for preserving Bactrian camel semen at chilled condition. This might be one of the reasons that previous researchers decided to inseminate the Dromedary camel females within 15 min after dilution of semen in lactose extender (Anouassi *et al*., 1992). Osmolality of the medium, being hypotonic or hypertonic, markedly affects sperm volume (Bredderman and Foote, 1969) via altering the transfer of water through the membrane, and disrupts the integrity and survival of sperm cell (Bredderman and Foote, 1969; Fiser *et al*., 1981; Gao *et al*., 1993; Kleinhans *et al*., 1993; Curry *et al*., 1994; Gao *et al*., 1995; Liu and Foote, 1998; Watson *et al*., 2001). It has shown that hyperosmotic environment can inhibit sperm motility, and decrease cell volume of sperm (Pommer *et al*., 2002). Either hypotonic or hypertonic extenders will reduce metabolic rate, but neither will extend the life of the spermatozoa (Bearden and Fuquay, 2000). Hypertonic diluents are less harmful than hypotonic diluents (Mann, 1964), perhaps because they result in a degree of dehydration of the cells, which is advantageous during freezing (Watson, 1979). This is the reason why the majority of diluents recommended for preservation of sperm are hypertonic with respect to seminal plasma (Watson, 1979; Fiser *et al*., 1981; Fiser *et al*., 1982). Blackshaw and Emmens (1951) stated that spermatozoal survival was greatest in isotonic diluents. Considering the suggestion that isotonic media may be superior for maintenance of the spermatozoa and the fact that the osmolality of freshly ejaculated Bactrian camel semen has fallen in the range of 300-348 (316.1 ± 1.48) mOsm/kg H_2O (Mosaferi *et al*., 2005), the lactose extender with the osmolality of 290-350 mOsm/kg H_2O (9-11%) seems to be more favorable for preserving Bactrian camel spermatozoa than other osmolalities. However, it should be considered that the significant reduction ($P<0.05$) of sperm viability occurs after 4 hrs incubation at 4°C in this extender.

In the second experiment of this study, the effect of different pH levels (5.9, 6.9, 7.5, 7.9 and 8.9) of lactose extender on the viability of Bactrian camel spermatozoa was investigated. Considering the result of the first experiment of this study as well as the fact that 10% lactose extender (330 mOsm/kg H_2O) has fallen within the range of the osmolality of Bactrian camel semen (300-348 mOsm/kg H_2O), we used 10% lactose extender as the base medium to prepare different levels of pH. PFM of Bactrian camel spermatozoa was totally terminated following immediate exposure of semen to the pH of 5.9. It is documented that lowering the pH of the extender might inhibit the motility of spermatozoa through decreasing its metabolic activity (Holt, 2000). In the present study, the PFM of spermatozoa reduced to less than 8% after semen dilution in extenders with 7.9 and 8.9 pH. This suggests that Bactrian camel spermatozoa may not be able to tolerate high alkaline conditions. Meanwhile, the PFM of spermatozoa was greatest in the pH of 6.9 (35.5%), which is close to that obtained in the first experiment of this study (34%). Green buffer extender has the pH of 6.9 and the osmolality of 330 mOsm/kg H_2O (unpublished data), which is similar to the specifications of 10% lactose extender used in this experiment. However, they exhibited different result even immediately after semen dilution (10% lactose: 35.5% vs Green buffer: 62.4%; Mosaferi *et al*., 2005). This indicates that not only osmolality and pH of the extender but also its nature are determinants on the viability of

spermatozoa. Irrespective of the diluent, dilution rate, temperature or conditions of storage, the spermatozoa deteriorated as the duration of storage increased. The main changes that occur during storage are the reduction in motility and morphological integrity of spermatozoa. These changes may be contributed to the accumulation of the toxic products of metabolism, mainly of reactive oxygen species (ROS) formed through lipid peroxidation of the membranes of spermatozoa. The above events are accompanied by a decline in transport and survival of spermatozoa in the female reproductive tract, and reduction in fertility (Salamon and Maxwell, 2000). Slight acidic (pH = 6.9) medium, similar to ram and bull, provide better protective condition for preservation of Bactrian camel spermatozoa compared to slight (7.5), moderate (7.9) and extreme (8.9) alkaline, and moderate acidic (5.9) extenders. Hassan *et al*., (1995), Rai *et al*., (1997), and Deen and Sahani (2000) used respective pH of 7.3, 6.8 and 6.75 for their proposed semen extender designed for Dromedary camel. These values conform to our data as they are close to the range of 6.9-7.5, which is suitable for preservation of Bactrian camel semen. The pH of a diluent can affect the metabolic rate and motility of spermatozoa (Donoghue and Wishart, 2000). Optimum activity range of the most enzymes in spermatozoa performs at the pH of about 7.0 (6.9 to 7.5 for different species; Bearden and Fuquay, 2000). Therefore, a higher metabolic rate is expected when the pH of semen is maintained near neutrality (7.0). If the pH of semen deviates toward alkalinity or acidity, metabolic rate will be reduced (Bearden and Fuquay, 2000). More recently, we have reported that the pH of the freshly ejaculated Bactrian camel semen was between 7.1 and 7.9 (7.4 ± 0.03; Mosaferi *et al*., 2005). The result of this experiment indicates that slightly acidic medium (pH = 6.9) provides more appropriate condition to preserve Bactrian camel spermatozoa than other media at chilled condition up to 4 hrs. This is in line with the optimum pH of the semen extenders for other mammalian species, where it is adjusted close to neutrality, between 6.9 and 7.1 (Watson, 1990).

Zhao (2000) has proposed a variety of extenders for preservation of Bactrian camel semen at chilled condition, as follows: (a) 12% sucrose and 7% glucose, (b) sucrose and amino acetic acid, (c) lactose and glucose, (d) sucrose and sodium citrate, and (e) glucose and skim milk. This study is suffering from the lack of information on the amount of ingredients and the viability of the spermatozoa exposed to these extenders. Chong (1995) used 12% sucrose (a) and a combination of equivalent volume of 12% sucrose and 6% glucose (b) for preservation of Bactrian camel semen. Likewise, there was no report on the viability of spermatozoa in this study. Hassan *et al*., (1995) reported the effect of three extenders: tris, citric acid, glucose and mannose (buffer A; osmolality: 400 mOsm/kg and pH = 7.3), buffer A plus 1% Orvus Es past (buffer B; osmolality: 400 mOsm/kg and pH = 7.3), and a combination of lactose and fructose (buffer C) on the viability of Dromedary camel semen at chilled condition. Respective rate of sperm motility (type of motility has not been indicated) at the time of dilution, 10 and 24 hrs after dilution were 60, 2 and 0% for buffer A, 60, 0 and 0% for buffer B, and 55, 55 and 0% for buffer C. There was no information on the concentration of ingredients used in this study. It seems very unlikely that the sperm could maintain its initial motility after 10 hrs incubation at chilled condition in buffer C. Anouassi *et al*., (1992) used 11% lactose medium for dilution of semen, and insemination of Dromedary camels after induction of ovulation. The semen was preserved at 37°C and artificial insemination was carried out within 15 min after semen dilution. Pregnancy rates out of ovulated females were 1/2 and 5/6 in two independent studies.

Glancing down reports and articles over Dromedary and Bactrian camel semen would probably convince us that there are no comprehensive studies for determination of osmolality and pH of extenders suitable for preservation of camel semen. In conclusion, the concentrations of lactose extender in range of 9-11% with the pH of 6.9 may be appropriate for short preservation of Bactrian camel semen but they are not appropriate extenders to preserve semen beyond 4 hrs. Therefore, more investigations

are necessary to elucidate an appropriate medium (or media) for preservation of semen in this species.

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References

1- Anchordoguy, TJ; Rudolph, AS; Carpenter, JF and Crowe, JH (1987). Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology. 24(4): 324-331.

2- Anouassi, A; Adnani, M and Raed, EL (1992). Artificial insemination in the camel requires induction of ovulation to achieve pregnancy. *Proceedings of the 1st. International Camel Conference*, Dubai, UAE. PP: 175-177.

3- Bearden, HJ and Fuquay, JW (2000). *Applied animal reproduction*. 5th. Edn., Prentice Hall. PP: 183-207.

4- Blackshaw, AW and Emmens, CW (1951). The interaction of pH, osmotic pressure and electrolyte concentration on the motility of ram, bull and human spermatozoa. J. Physiol., 114(1-2): 16-26.

5- Bravo, PW; Skidmore, JA and Zhao, XX (2000). Reproductive aspects and storage of semen in Camelidae. Anim. Reprod. Sci., 62(1-3): 173-193.

6- Bredderman, PJ and Foote, RH (1969). Volume of stressed bull spermatozoa and protoplasmic droplets, and the relationship of cell size to motility and fertility. J. Anim. Sci., 28(4): 496-501.

7- Chong, S (1995). The order of performing winter artificial insemination in Bactrian camels. Camel Newsletter. 11: 25-32.

8- Comhaire, F and Vermeulen, L (1995). Human semen analysis. Hum. Reprod. Update. 1(4): 343-362.

9- Curry, MR; Millar, JD and Watson, PF (1994). Calculated optimal cooling rates for ram and human sperm cryopreservation fail to conform with empirical observations. Biol. Reprod., 51(5): 1014-1021.

10- Deen, A and Sahani, MS (2000). Preliminary attempts to collect and cryopreserve camel semen. J. Camel Pract. Res., 7(2): 181-186.

11- Donoghue, AM and Wishart, GJ (2000). Storage of poultry semen. Anim. Reprod. Sci., 62(1-3): 213-232.

12- England, GWC (1993). The cryopreservation of dog semen: a review, J. Reprod. Fertil., (Suppl.), 47: 243-255.

13- Fiser, PS; Ainsworth, L and Fairfull, RW (1982). Cryosurvival of ram spermatozoa in hypertonic and isotonic diluents. Can. J. Anim. Sci., 62: 425-428.

14- Fiser, PS; Ainsworth, L and Langford, GA (1981). Effect of osmolality of skim-milk diluents and thawing rate on cryosurvival of ram spermatozoa. Cryobiology. 18(4): 399-403.

15- Gao, DY; Ashworth, E; Watson, PF; Kleinhans, FW; Mazur, P and Critser, JK (1993). Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biol. Reprod., 49(1): 112-123.

16- Gao, DY; Liu, J; Liu, C; McGann, LE; Watson, PF; Kleinhans, FW; Mazur, P; Critser, ES and Critser, JK (1995). Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Hum. Reprod., 10(5): 1109-1122.

17- Hassan, MM; Saeed, M and Ul-Moqtadir, R (1995). Semen collection by artificial vagina and cryopreservation of camel (*Camelus dromedaries*) spermatozoa. Pak. Vet. J., 15(3): 105-108.

18- Holt, WV (2000). Basic aspects of frozen storage of semen. Anim. Reprod. Sci., 62(1-3): 3-22.

19- Kleinhans, FW; Mazur, P and Critser, JK (1993). Osmotic behavior of human spermatozoa studied by ESR. Cryo-Lett., 14: 285-294.

20- Kumi Diaka, J (1993). Subjecting canine semen to the hypo-osmotic test. Theriogenology. 39(6): 1279-1289.

21- Liu, Z and Foote, RH (1998). Bull sperm motility and membrane integrity in media varying in osmolality. J. Dairy. Sci., 81(77): 1868-1873.

22- Loskutoff, NM (1999). Generalized procedures for harvesting sperm post-mortem for cryobanking. In: Loskutoff, NM and Crichton, EG (Eds.), *Standard operating procedure: genome resource banking and assisted reproduction*. The Bill and Berniece Grewcock Center for Conservation and Research Omaha's Henry Doorly Zoo, PP: 8-

16.
23- Mann, T (1964). *The biochemistry of semen and of the male reproductive tract*. 2nd. Edn., London, Methuen, Press. PP: 265-364.
24- Mann, T and Lutwak-Mann, C (1981). *Male reproductive function and semen. Themes and trends in physiology, biochemistry and investigative andrology*. Berlin, Springer-Verlag. PP: 299-306.
25- Melrose, DR (1962). Artificial insemination in cattle. In: Maule, JP (Ed.), *The semen of animals and artificial insemination. Commonwealth agricultural Bureaux*. England, Farnham Royal. PP: 67-88.
26- Mosaferi, S; Niasari-Naslaji, A; Abarghani, A; Gharahdaghi, AA and Gerami, A (2005). biophysical and biochemical characteristics of Bactrian camel semen collected by artificial vagina. Theriogenology. 63(1): 92-101.
27- Nagase, H; Yamashita, S and Irie, S (1968). Protective effects of sugars against freezing injury to bull spermatozoa. sixth international congress on animal reproduction and artificial insemination, Paris, Vol 2, PP: 1111-1113.
28- O'Dell, WT; Almquist, JO and Flipse, RJ (1959). Metabolism of bovine semen. Effect of fructose and arabinose on the uptake and metabolic utilization of glycerol-l-C^{14} by bovine spermatozoa. J. Dairy Sci., 42: 89-93.
29- Pommer, AC; Rutllant, J and Meyers, SA (2002). The role of osmotic resistance on equine spermatozoal function. Theriogenology. 58(7): 1373-1384.
30- Rai, AK; Sharma, N; Mannivannan, B and Khanna, D (1997). Camel semen during breeding and non-breeding seasons. Indian J. Anim. Sci., 67(5): 397-399.
31- Salamon, S and Lightfoot, RJ (1969). Freezing of ram spermatozoa by the pellet method. 1. The effect of diluent composition on the survival of spermatozoa. Aust. J. Biol. Sci., 22: 1527-1546.
32- Salamon, S and Maxwell, WMC (1995). Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci., 37(3-4): 185-249.
33- Salamon, S and Maxwell, WMC (2000). Storage of ram semen. Anim. Reprod. Sci., 62(1-3): 77-111.
34- SAS/STAT, (1993). *User's Guide* (version 6.12) SAS Institute Inc, Cary, NC. PP: 551-640.
35- Steel, RGD and Torrie, JH (1980). *Principles and procedure of statistics a biometrical approach*. 2nd. Edn., McGraw-Hill. PP: 544-545.
36- Stoss, J (1983). Fish gamete preservation and spermatozoan physiology. In: Hoar, WS; Randall, DJ and Donaldson, EM (Eds.), *Fish physiology*. Vol IX, Reproduction PtB, Behavior and Fertility Control. Academic Press, New York. PP: 305-350.
37- Watson, PF (1979). The preservation of semen in mammals. In: Finn, CA (Ed.), *Oxford reviews of reproductive biology*. Oxford University Press. Vol l, PP: 283-350.
38- Watson, PF (1990). Artificial insemination and the preservation of semen. In: Lamming, GE (Ed.), *Marshall's physiology of reproduction*, Vol. 2, *Reproduction in the Male*. Churchill Livingston. PP: 746-869.
39- Watson, PF; Kunze, E; Cramer, P and Hammerstedt, RH (2001). A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. J. Androl., 13(2): 131-138.
40- White, IG; Blackshaw, AW and Emmens, CW (1954). Metabolic and motility studies relating to low temperature storage of ram and bull spermatozoa. Aust. Vet. J., 30: 85-94.
41- Zhao, XX (2000). Reproduction in the Bactrian camel. In: Gahlot, TK and Singh, J (Eds.), *Selected topics on Camelids*. The Camelid publishers, Bikaner. PP: 499-538.