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USE OF COLLAGENASE TYPE-1 TO IMPROVE THE SEMINAL CHARACTERISTICS OF DROMEDARY CAMEL SEMEN

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Abstract: In this study, a total of forty semen ejaculates were collected during the breeding season from eight different stud camels using artificial vagina. All ejaculates were spilt into 3 equal parts of aliquots. One aliquots was kept as control (A1) and two of these were diluted with tris buffer media in 1:1 with (A3) or without (A2) addition of 0.1% collagenase type-1 enzyme and evaluated for macroscopic and microscopic semen characteristics after being kept at room temperature for 20 min. All aliquots were pipetted to observe the macroscopic examination (Consistency and rheological (Thread formation) properties). Aliquot (A3) did not form thread when pipetted and showed thin watery consistency while the other two aliquots (A1 and A2) did evidence thick viscid, thick and thin watery consistency in different proportions. Only aliquot (A3) showed initial individual sperm motility and functional activity (HOST) curled tailed spermatozoa with overall average over 70%. There were significant differences ($p<0.01$) between all the aliquots for sperm motility and sperm with functional membrane where as non-significant differences ($p>0.01$) were observed between all the aliquots for live spermatozoa and sperm abnormalities percentage. An overall mean of sperm concentration in the camel semen treated with collagenase enzyme

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was 331.75 ± 13.71 million/ml. The results showed that treating semen with 0.1% collagenase in tris buffer media improves semen macroscopic and microscopic seminal characteristics and also facilitates the separation of spermatozoa from seminal plasma in dromedary camel semen.

Keywords: Camel, semen, collagenase type-1, tris buffer.

1. INTRODUCTION

Assisted reproductive technologies such as artificial insemination (AI), in vitro fertilization (IVF), embryo transfer (ET) and cryopreservation of gametes allow exchange of genetic material between populations without the need of transporting animals [1]. It also eliminates the problems of behavioral incompatibility, overcomes physical conditions that limit breeding, and reduce opportunities for disease transmission [2, 3]. Artificial insemination (AI) has been the most powerful tool for livestock improvement ever available to the breeder; however, this technique has not been well developed as a routine method for breeding camelids compared with its fast and universal application in other farm animals. The judicious use of artificial insemination (AI) and embryo transfer (ET) could be used to increase overall reproductive efficiency and accelerate genetic improvement in this species.

Interest in the use of artificial insemination in the camel has been stressed [4] and conservation of dromedary camel semen on a global basis attached importance [5]. Opportunities to improve reproductive efficiency of dromedary camels are limited by different constraints such as semen characteristics, long gestation, late sexual puberty and maturity, limited breeding season and the mechanism of estrous cycle and ovulation of she-camel [6]. The highly viscous nature of camel semen is one of the major constraints facing semen packing and freezing process. Vyas [7] had reported an absence of mass motility in freshly collected camel semen ejaculates mainly due to the presence of a thick gel in semen. They had also observed that the motility improved after extension of ejaculates in Tris egg yolk citrate buffer. The absence of initial motility hampers the qualitative evaluation of the semen ejaculate. Various methods of liquefaction of semen, such as the use of enzymes [8] and mechanical stirring [9] have been investigated in camelids with varied success. The results of collagenase enzyme addition in improvement of seminal characteristics have been encouraging in Llama (*Lama glama*) [10].

Success of artificial insemination is dependent on the quality of semen obtained and its capacity for dilution and storage with minimum loss of fertilizing ability. The present study was therefore planned to evaluate the effect of addition of 0.1 % collagenase enzyme type -1(in Tris buffer media) on seminal characteristics of dromedary camel semen.

2. MATERIAL AND METHODS

2.1 Experimental animals

Eight sexually mature male stud camels aged between 5-10 years and stationed at National Research Centre on Camels, Jorbeer, Bikaner were included in the present study. All the stud camels were reared under uniform conditions of feeding, management and housing. All the animals were in good health throughout the study. The brand numbers of the stud camels were B-480, B-592, B-600, J-218, J-242, J-244, J-246, and K-138.

2.2 Experimental design

A total of 40 semen ejaculates from 8 male camels (5 from each) were collected using artificial vagina according to the technique described previously [7,11,12] for the present experiment in order to study the improvement in seminal characteristics by addition of 0.1% collagenase Type-1 enzyme (Sigma chemicals, USA). Each ejaculate was divided into 3 equal aliquots. One aliquot served as control (A1) and each one of the other aliquot was extended 1:1 in both Tris buffer media [13] alone (A2) kept at 37^0 C for 10 min, and Tris buffer media and 0.1% collagenase type -1 enzyme (A3) kept at 37^0 C for 20 min respectively. Macroscopic and microscopic semen evaluations were made for all the 3 aliquots and were computed separately.

2.3 Macroscopic and microscopic semen evaluation

All the three groups (aliquots A1, A2 and A3) of semen were subjected to macroscopic and microscopic evaluation as follows. Macroscopic evaluation included the evaluation of consistency and rheological properties of aliquots. Viscosity of the semen was assessed by pipetting the semen and was graded as thick viscid (T.V.), thick (T), thin watery (T.W.), respectively as per method described by Agarwal [14]. The semen thread was pulled slowly and

thread formation was observed directly by naked eye as per the method described previously [15]. Microscopic evaluation included the evaluation of spermatozoa motility, percent of live sperms, sperm morphological abnormalities, sperm concentration and hypo osmotic swelling test. Initial individual motility of spermatozoa was checked by taking a small drop of semen diluted in either Tris –buffer media or in Tris –buffer media with 0.1% collagenase enzyme on a pre-warmed dry and clean glass slide under high power objective (40X) of the inverted phase contrast microscope (Nikon). It was observed that in the aliquot (A1) spermatozoa were entrapped in the gel so the estimation of motility was not possible. To determine the percent of live spermatozoa in the three groups of semen, the differential staining technique was carried out with the Eosin-Nigrosine stain [16]. The slides prepared for live sperm percentage count were also used for the determination of percent sperm showing morphological abnormalities. The abnormal spermatozoa were classified as per the method described previously [17]. The morphological abnormalities were graded as head abnormalities, mid-piece and tail abnormalities.

Concentration of the spermatozoa (million/ml) in the enzyme treated neat semen was determined by the haemocytometer method adopting WBC counting procedure. The Hypo-osmotic swelling test was performed by mixing 0.1 ml of semen from all the three aliquots with 1.0 ml of a hypo-osmotic solution separately prepared by mixing 0.735 gm of sodium citrate and 1.351gm of fructose in 100 ml of distilled water (incubated for 10 min at 37 ℃) in accordance with a previously described technique [18].

2.4 Statistical analysis

Semen collected from 8 males was replicated 5 times each. Sperm initial individual motility was analyzed using a student paired‘t’ test. Live spermatozoa, morphological abnormalities and curled tail spermatozoa were analyzed using F table ANOVA method.

3. RESULTS

3.1 Influence of 0.1% collagenase Type-1 enzyme addition on macroscopic seminal characteristics.

The consistency of the semen was recorded for all the three aliquot groups. The proportions of grades of consistency recorded are presented (Table-1). The consistency of semen ejaculates improved in A2 group with 80% of the ejaculates evidencing a thick and remaining 20% thin watery consistency. The consistency of semen further improved in the A3 group with no ejaculates evidencing a thick viscid or thick consistency and 100% of ejaculates showing a thin watery consistency. The rheological property (Thread formation) of the semen was recorded for all the three aliquots and it was found that 100% of ejaculates of A2 group showed moderate thread formation, and enzyme treated A3 aliquots showed no thread formation (Table 1).

3.2 *Influence of 0.1% collagenase Type-1 enzyme addition on microscopic seminal characteristics (spermatozoa motility, percent of live sperms, sperm morphological abnormalities, sperm concentration and hypo osmotic swelling test).*

Initial individual motility in control semen aliquot (A1) was examined and it was observed that spermatozoa were entrapped in the gel so the estimation of motility was not possible. Thus a significantly higher ($P< 0.01$) motility could be observed for semen in A3 group compared to A2 group indicating that addition of 0.1% collagenase improves the sperm motility, which is otherwise entrapped in a thick gel in undiluted semen. When the raw data was subjected to statistical analysis (Paired t test), significant difference ($P<0.01$) was found between the initial individual motility percentages of A2 and A3 groups.

Live spermatozoa percentage was computed for all the three aliquots separately (Table2). When the raw data was subjected to analysis of variance (F test), non significant difference ($P>0.01$) was found between the live sperm percentages of group A1, A2 and A3 indicating that addition of either tris buffer alone or tris buffer and 0.1% collagenase did not have detrimental effects on spermatozoa. Similarly morphological abnormalities of head, mid-piece, tail were examined in all the three aliquot groups (Table 2). When the raw data was subjected to analysis of variance (F test), non significant ($P>0.01\%$) difference was found between the total sperm morphological abnormalities of groups A1, A2 and A3 indicating that addition of neither tris buffer nor collagenase increased sperm morphological abnormalities although both treatments increased sperm functions.

The concentration of the spermatozoa (million/ml) in the semen collected from eight stud camels ranged between 150-480 million/ml with an overall mean of 331.75 ± 13.71 million/ml.

Curled tail percentage of spermatozoa was observed for all the three aliquots (Table 2). When the raw data was subjected to analysis of variance (F test), significant difference ($P<0.01$) was found between the sperm hypo osmotic swelling test (curled tail) of control (A1), diluted (A2), and 0.1% collagenase treated (A3) camel semen. The curled tails were highest for the 0.1% collagenase treated camel semen indicating that curled tailed sperms have higher functional activity.

4. DISCUSSION

The camel's semen is highly viscous and forms coagulum soon after copulation [19-21] which presents difficulties in separating sperm cells from seminal plasma by conventional methods to assess the semen quality parameters, especially the motility [22], sperm concentration and morphology which are considered as prerequisites to semen processing. Gel in the camel semen is the main hindrance to the sperm motility and the fibrous network formed by the gel allow little chance for the sperm to move till liquefaction is complete [23, 24]. According to Wani, [25] the viscosity in camel semen does not allow the semen to mix well with the extenders until it is completely liquefied. All the extended semen samples were liquefied with in 1.5 hrs at 37 ℃. However, there was slow liquefaction in the sample without added extender (control). Similarly, Ghoneim [26] studied the effect of extenders (Citrate yolk, Tris fructose yolk, Androhep, Laciphos, Green buffer) on the semen viscosity and sperm viability of the dromedary camel and found a beneficial effect of adding extenders on liquefaction of camel semen and sperm motility.

Different enzymes (Trypsin, collagenase, fibrolysin, hyaluronidase and papain) have been used to decrease seminal plasma viscosity in South American camelids [8, 12, 27, 28]. The favorable outcomes demonstrated by the use of enzymes also proved that the viscous component of seminal plasma was highly susceptible to liquefaction by proteolytic enzymes, thus proving that it has a major protein component. Addition of Tris buffer decreased the rheological properties of semen from a thick to moderate thread formation and addition of 0.1% collagenase

enzyme prevented thread formation. Thus, this treatment improved the semen quality by preventing the thread formation. The results of the present study resemble to those of Giuliano [10] who observed that incubation of semen from one llama male with a solution of collagenase 0.1% in H-TALP-BSA prevented thread formation.

Initial individual motility in control semen aliquot (A1) was examined and it was observed that spermatozoa were entrapped in the gel so the estimation of motility was not possible. Thus a significantly higher ($P< 0.01$) motility could be observed for semen in A3 group compared to A2 group indicating that addition of 0.01% collagenase improves the sperm motility which is otherwise entrapped in a thick gel in undiluted semen.

Non significant difference was found between the live sperm percentages of group A1, A2 and A3 indicating that addition of either tris buffer alone or tris buffer and 0.1% collagenase did not have detrimental effects on spermatozoa viability. The results are in accordance with those of Giuliano [10] who reported that there is no significant detrimental effect of collagenase enzyme on the live spermatozoa percentage in the llama semen. In abnormal spermatozoa percentage non significant differences were observed between the total sperm morphological abnormalities of groups A1, A2 and A3 indicating that addition of neither tris buffer nor collagenase increased sperm morphological abnormalities. Sperm morphological abnormalities are found to be affected by the age of camel stud [23] and are found to be high in old males. Season is also having effect on the percentage of sperm abnormalities of camel semen [29]. Decapitated head, proximal droplets in middle piece and coiled tail are most commonly encountered abnormalities in dromedary semen. Complete liquefaction of gel in the semen by action of proteolytic enzyme collagenase facilitates the release of entrapped spermatozoa from semen by which sperm concentration was estimated in the camel semen. Incomplete and dribbling ejaculation is known to be one reason for variation in sperm concentration in the camel [11].

Significant difference was found between the sperm hypo osmotic swelling test (curled tail) of control (A1), diluted (A2), and 0.1% collagenase treated (A3) camel semen. Collagenase enzyme released the live sperm which are functionally active from the gel in the camel semen.

5. CONCLUSIONS

The current study indicated that treating dromedary camel semen with 0.1% collagenase in tris buffer media improves semen macroscopic and microscopic seminal characteristics and also facilitate the separation of spermatozoa from seminal plasma.

REFERENCES

[1] Durrant, B. S., The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). Theriogenology, 71 (2009):113–122.

[2] Loskutoff, N.M., Role of embryo technologies in genetic management and conservation of wild life. In:Holtz, W.V., Pickard,A.R., Rodger,J.C., Wildt,D.E.(Eds.),Reproductive Science and Integrated Conservation. Cambridge University Press, Cambridge. (2003). pp. 183-194.

[3] Wildt,D.E., Potential application of IVF technology for species conservation. In: Bavister, B.D., Cummins, J.,Roldan. E.R.S. (Eds.), Fertilization in mammals, Serono Symposium. Norwell, (1990); pp:49-364

[4] Purohit, G.N., Biotechnologies in camelid reproduction: Current status and future prospective. J. Camel Pract. Res., 6 (1999): 1-13.

[5] Purohit, G. N. and Pareek, P.K., Research on dromedary reproduction. The past two decades amd future prospective. Vet. Bulletin, 70 (2000):1235-74.

[6] Deen, A., Why artificial insemination in dromedaries is not successful? Proceed of the WBC / ICAR, Satellite Meet. Camelid Reprod., Budapest, Hungary, (2008); pp: 28-32

[7] Vyas, S., Goswami, P., Rai, A.K. and Khanna, N.D., Use of tris and lactose extenders in preservation of camel semen at refrigerated temperature. Indian Vet. J., 75 (1998):810-812.

[8] Bravo, P.W., Skidmore, J.A., Zhao, X.X., Salamon, S. (ed.); Maxwell, W.M.C., Reproductive aspects and storage of semen in Camelidae. Special Issue: Storage of Semen of Domestic Animals. Anim. Reprod.Sci., 62 (2000): 173-193.

[9] Mosaferi, S., Niasari-Naslaji, A., Abarghani, A., Gharahdaghi, A. A. and Gerami, A., Biophysical and biochemical characteristics of Bactrian camel semen collected by artificial vagina. Theriogenology, 63 (2005): 92-101.

[10] Giuliano, S., Carretero, M., Gambarotta, M., Neild, D,. Trasorras, V., Pinto, M. and Miragaya, M., Improvement of llama (*Lama glama*) seminal characteristics using collagenase. Anim. Reprod. Sci., 118 (2010):98-102.

[11] Hassan, M.M., Saeed, M. and Rizwan-ul, M., 'Semen collection by artificial vagina and cryopreservation of camel (*Camelus dromedarius*) spermatozoa', Pakistan Vet. J., 15 (1995): 105-108.

[12] Maxwell, W.M.C., Evans, G. and Morton, K.M., The development of collection, processing and storage technologies for alpaca semen. In: proceedings, International Congress on Animal Reproduction and Satellite Symposium on Camelid Reproduction. (2008); pp: 19-22.

[13] Foote, R.H., Fertility of bull semen at high extensive rates in tris buffered extenders. J. Dairy Sci., 53 (1970): 1475-1477.

[14] Agarwal, V.K., Ram L., Rai A.K., Khanna, N.D. and Agarwal S.P., Physical and biochemical attributes of camel semen. J. Camelid Sci., 1 (2004):25-30.

[15] Vaughan J., Galloway D., Hopkins D., Artificial insemination in alpacas (*Lama pacos*); A report for the Rural Industries Research and Development Corporation. (2003); pp: 67-73.

[16] Campbell, R.G., Hancock J.L. and Rothscild L., Counting live and dead spermatozoa. J. Exptl. Biol., 30 (1953):44.

[17] Blom E.A., One –minute live dead sperm stain by mean of eosin – nigrosin. Fertil Steril., 1 (1950):176-177.

[18] Jeyendran, R.S., Vander ven H.H., Perez-pelaez M., Carbo B.G. and Zaneveld L.J.D., Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fert., 70 (1984):219-228.

[19] Lichtenwalner, A.B., Woods, G.L. and Weber, J.A., Seminal collection, Seminal characteristics and pattern of ejaculation in the Llamas. Theriogenology, 46 (1996):293-305.

[20] Bravo, P.W., Flores, D. and Ordonez, C., Effect of repeated collection on semen characteristics of alpacas. Biol. Reprod., 57 (1997): 520 – 524.

[21] Zeidan, A.E.B., Habeeb, A.A.M., Ahmadi, E.A.A., Amer, H.A. and Abd El-Razik, A., Testicular and physiological changes of the male dromedary camels in relation to different ages and seasons of the year. Proceedings of 2nd International Conference on Animal Production and Health in Semi- Arid Areas, El-Arish, North Sinai, Egypt (2001); pp: 147-160.

[22] Deen, A., Vyas, S., Jain, M. and Sahani, M. S., Explanation of no or low sperm motility in camel semen. Israel J. Vet. Med., 59 (2004): 1-2.

[23] Tibary, A. and Anouassi, A., Theriogenology in Camelidae, Anatomy, Physiology, Pathology and Artificial Breeding. First Edition. Veterinary Research Centre, Ministry of Culture and Information. Abu Dhabi, U.A.E. (1997); pp: 79-113

[24] Aminu Deen and Sahani, M.S., Preliminary attempts to collect and cryopreserve camel semen. J. Camel Pract. Res., 7 (2000): 181-186.

[25] Wani, N. A., In vitro embryo production in camel (Camelus dromedarius) from in vitro matured oocyte fertilized with epididymal spermatozoa stored at 4 ℃. Anim. Reprod. Sci., 111 (2009):69-79.

[26] Ghoneim, I., Al-Eknah M., Waheed M.M., Alhaider A.K., Homeida A.M., Effect of some extenders and enzymes on semen viscosity and sperm viability in dromedary camels. J. Camel Pract. Res., 17 (2010):85-89.

[27] Bravo, PW, Pacheco, C, Quispe, G, Vilcapaza, L and Ordonez, C., 'Degelification of alpaca semen and the effect of dilution rates on artificial insemination outcome', Arch. Androl., 43 (1999): 239-246.

[28] Giuliano SM, Spirito SE, Miragaya MH, Capdevielle EF, Aguero A, Boquet MD, Ferrari MR., Electro ejaculation and seminal parameters in vicuna (*Vicugna vicugna*). Theriogenology, 57(2002):583.

[29] Farrag, A.A.; Serur, B.H. and Gomaa, A.A., Seasonal variations of the epididymal spermatozoa in camels (*Camelus dromedarius*). Assiut Vet. Med. J., 17(1986): 163-166.

Table.1. ***Comparison of macroscopic seminal characteristics (with different grades) in untreated (A1), tris buffer treated (A2) and tris buffer and enzyme treated (A3) camel semen.***

Seminal characteristics	Grades	A 1	A 2	A 3
Consistency	Thin Watery	6 (15%)	8 (20%)	40 (100%)
	Thick	14(35%)	32 (80%)	0 (0%)
	Thick viscid	20 (50%)	0 (0%)	0 (0%)
Thread formation	Thick	19 (47.5%)	0 (0%)	0 (0%)
	Moderate	21 (52.5%)	40 (100%)	0 (0%)
	Nil	0 (0%)	0 (0%)	40 (100%)

Table.2 ***Comparison of microscopic seminal characteristics in untreated (A1), tris buffer treated (A2) and tris buffer and enzyme treated (A3) camel semen***

Microscopic seminal characteristics	A 1	A 2	A 3
Initial individual motility (%)	0	20.25 ± 1.51	71.75 ± 1.28
Live sperm (%)	67.72 ±1.28	70.37 ±1.23	71.72 ±1.33
Morphological abnormalities (%)	10.77 ±0.34	10.8 ±0.36	11.05 ±0.32
Curled tailed (%)	55.77 ±1.99	66.22 ±1.00	73.07 ±1.03