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Evaluation of viability and cytoplasmic droplets in sperm cells harvested from the dromedary cauda epididymis

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Target Audience: *Inseminators, researchers and reproductive physiologists*

Abstract

The objective of the present study was to evaluate viability and cytoplasmic droplets (CD) in camel epididymal sperm cells. Epididymal samples from 7 adult male camels were utilized. The cauda epididymis was immersed in saline, minced and allowed to stand. It was filtered and an aliquot was stained with Eosin-Nigrosin stain. A smear was made on two slides, one each for the left and right epididymides. Twenty different fields per slide were examined using a microscope equipped with an eyepiece camera. The numbers of stained (dead) and unstained (living) cells were counted and reported as paired live-dead counts. Chi-square test for independence was carried out and viability was found to be associated with presence of CD. A reasonable proportion of live sperm cells (1824/2362) were recorded. Live spermatozoa with distal CD were higher in number (969/1824) than those with no droplets (641/1824) and those with proximal CD (214/1824). For the dead spermatozoa, a higher count of spermatozoa with no CD (346/538) followed by those with distal CD (143/538) and lastly those with proximal CD (49/538) was observed. It can be concluded that viability of camel spermatozoa was encouraging; hence, the possibility of its use in assisted reproductive technologies.

Keywords: *Assisted reproduction, cytoplasmic droplets, epididymal sperm, male camel*

Description of the Problem

The camel is gaining popularity in many countries of the world because of its ability to survive well under arid and semi-arid conditions (1). The camel is special in the desert and semi-desert for its ability to survive the severe weather or drought condition by its many and varied physiological mechanisms. Although other ruminants have large quantities of water in their digestive tract that is needed for normal digestive processes their water turnover is far greater than that of the camel (2). This low water turnover enables the camel to graze relatively far from water source (3) and to replenish losses in a very short time (4). Camels not only sustain life on a day-to--day basis for many people living on the fringe of subsistence, but serve as depository of wealth and security against unknown future (5).

The number of spermatozoa stored in the epididymis has been said to be related to the rate of sperm production by the testes, although there are reports in some literature to the contrary (6). Knowledge about gonadal and extragonadal sperm reserves seem to be essential for a careful assessment of male fertility (7). Epididymal sperm has been used in many laboratories because it is easier to get in many species (8). Cryopreserved epididymal sperm is now used for intracytoplasmic sperm injection (ICSI) in human insemination (9,10). Epididymal sperms have been obtained and

individual variations in cryoprotectant toxicities have been studied from African antelope (11). *In vitro* fertilization (IVF) was carried out in goats using epididymal sperms obtained at necropsy (12). Motile and membrane-intact spermatozoa from canine epididymis were successfully stored and recovered for 8 days at 4^{o}C (13). Equine sperm in the epididymis was stored at 4^{o}C for 24, 48, 72 and 96 hours (14). Similar experiments have also been done by (15) on the quality of cauda epididymal ram spermatozoa. On the other hand, IVF and artificial insemination as well as embryo transfer have been used for camelids (16,17,18,19,20). Other researchers (21) revealed that no offspring were obtained from epididymal sperm in South American camelids.

Ejaculated camelid semen has inherent viscosity which leads to low concentration of motile sperm cells. This is an impediment to the application of artificial reproductive technologies (ART) in this species of animal. Hence, the use of epididymal spermatozoa in recent times which could be a likely alternative to ejaculated semen. The current work, therefore, adds to existing knowledge in this regard by evaluating viability and distribution of cytoplasmic droplets in sperm cells harvested from the dromedary epididymis.

Materials and Methods

The study was conducted in the Kano metropolitan abattoir located at Kofar Mazugal. Kano State lies between latitude 9^{o} 30$^{'}$ and 12 ^{o} 30' N and longitude 8 ^{o} 42’ and 9 ^{o} 30' E. It is within the semi-arid Sudan savannah zone of West Africa. Kano is in the dry sub-humid agro-ecological zone of Nigeria (22). It belongs to tropical and dry or Savannah climate as classified by W. Koppen (23). It is about 840 kilometers from the edge of Sahara desert. Kano city and its metropolis is the third largest in Nigeria with population of 9,401,288 according to 2006 census (24).

A total of 7 male camels were used in the study. The camels’ individual age was determined using the dentition method as described by Misk *et al.*(25) with minor modifications. Weight was determined using Indian Army Veterinary Corps method for dromedaries (26). Briefly, the girth (G) was measured from behind the elbow over the ribs cranial to the hump; the length (L) was then measured from the point of shoulder to the caudal aspect of the thigh. Measurements were made in cm and the base weight figure obtained from the conversion table. Where the girth and length of the camel exceeds the figure obtained from conversion table, the highest figure on the table was used. The hump factor was calculated using the formula (A + B)/70, where A is the circumference of the base of the hump (cm) and B is the distance from the lateral base of the hump over its highest point to the base on the opposite side (cm). The ´hump factor' was added to the figure obtained from the conversion table to give the approximate weight in kg.

Scalpel blade was used to make an incision at the median raphe of the scrotal sac. The testes with attached epididymides were pulled outside the scrotum and detached from the spermatic cord. The sample was wrapped in tissue paper and place in Styrofoam box with ice pack to avoid death of sperm cells and then taken to the laboratory.

In the laboratory, the epididymis was separated from the testis. The cauda epididymis was detached from the whole epididymis. It was then immersed in 20 ml of phosphate buffered saline, minced into smaller pieces with scissors, and allowed to stand for 5 minutes to enable sperm cells swim out. The suspension was filtered through soft tissue paper embedded in a plastic funnel. An aliquot of the filtrate was stained by mixing with Eosin-Nigrosin stain and incubated at room temperature for 30 seconds. A smear was made on a glass slide. Two slides were produced,

one each for the left and right cauda epididymides. The slides were examined using a microscope equipped with a camera at a total magnification of ×400. Photographs were taken from 20 different fields per slide. The numbers of stained (dead) and unstained (living) cells were counted for each slide and the total for both slides was reported as paired live-dead counts.

To determine the live-dead ratio, proportion of sperm cells with proximal or distal or no cytoplasmic droplets and the distribution of cytoplasmic droplets according to live-dead categories, counts were analyzed using descriptive statistics. To determine association between viability and occurrence of cytoplasmic droplets, Chi-square test for independence was carried out. The effect of camel bull on viability and occurrence of cytoplasmic droplets was tested using Kruskal-Wallis Test and significant mean ranks were separated using Dunn's multiple comparison test. All statistical tests were carried out using GraphPad Instat Version 3.05 for Windows.

Results

The distribution of live and dead spermatozoa according to cytoplasmic droplets is presented in Table 1. Live spermatozoa constituted 77.22% (1824/2362) of total spermatozoa count while dead spermatozoa accounted for the remaining 22.78% (538/2362). For live spermatozoa, there was a preponderance of distal cytoplasmic droplets (969/1824) followed by those without droplets (641/1824) and finally proximal droplets (214/1824). For dead spermatozoa, higher counts (346/538) were recorded for spermatozoa without cytoplasmic droplets followed by distal droplets (143/538) and lastly proximal droplets (49/538). There was a very highly significant ($P<0.001$; $\chi^2 = 149.35$) association between viability and presence of droplets in camel cauda epididymal spermatozoa.

The summary statistics (including KW statistic) for live cauda epididymal spermatozoa count is presented in Table 2. The result revealed that camel number 1 had a median of 3 with values ranging from 0 to 13 with sum and mean rank of 4402.50 and 110.06, respectively. The sum and mean rank values for camel number 2 were 6663.00 and 166.58, respectively with a median of 6 and values ranging from 1 to 18. Camel number 3 had a median of 2 with values ranging from 0 to 25 with a sum and mean rank values of 3604.50 and 90.11, respectively. Values for camel number 4 ranges from 0 to 21 with a median of 8 with sum and mean rank of 7234.50 and 180.86, respectively. The sum and mean rank values for camel number 5 were 3228.00 and 80.70, respectively, with median of 3 and values ranging from 0 to 12. Camel number 6 had a median of 7 with values ranging from 0 to 26 with sum and mean rank values of 6959.50 and 173.99 respectively. The sum and mean rank values for camel number 7 were 7248.00 and 181.20, respectively, with a median of 8 and values ranging from 0 to 19. There was an extremely significant ($P<0.0001$; KW = 74.441) difference in live cauda epididymal spermatozoa counts among dromedary bulls.

Table 1: Distribution of live and dead spermatozoa according to cytoplasmic droplets

Cytoplasmic droplet	Viability		Total
	Live	Dead	
Proximal	214	49	263 (11.13%)
Distal	969	143	1112 (47.08%)
None	641	346	987 (41.79%)
Total	1824 (77.22%)	538 (22.78%)	2362 (100.00%)

$P<0.001$, $\chi^2 = 149.35$

Table 2: Summary statistics (including KW statistic) for live cauda epididymal spermatozoa count

Camel number	Observations	Median	Minimum	Maximum	Sum of Ranks	Mean of Ranks
1	40	3	0	13	4402.5	110.06
2	40	6	1	18	6663.0	166.58
3	40	2	0	25	3604.5	90.113
4	40	8	0	21	7234.5	180.86
5	40	3	0	12	3228.0	80.700
6	40	7	0	26	6959.5	173.99
7	40	8	0	19	7248.0	181.20

Kruskal-Wallis Statistic (KW) = 74.441 (corrected for ties), $P<0.001$

The effect of camel bull on live cauda spermatozoa counts is highlighted in Table 3. Comparisons among camels shows that there was no significant ($P>0.05$) differences between Camel 1 and Camel 3, Camel 1 and Camel 5, Camel 2 and Camel 4, Camel 2 and Camel 6, Camel 2 and Camel 7, Camel 3 and Camel 5, Camel 4 and Camel 6, Camel 4 and Camel 7 and Camel 6 and Camel 7. However, significant ($P<0.05$) differences were observed between Camel 1 and Camel 2, Camel 1 and Camel 4, Camel 1 and Camel 6, Camel 1 and Camel 7, Camel 2 and Camel 3 Camel 2 and Camel 5, Camel 3 and Camel 4, Camel 3 and Camel 6, Camel 3 and Camel 7, Camel 4 and Camel 5, Camel 5 and Camel 6 and Camel 5 and Camel 7 in live cauda spermatozoa counts.

Summary statistics (including KW statistic) for dead cauda epididymal spermatozoa counts is illustrated in Table 4. The result shows that camel number 1 had median of 1 with values ranging from 0 to 5 with sum and mean ranks of 5703.0 and 142.58, respectively. The sum and mean rank values for camel number 2 were 5120.0 and 128.00, respectively, with a median of 1 and values ranging from 0 to 6. Camel number 3 had median of 0 with values ranging from 0 to 6 with sum and mean of rank values of 4491.5 and 112.29, respectively. Values for camel number 4 ranges from 0 to 12 and a median of 1 with sum and mean ranks of 5967.5 and 149.19, respectively. The sum and mean rank values for camel number 5 were 5687.0 and 142.18, respectively, with a median of 1 and values ranging from 0 to 8. Camel number 6 had a median of 2 with values ranging from 0 to 40 with sum and mean rank values of 6550.5 and 163.76, respectively. For camel number 7, the sum and mean rank values were

5820.5 and 145.51, respectively, with a median of 1 and values ranging from 0 to 5. There was no significant (P>0.05) different in dead cauda epididymal spermatozoa counts between camels.

Table 3: Dunn's multiple comparison tests for the effect of camel bull on live cauda spermatozoa count

Comparison	Mean Rank Difference	P Value
Camel 1 *vs*. Camel 2	-56.513	P<0.05
Camel 1 *vs*. Camel 3	19.950	P>0.05
Camel 1 *vs*. Camel 4	-70.800	P<0.01
Camel 1 *vs*. Camel 5	29.363	P>0.05
Camel 1 *vs*. Camel 6	-63.925	P<0.01
Camel 1 *vs*. Camel 7	-71.138	P<0.01
Camel 2 *vs*. Camel 3	76.463	P<0.001
Camel 2 *vs*. Camel 4	-14.288	P>0.05
Camel 2 *vs*. Camel 5	85.875	P<0.001
Camel 2 *vs*. Camel 6	-7.413	P>0.05
Camel 2 *vs*. Camel 7	-14.625	P>0.05
Camel 3 *vs*. Camel 4	-90.750	P<0.001
Camel 3 *vs*. Camel 5	9.413	P>0.05
Camel 3 *vs*. Camel 6	-83.875	P<0.001
Camel 3 *vs*. Camel 7	-91.088	P<0.001
Camel 4 *vs*. Camel 5	100.16	P<0.001
Camel 4 *vs*. Camel 6	6.875	P>0.05
Camel 4 *vs*. Camel 7	-0.3375	P>0.05
Camel 5 *vs*. Camel 6	-93.288	P<0.001
Camel 5 *vs*. Camel 7	-100.50	P<0.001
Camel 6 *vs*. Camel 7	-7.212	P>0.05

Table 4: Summary statistics (including KW statistic) for dead cauda epididymal spermatozoa count

Camel bull No.	Observations	Median	Minimum	Maximum	Sum of Ranks	Mean of Ranks
1	40	1	0	5	5703.0	142.58
2	40	1	0	6	5120.0	128.00
3	40	0	0	6	4491.5	112.29
4	40	1	0	12	5967.5	149.19
5	40	1	0	8	5687.0	142.18
6	40	2	0	40	6550.5	163.76
7	40	1	0	5	5820.5	145.51

Kruskal-Wallis Statistic (KW) = 10.564 (corrected for ties)

Discussion

The current study evaluated the viability and cytoplasmic droplets in cauda epididymal sperm cells in the dromedary bull. According to (27), percentage live spermatozoa averaged 55%. In the bactrian camel, 5% of ejaculatory sperm were dead while 4.9% were abnormal (28). Semen collected by electro-ejaculation, artificial vagina using teaser and camel dummy constituted 25, 21 and 12% of dead spermatozoa, respectively (29). Also, a proportion of 22, 19 and 11% of abnormal spermatozoa were reported, respectively, for the three methods (29).

Ejaculated camelid semen is highly viscous with low concentration and motility of spermatozoa (30). To efficiently use this semen for artificial reproductive technologies, there is a need to counteract the inherent viscosity which may improve the inherent low sperm motility observed in this species. Some authors have suggested the use of mucolytic agents or substances capable of reducing viscosity to improve on the physical properties and homogeneity of camelid semen (30, 31). In Kano abattoir, an average of 70 camels are slaughtered on a daily basis. A substantial proportion of males from this population could be used to develop an epididymal sperm cryobank for subsequent use in assisted reproduction.

Assisted reproductive technologies comprise AI, *in vitro* fertilization, embryo transfer and cryopreservation of gametes, all of which allow exchange of genetic materials between populations without the need for transporting the animal (32). Semen packing and freezing processes are greatly affected due to high viscosity of the camel semen (33). This implies that in order to use camel semen for assisted reproduction, the viscosity issue must be addressed or an alternative method should be used. Collection of spermatozoa from cauda epididymis is a viable option to preserve genetic material from threatened species and for use in assisted reproduction (34).

Ejaculated camelid sperm have been shown to be less tolerant to freezing and thawing procedures (35), hence liquid storage of sperm may facilitate the development of AI technology in camelids (36). According to Morton *et al*.(36), epididymal alpaca and llama sperm unlike their ejaculated counterparts tolerates liquid storage procedures. This could be as a result of lack of exposure to seminal plasma in epididymal sperm which is abundant in ejaculated sperm. Epididymal alpaca sperm has been reported to tolerate freezing and thawing better than their ejaculated counterparts, remaining approximately 30-40% of their original motility after thawing (35, 37). Also, 35-40% motility was retained by epididymal alpaca sperm when transported overnight to the laboratory or when liquid stored at 4^{o}C for 24 hours and then frozen and thawed (35). The afore-mentioned evidences point to the robustness of epididymal camelid sperm; hence, their recent use as models in the development of semen preservation techniques in alpacas (36). This further exposes the camelid viscous seminal plasma as a major hindrance to the use of assisted reproductive technologies in alpacas and other camelids (36).

In the current study, the proportion of live cauda epididymal spermatozoa was substantial compared to dead ones. This implies good quality of the spermatozoa for possible use in assisted reproduction. Also, in this proportion, majority of spermatozoa were shown to harbor distal cytoplasmic droplets followed by those with no droplets and those with proximal droplets. According to (38), higher proximal cytoplasmic droplets influence spermatozoa quality traits in the bovine bull. Previously, (39) related spermatozoa with high percentage of proximal cytoplasmic droplets to lower motility in humans. There was a significant negative correlation between motility and proximal cytoplasmic droplets (38). According

to (40), mouse epididymal spermatozoa exhibiting progressive motility mostly possess cytoplasmic droplets, whereas those without cytoplasmic droplets were rarely motile. They, therefore, suggested that cytoplasmic droplets have a role in motility development during sperm epididymal maturation. According to the same authors, a complete lack of cytoplasmic droplets could be indicative of defective spermiogenesis in the mouse. (41), however, reported that there is no direct correlation between the percentage of distal cytoplasmic droplets and fertility in boars. Therefore, the preponderance of distal cytoplasmic droplets among live spermatozoa in the current study cannot be fully explained even though there was an extremely significant association between viability and presence of cytoplasmic droplets.

The significant effect of camel bull on viability and occurrence of cytoplasmic droplets in the current study could not be traced to the ages of the camel bulls. There was an inconsistent pattern when age was considered as a likely explanation. Therefore, it is more of an individual difference than age. According to (42), it is commonly usual to find differences between individuals even within the same breeds anytime semen from different males is obtained for experimental purposes.

Conclusions and Applications

From results of the present study, the following conclusions were made:

1. Live spermatozoa with distal cytoplasmic droplets were higher in number than those with no droplets as well as those with proximal droplets.
2. For the dead spermatozoa, those with no cytoplasmic droplets had a higher count followed by those with distal droplets and lastly those with proximal droplets.
3. The proportion of live cauda epididymal spermatozoa was substantial compared to dead ones.
4. Cauda epididymal spermatozoa could have potential application in artificial insemination in the dromedary camel. With the daily volume of slaughter in the Kano abattoir, an epididymal sperm cryobank could be maintained for artificial breeding long after the camel bulls might have been slaughtered. The cryopreserved epididymal sperm could be inseminated into camel cows in case of death by slaughter or natural causes or in the event of incapacitation of a breeding camel bull with high performance traits.
5. The staining method (Eosin-Nigrosin staining) used in this study constitutes a drawback due to the unclear background it presents. This could lead to over- or under-estimation in counts. Therefore, it is suggested that a comparison with other stains that measure viability should be done in future studies.

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