DOI : 10.5958/2277-8934.2020.00033.8 *Vol 27 No 2, p 217-224*

In-vitro CAPACITATION OF SPERMATOZOA AS ASSESSED BY CHLORTETRACYCLINE STAINING IN CAMELS (*Camelus dromedarius*)

M.M. Waheed[1,2], S.M. El-Bahr[3,4], A.K. Alhaider[1], I.M. Ghoneim[1,2] and A.M.A. Meligy[5,6]

[1]Department of Clinical Sciences, [3]Department of Physiology, Biochemistry and Pharmacology (Biochemistry), [5]Central Diagnostic Lab., College of Veterinary Medicine, King Faisal University, P.O. Box 400, Al-Ahsa 31982, Saudi Arabia
[2]Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza 12515, Egypt
[4]Department of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Egypt
[6]Physiology Department, Plant Protection Research Institute (PPRI), Agricultural Research Centre (ARC), Giza, Egypt

ABSTRACT

Twelve ejaculates were collected from 6 adult healthy dromedary camels during the rutting season to study the effect of heparin, caffeine and calcium-ionophore on the induction of capacitation in dromedary spermatozoa. Each semen sample was evaluated (sperm progressive motility % and sperm concentration x 10^6/mL). Nine ejaculates out of twelve were diluted with Shotor buffer to obtain 15 aliquots of 5-10 x 10^6 motile spermatozoa/990µl. Five aliquots were mixed with 10 µl of heparin in concentrations of 0 control, 10 IU (2.5 µl/mL), 25 IU (5 µl/mL), 50 IU (10 µl/mL) and 100 IU (20 µl/mL). Caffeine (10 µl) was added to another 5 aliquots in concentrations of 0 control, 2.5 mM (0.00485g/mL), 5 mM (0.0097g/mL), 10 mM (0.0194g/mL) and 20 mM (0.0388g/mL). The last 5 aliquots were mixed with 10 µl of calcium-ionophore A23187 in concentrations of 0 control, 0.05 mM (3.75 µl/mL), 0.1 mM (7.53 µl/mL), 0.2 mM (14.95 µl/mL) and 0.3 mM (20 µl/mL). All aliquots were incubated at 38ºC in a 5% CO_2 atmosphere and 90% relative humidity for 60 min. Aliquots from replications were taken at 0, 5, 15, 30 and 60 min and evaluated for percentages of sperm motility, live sperm and spermatozoa with reacted acrosomes using eosin nigrosin and Chlortetracycline staining. Results revealed differences in viability indices (VI) between camel semen incubated with calcium-ionophore and both semen incubated with heparin and caffeine. Heparin 100 IU, caffeine 5 mM and calcium-ionophore 0.05 mM were the best capacitating factors. A marked increase existed in B (capacitated and acrosome intact) and AR (capacitated and acrosome reacted) patterns cells accompanied with a large decrease in F pattern (uncapacitated and acrosome intact) cells in aliquots with the capacitating factors than in control. In conclusion, heparin (100 IU), caffeine (5 mM) and calcium-ionophore A23187 (0.05 mM) are convenient capacitating factors for dromedary camels' semen. CTC fluorescent staining technique can be used for assessing capacitation status and acrosome reaction in dromedary camels.

Key words: Caffeine, camel, capacitation, chlortetracycline, heparin

Assisted reproductive technologies such as artificial insemination (AI), embryos transfer (ET) and *in-vitro* embryos production (Torner *et al,* 2003; Skidmore and Billah, 2006; Tibary *et al,* 2007; Wani, 2009) could improve the well-known poor reproductive efficiency of the camel. Several areas of *in-vitro* embryo production in dromedary, including sperm capacitation, need to be resolved before this technology could be used regularly. Capacitation is defined as a series of biochemical and biophysical changes prior to fertilisation (Wani, 2002). There is limited information on *in vitro* induction of capacitation in camels. When mammalian spermatozoa are first released from the male reproductive tract, they are unable to fertilise oocytes immediately, despite being morphologically mature and independently motile (Das Gupta *et al,* 1993). The spermatozoa must undergo a post-release maturation phase termed capacitation which is obligatory for mammalian spermatozoa to attain full fertilising potential (Sebkova *et al,* 2012; Tulsiani and Abou-Haila, 2012; Kwon *et al,* 2014). Chlortetracycline (CTC) fluorescence technique was first used to assess the functional status of mouse spermatozoa (Ward and Storey, 1984). CTC is a fluorescent antibiotic that binds to the surface of sperm cells in a Ca^{2+} dependent manner (Tsien, 1989). CTC staining is currently the assay of choice because it distinguishes 3 different stages of sperm activation; non-capacitated, capacitated acrosome-

SEND REPRINT REQUEST TO M.M. WAHEED email: mmwaheed@kfu.edu.sa

intact and capacitated acrosome-reacted spermatozoa (Rathi *et al,* 2001; Nakai *et al,* 2012). CTC staining has been used previously to assess the capacitation state of spermatozoa in mouse (Saling and Storey, 1979; Ward and Storey, 1984), stallion (Varner *et al,* 1987), bull (Fraser *et al,* 1995), dog (Guerin *et al,* 1999), ram (Paulenz *et al*, 2002) and camels (Crichton *et al*, 2015). However, unfortunately, a clear understanding of how CTC interacts with the sperm surface at the molecular level is lacking and the evaluation of CTC staining has been performed on fixed sperm cells (Rathi *et al,* 2001). Present investigation was done to study the effect of different concentrations and time of incubation of heparin, caffeine and calcium-ionophore A23187 on the *in vitro* capacitation of ejaculated dromedary spermatozoa and to assess this effect by aid of CTC stain.

Materials and Methods

Chemical reagents preparation

A Shotor buffer (Niasari-Naslaji *et al,* 2007) was made by dissolving 2.60 g Tris (Sigma, T8793), 1.35 g Citric acid (Sigma, C1857), 1.20 g Glucose (Sigma, G7528) and 0.90 g Fructose (Sigma, F2543) in 100 ml deionised water with an osmolality of 330 mOsm/kg and pH of 6.9. Heparin concentrations were made by dissolving heparin sodium salt (Sigma, H4784) in deionised water and preparing serial dilutions with final heparin concentrations of 0, 10, 25, 50 and 100 IU. Caffeine concentrations were made by dissolving caffeine powder (Sigma, C0750) in deionised water and preparing serial dilutions of caffeine as 0, 2.5, 5, 10 and 20 mM. Calcium-ionophore A23187 concentrations were prepared by dissolving calcium-ionophore A23187 (Sigma, C7522) in Dimethyl sulfoxide (DMSO; Sigma, D2650) and serial dilutions were done to obtain concentrations of 0, 0.05, 0.1, 0.2 and 0.3 mM. The CTC fluorescence stain was adopted from a previously stated method (Hewitt and England, 1998). A fixative buffer was prepared by dissolving 12.11 g Tris (Sigma, T8793) in 100 ml deionised water. The fixative was made by mixing glutaraldehyde 25% (Sigma, G5882) with the fixative buffer 1:1 (v/v) and the pH was adjusted to 7.4. A CTC buffer was prepared by dissolving 240 mg Tris (Sigma, T8793) and 760 mg sodium chloride (Sigma, S3014) in 100 ml deionised water. The buffer was filtered through Amicon® Ultra-15 Centrifugal Filter Device with 100,000 MWCO (UFC 910024, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) using a swinging bucket rotor at 4,000 x g for 30 min and stored at 4°C. A CTC solution was made by mixing 2 mg of CTC (Sigma, 26430) and 4.4 mg of DL-Cysteine (Sigma, 861677) with 5 ml of CTC buffer. The pH of the solution was adjusted to 7.8 and stored at 4°C.

Camels and semen collection

Six adult healthy camels with sound history of fertility in the herd and an average age of 7 years (range, 5–13 years) were used in this study during the rutting season (December to April; Arthur *et al,* 1985). These males were maintained under standard conditions of feeding and management at the Camel Research Centre, King Faisal University, Saudi Arabia and served as sires for breeding females. Twelve ejaculates were collected (one ejaculate/week) from these camels using electro-ejaculation method (Tingari *et al,* 1986). Quality of semen samples was established by evaluating sperm motility and concentration by the same trained individual using Sperm Vision® 3.5 (Minitube of America, Inc) and NucleoCounter® SP-100TM (Chemometec, Ser. no. 1110-020-03, Gydevang 43, DK-3450 Allerod, Denmark), respectively. Percentages of sperm abnormalities were determined using eosin nigrosin stain (Eosin G, 2% Ref. 15405/0025, miniTüb, GmbH, Germany; Nigrosin, 4% Ref. 15405/0029, miniTüb, GmbH, Germany).

Semen dilution and capacitation

Only semen samples that had sperm progressive motility equal to or more than 50% (9 out of 12) were used. Semen samples and all chemical reagents were put in a water bath at 34°C. Immediately after semen evaluation, semen samples were diluted with an appropriate volume of Shotor buffer to obtain 15 aliquots with a final concentration of 5-10 x 10^6 motile spermatozoa/aliquot (each aliquot was 990 µl).

One aliquot of Sperm-Shotor's suspension of 5-10 x 10^6 spermatozoa (990 µl) was mixed with 10 µl dose of 0 heparin (heparin control) and 4 aliquots were mixed with 10 µl of 1 of 4 concentrations of heparin (10 IU (2.5 µl/mL), 25 IU (5 µl/mL), 50 IU (10 µl/mL) and 100 IU (20 µl/mL). Another aliquot of Sperm-Shotor's suspension was mixed with 10 µl dose of 0 caffeine (caffeine control) and 4 aliquots were mixed with 10 µl of 1 of 4 concentrations of caffeine (2.5 mM (0.00485g/mL), 5 mM (0.0097g/mL), 10 mM (0.0194g/mL) and 20 mM (0.0388g/mL). Also, one aliquot of Sperm-Shotor's suspension was mixed with 10 µl dose of 0 calcium-ionophore A23187 (calcium-ionophore A23187 control) and 4 aliquots were mixed with 10 µl dose of 4 concentrations of

calcium-ionophore A23187 (0.05 mM (3.75 µl/mL), 0.1 mM (7.53 µl/mL), 0.2 mM (14.95 µl/mL) and 0.3 mM (20 µl/mL). All aliquots were incubated at 38°C in a 5% CO_2 atmosphere and 90% relative humidity for 60 min. Aliquots were taken at 0, 5, 15, 30 and 60 min and evaluated for percentage of sperm motility. The viability indices (VI, Change in sperm motility with time; Milovanov *et al,* 1964) were calculated from the following equation:

$$VI = \Sigma [M \times (T-R/2)]$$

where; VI is the viability index, Σ is a sign for the sum total, M is the percentage of sperm motility, T is the time of next determination of motility and R is the time of previous determination of motility.

Moreover, aliquots were taken at 0 and 60 min and percentages of live sperm were determined using eosin nigrosin exclusion technique (Björndahl *et al,* 2003; Cecere, 2014; Agarwal *et al,* 2016) and maturational state was evaluated by CTC staining assay (Wang *et al,* 1995). Aliquots from replications (20 µl) were mixed with an equal volume of eosin solution and 40 µl of nigrosin solution and smeared onto a pre-warm microscope slide (37°C). After drying of the slide, sperm were scored (at least 100 cell/slide) under light microscope (oil-immersion 100x). Live sperm showed no staining and dead cells showed pink colouration. The presence of spermatozoa with a partial colouration were considered as dead cell. In the CTC staining assay, the microscope slide of the CTC stain was prepared by mixing 45 µl of each of replications with 45 µl of the CTC solution and 8 µl of fixative. A droplet (10 µl) of the stained replication was placed on a prewarmed (37°C) microscope slide and a droplet of vectrashield (Vector Laboratories, Peterborough, UK) was added to retard fading of the fluorescence. These droplets were mixed on the slide using a pipette tip, a coverslip was applied and gently compressed using a tissue paper. The coverslip was sealed on the slide by colourless nail varnish (Hewitt and England, 1998). The slides were examined with an Olympus corporation microscope (TH4-200, Tokyo, Japan) equipped with epifluorescence optics (excitation at 405 nm BP filter and CTC fluorescence emission at 455 DM), Olympus optical high pressure mercury burner (BH2-RFL-T3, Ser no. 2206115, Japan) and Thermo plate (MATS-U55RH20, Ser.no. 120997, Tokai Hit Co., Ltd, Japan). The characters of the CTC stained sperms were determined, photographed and described. For each replication, 50 spermatozoa were evaluated.

Statistical analysis

Data are presented as means ± SEM for camel sperm parameters, motility, viability index, percentage of live sperm and sperm staining pattern with CTC. These parameters were compared by t-test using SPSS program, version 24.0 (SPSS, 2016).

Results and Discussion

The initial semen parameters (mean ± SEM) of camels' ejaculate volume, percentage of motile sperm, sperm concentration and percentage of sperm abnormalities were 3.90 ± 0.71 ml, 55.33 ± 2.72, 342.53 ± 86.82 x 10^6/ml and 25.33 ± 1.00, respectively. As shown in table 1, there are significant (P<0.05 - P<0.001) differences in viability indices among camel semen incubated with heparin. Heparin 100 IU resulted in the best result of viability indices of camel semen as a capacitating factor (Table 1). Significant (P<0.05 - P<0.001) differences in viability indices of camel semen incubated with caffeine are shown in table 2. Caffeine 5 mM is the best concentration of caffeine that exerted the highest viability index of the incubated camel semen (Table 2). Table 3 declares that calcium ionophore 0.05 mM is the best concentration resulted in good viability index of camel semen in comparison to the other calcium ionophore concentrations. Significant differences in live sperm percent between the 0 min and 60 min of incubation in all semen aliquots except the part incubated with calcium-ionophore 0.05 mM is shown in table 4. Three CTC fluorescent staining patterns are observed: F pattern with fluorescence is

Table 1. Sperm motility (%) and viability indices of incubated camel semen (n=9) with heparin (mean ± SEM).

Heparin concentrations	Motility %					Viability Indices
	0 min	5 min	15 min	30 min	60 min	
Heparin 100 IU	60.83^{a} ± 2.69	51.67^{a} ± 4.54	48.33^{a} ± 4.54	33.33^{a} ± 3.97	18.33 ± 4.54	2443.75^{a} ± 294.89
Heparin 50 IU	44.17^{ab} ± 6.79	38.33^{b} ± 6.91	35.83^{b} ± 6.89	26.67^{a} ± 5.69	16.67 ± 3.60	1945.83^{ab} ± 386.66
Heparin 25 IU	40.00^{b} ± 4.71	38.33^{b} ± 4.54	35.00^{b} ± 4.93	26.67^{ab} ± 3.70	14.17 ± 3.38	1850.00^{b} ± 258.36
Heparin 10 IU	41.67^{b} ± 4.84	38.33^{b} ± 4.22	30.00^{b} ± 4.41	28.33^{a} ± 3.88	15.00 ± 3.63	1854.17^{b} ± 278.34
Heparin 0 IU (Control)	40.00^{b} ± 7.07	40.00^{ab} ± 7.07	31.67^{b} ± 4.84	22.50^{b} ± 5.12	16.67 ± 3.60	1802.08^{b} ± 343.00

Means with dissimilar superscripts in the same column are significantly different from P<0.05 - P<0.001.

visible over the whole sperm head (uncapacitated and acrosome intact); B pattern with bright anterior head and faint fluorescence in the post-acrosomal region (capacitated and acrosome intact); AR pattern with dull fluorescence stain over the whole sperm head (capacitated and acrosome reacted). The capacitation and acrosome reaction of camel spermatozoa was recorded using CTC pattern at the start of incubation (0 min) and after 60 min in the presence or absence of the capacitating factors. As illustrated in Fig 1, just after the start of incubation (0 min), it was recorded that a marked increase in B and AR patterns cells accompanied with a large decrease in F pattern spermatozoa in aliquots with the capacitating factors than in control aliquots. The percentages of F, B and AR cells at 0 and 60 min were 32.75 and 25.75%, 40.12 and 45.50%, and 22.13 and 28.75%, respectively, in aliquots treated with heparin; 33.60 and 27.75%, 37.75 and 42.25%, and 28.65 and 30.00%, respectively, in aliquots treated with caffeine; 30.75 and 22.50%, 39.15 and 41.38%, and 30.10 and 36.12%, respectively, in aliquots treated with calcium-ionophore; 13.42 and 17.67%, 21.8 and 23.23%, and 12.78 and 17.10%, respectively, in control aliquots (Fig 1).

In the present study, semen parameters of the freshly collected ejaculates differed slightly from the previously reported parameters in dromedary camels. In 14 dromerary ejaculates (Waheed *et al,* 2018), the ejaculate volume, percentages of sperm motility, sperm cell concentration and percentages of sperm abnormalities were 4.72±0.72 ml, 66.11±2.32 %, 268.56±29.10 x 10^6/ml and 18.78±2.67 %, respectively. Higher values of the ejaculate volume and sperm cell concentration (5.4±4.7ml and 520.3±388.2 x 10^6/ml) have been found in 5 ejaculates of dromedaries (Monaco *et al,* 2013). In this study, by using eosin nigrosin stain, The percentages of live spermatozoa decreased significantly between the 0 min and 60 min of incubation period except in one replicate. However, by using Trypan blue and Giemsa stain,

n= number of ejaculates.

Table 2. Sperm motility (%) and viability indices of incubated camel semen (n=9) with caffeine (mean ± SEM).

Caffeine concentrations	Motility %					Viability Indices
	0 min	5 min	15 min	30 min	60 min	
Caffeine 20 mM	50.00^{a} ± 3.33	43.33^{a} ± 2.72	36.67^{aa} ± 2.72	26.67^{a} ± 3.97	11.67^{ab} ± 2.45	1858.33^{a} ± 185.90
Caffeine 10 mM	42.50^{b} ± 4.98	40.83^{ab} ± 4.60	36.67^{ab} ± 5.69	30.00^{a} ± 4.71	11.83^{ab} ± 4.10	1900.83^{a} ± 336.51
Caffeine 5 mM	50.83^{a} ± 4.89	45.83^{a} ± 4.60	36.67^{ab} ± 4.91	29.17^{a} ± 3.58	12.50^{ab} ± 3.31	1960.42^{a} ± 256.01
Caffeine 2.5 mM	45.00^{ab} ± 7.22	40.83^{ab} ± 6.40	34.17^{ab} ± 5.17	26.67^{a} ± 5.18	15.00^{a} ± 4.64	1895.83^{a} ± 331.18
Caffeine 0 mM (Control)	38.33^{b} ± 5.38	34.17^{b} ± 5.43	29.17^{b} ± 3.58	16.67^{b} ± 3.30	6.83^{b} ± 1.94	1293.67^{b} ± 200.61

Means with dissimilar superscripts in the same column are significantly different from P<0.05 - P<0.001.
n= number of ejaculates.

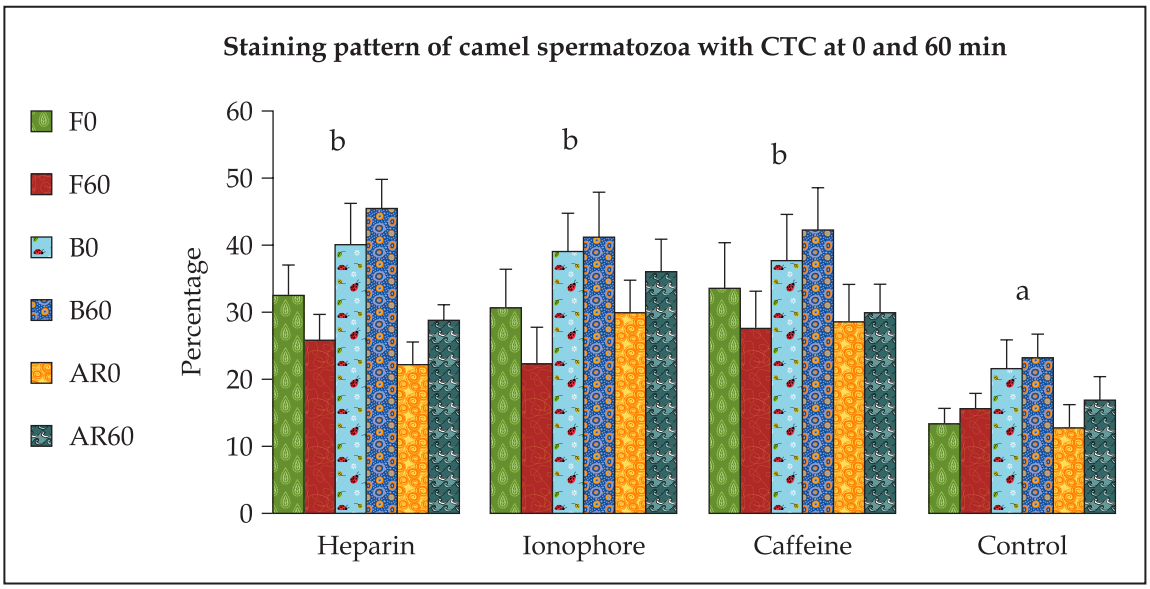

Fig 1. Staining pattern of camel spermatozoa with CTC at 0 and 60 min.

Table 3. Sperm motility (%) and viability indices of incubated camel semen (n=9) with Calcium ionophore (mean ± SEM).

Calcium ionophore concentrations	Motility %					Viability Indices
	0 min	5 min	15 min	30 min	60 min	
Calcium ionophore 0.3 mM	$9.17^{a} \pm 3.05$	$2.50^{a} \pm 1.10$	$0.17^{a} \pm 0.11$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$43.75^{a} \pm 15.42$
Calcium ionophore 0.2 mM	$13.33^{ab} \pm 3.08$	$6.00^{a} \pm 3.14$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$78.33^{a} \pm 29.39$
Calcium ionophore 0.1 mM	$17.50^{a} \pm 4.70$	$4.33^{a} \pm 2.09$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$76.25^{a} \pm 27.00$
Calcium ionophore 0.05 mM	$20.00^{b} \pm 4.71$	$11.67^{b} \pm 4.14$	$0.83^{a} \pm 0.54$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$147.92^{b} \pm 47.21$
Calcium ionophore 0 mM (Control)	$40.00^{c} \pm 4.41$	$33.33^{c} \pm 6.16$	$28.33^{b} \pm 6.10$	$24.17^{b} \pm 5.17$	$7.67^{b} \pm 2.56$	$1477.92^{c} \pm 302.97$

Means with dissimilar superscripts in the same column are significantly different from P<0.05 - P<0.001. n= number of ejaculates.

Table 4. Percentages of live sperm in incubated camel semen (n=9) with the capacitating factors using eosin nigrosin stain (mean ± SEM).

Capacitating factor	Live sperm % 0 min P< 0.05-0.001	Live sperm % 60 min P< 0.05
Heparin 100 IU	$68.78^{Aa} \pm 1.35$	$40.11^{Bac} \pm 4.38$
Heparin 50 IU	$54.11^{Abdgh} \pm 3.68$	$41.89^{Babc} \pm 5.46$
Heparin 25 IU	$60.67^{Adeh} \pm 2.74$	$41.00^{Babc} \pm 3.74$
Heparin 10 IU	$57.22^{Abegh} \pm 2.83$	$39.44^{Babc} \pm 3.96$
Heparin 0 IU	$56.11^{Abdgh} \pm 3.78$	$39.89^{Bac} \pm 4.23$
Calcium ionophore 0.3 mM	$41.11^{Ac} \pm 2.38$	$34.89^{Bab} \pm 2.23$
Calcium ionophore 0.2 mM	$51.11^{Abdgh} \pm 4.69$	$35.44^{Bab} \pm 4.40$
Calcium ionophore 0.1 mM	$49.78^{Acg} \pm 3.23$	$40.67^{Bac} \pm 3.93$
Calcium ionophore 0.05 mM	$49.00^{ch} \pm 3.96$	$36.33^{abc} \pm 6.86$
Calcium ionophore 0 mM	$62.00^{Abdg} \pm 3.23$	$28.33^{Bb} \pm 3.25$
Caffeine 20 mM	$59.67^{Aabdgh} \pm 3.70$	$35.44^{Babc} \pm 4.66$
Caffeine 10 mM	$57.89^{Abdgh} \pm 4.04$	$39.22^{Babc} \pm 4.88$
Caffeine 5 mM	$61.89^{Aabdgh} \pm 4.03$	$40.78^{Babc} \pm 4.90$
Caffeine 2.5 mM	$63.11^{Aabd} \pm 4.81$	$43.44^{Bc} \pm 2.58$
Caffeine 0 mM	$62.56^{Adf} \pm 1.48$	$39.56^{Bac} \pm 3.34$

Means with dissimilar superscript capital letters in the same row of each parameter and means with dissimilar superscript small letters in the same column are significantly different from P<0.05 - P<0.001.

n= number of ejaculates

the addition of calcium-ionophore to the bovine and caprine semen resulted in a significantly improved percentage of live spermatozoa with true acrosome reaction at all stages of incubation (Pereira *et al,* 2000). In the present study, heparin 100 IU and caffeine 5 mM exerted a significant beneficial effect on viability indices of camel semen. However, calcium-ionophore A23187 had very low values of viability indices resulted from the dramatic decrease in sperm motility as no spermatozoa were scored as motile after 15 min of incubation with all calcium-ionophore concentrations, even though approximately 35-40% of the cells were still live at 60 min of incubation. This is might be explained by ionophore A23187 might affect sperm metabolism and has less effect on sperm plasma membrane. Heparin, caffeine and calcium-ionophore have been used in initiation of acrosome reaction in several species including bovine and caprine (Pereira *et al,* 2000). In rams, heparin 250 IU, caffeine 5.15 mM and calcium-ionophore 1.55 mM have been used for *in-vitro* capacitation and acrosome reaction (El-Shahat *et al,* 2016). These concentrations of heparin and calcium-ionophore are much more than the concentrations used in the present study. However, in bovine and caprine, the effect of incubation with heparin, caffeine and calcium-ionophore on sperm motility is negligible (Pereira *et al,* 2000). Similar to the present study, calcium-ionophore has a negative effect on stallion sperm motility during incubation for 3.5 h (Rathi *et al,* 2001). Nevertheless, an alternative means of improving the *in-vitro* fertilising capacity of spermatozoa, in bovine (Byrd, 1981; Jiang *et al,* 1991; Pereira *et al,* 2000), caprine (Shorgan, 1984; Pereira *et al,* 2000), equine (Zhang *et al,* 1991; Rathi *et al,* 2001), dogs (Hewitt and England, 1998), sheep (El-Shahat *et al,* 2016), and mouse (Tateno *et al,* 2013; Navarrete *et al,* 2016) is the use of calcium-ionophore A23187. Concerning caffeine, It inhibits nucleotide-phosphodiesterase that is responsible for cAMP degradation. Hence caffeine treatment induces an increase in intracellular cAMP concentration (Niwa and Ohgoda, 1988). The

Talp Stock medium supplemented with 25.7 mM caffeine induced sperm capacitation and *in-vitro* fertilisation in swine (de Oliveira *et al,* 2011). The addition of caffeine 12.87 mM to the Whittingham's T6 medium containing human serum (T6 + 10% HS) promoted the sperm's motility and vitality and enhanced fertilisation in mouse (Nabavi *et al,* 2013). On using caffeine 5 mM with the universal IVF medium, there is hyperactivation efficacy of frozen bovine semen (Barakat *et al,* 2015). As a result of their hydrophobic characteristics, calcium-ionophores are able to transport ions across membranes. The increase in free calcium-ionophore within the cell directly induces the acrosome reaction and bypasses capacitation (First and Parrish, 1987). The CTC staining patterns (F, B and AR) have been found in human (DasGupta *et al,* 1993), bovine (Fraser *et al,* 1995) and dogs' spermatozoa (Hewitt and England, 1998). In the present study, 3 staining patterns were recorded in camels' spermatozoa and this method could be useful for identification of capacitation and acrosome reaction in this species. In this study, at 0 min of incubation, the percentages of F, B and AR cells in calcium-ionophore treated aliquots were 30.75, 39.15 and 30.10%, respectively and in control aliquots were 13.42, 21.8 and 12.78%, respectively. In stallions, the average percentages of spermatozoa showing F and AR patterns are 50.2±0.8% and 9.5±3.2%, respectively, in samples diluted with Tyrode medium + bicarbonate at 0 min of incubation (Rathi *et al,* 2001). In the present study, the percentages of F, B and AR cells after 60 min were 17.67, 23.23 and 17.10%, respectively in control aliquots and they were 22.50, 41.38 and 36.12%, respectively in calcium-ionophore treated aliquots. In dogs, mean percentages of F, B and AR cells after 1 h incubation were 58.75, 37.25 and 4.00%, respectively in control samples and they were 25, 52.5 and 22.5%, respectively in the ionophore (10 mM) treated samples (Hewitt and England, 1998).

Heparin (100 IU), caffeine (5 mM) and calcium-ionophore A23187 (0.05 mM) are convenient capacitating factors for dromedary camels' semen. CTC fluorescent staining technique can be used for assessing capacitation status and acrosome reaction in dromedary camels, and it may be useful in future studies of *in-vitro* culture and *in-vitro* fertilisation.

Acknowledgements

The authors would like to thank the Camel Research Centre, King Faisal University for support and for approving by the ethical committee.

Deanship of Scientific Research, King Faisal University, Kingdom of Saudi Arabia (Project # 140057) is thanked for providing necessary grants.

References

Agarwal A, Gupta S and Sharma R (2016). Eosin-Nigrosin Staining Procedure. In: Andrological Evaluation of Male Infertility (Agarwal A., Gupta S., Sharma R. eds). Springer. pp 73-77.

Arthur HG, Rahim AT and Hindi A (1985). Reproduction and genital diseases of the camel. British Veterinary Journal 141:650-659.

Barakat IAH, Danfour MA, Galewan FAM and Dkhil MA (2015). Effect of Various Concentrations of Caffeine, Pentoxifylline, and Kallikrein on Hyperactivation of frozen bovine semen. BioMed Research International 7 pages. http://dx.doi.ogr/10.1155/2015/948575.

Björndahl L, Söderlund I and Kvist U (2003). Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Human Reproduction 18(4): 813-816.

Byrd W (1981). *In vitro* capacitation and the chemically induced acrosome reaction in bovine spermatozoa. Journal of Experimental Zoology 215:35-46.

Cecere JT (2014). Eosin-Nigrosin Staining in the Evaluation of Sperm. In: Dascanio JJ, McCue PM (eds). Equine Reproductive Procedures, John Wiley and Sons, Inc.

Crichton EG, Pukazhenthi BS, Billah M and Skidmore JA (2015). Cholesterol addition aids the cryopreservation of dromedary camel (*Camelus dromedarius*) spermatozoa. Theriogenology 83(2):168-174.

Das Gupta S, Mills CL and Fraser LR (1993). Ca^{2+} related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. Journal of Reproduction and Fertility 99:135-143.

de Oliveira VP, Marques MG, Simöes R, Assumpção MEOD and Visintin JA (2011). Influence of caffeine and chondroitin sulfate on swine sperm capacitation and *in vitro* embryo production. Acta Scientiae Veterinariae 39(2):960-965.

El-Shahat KH, Taysser MI, Badr MR and Zaki KA (2016). Effect of heparin, caffeine and calcium ionophore A23187 on *In vitro* induction of the acrosome reaction of fresh ram spermatozoa. Asian Pacific Journal of Reproduction 5: 148-155.

First NL and Parrish JJ (1987). *In-vitro* fertilisation of ruminants. Journal of Reproduction and Fertility Supplement 34: 151-165.

Fraser LR, Abeydeera LR and Niwa K (1995). Ca 21-Regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Molecular Reproduction and Development 40: 233-241.

Guerin P, Ferre M, Fontbonne A, Bénigni L, Jacquet M and MénézoIn Y (1999). *In vitro* capacitation of dog spermatozoa as assessed by chlortetracycline staining. Theriogenology 52:217-228.

Hewitt DA and England GCW (1998). An investigation

of capacitation and the acrosome reaction in dog spermatozoa using a dual fluorescent staining technique. Animal Reproduction Science 51:321-332.

Jiang S, Yang X, Chang S, Heuwieser W and Foote RH (1991). Effect of sperm capacitation and oocyte maturation procedures on fertilisation and development of bovine oocytes *in vitro*. Theriogenology 35:218.

Kwon W, Rahman MS, Lee J, Kim J, Yoon S, Park Y, Hwang YY and Pang M (2014). A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics 15(1):897. doi: 10.1186/1471-2164-15-897

Milovanov VK, Trubkin GD, Chubenko NS, Tsvetkov IV, Erzin ZK and Meschankin AB (1964). Artificial insemination of livestock in the U.S.S.R. Israel Program For Science and Translation, Jerusalem. pp 102-104.

Monaco D, Fatnassi M, Padalino B, Kchira B, El Bahrawy K, Rateb S, Khorchani T, Hammadi M and Lacalandra GM (2013). The experimental semen collection centers for dromedary camels in Egypt and Tunisia: current situation and future developments. In: 11th Congress of the Italian Society of Animal Reproduction, June 19-22. pp 132-136.

Nabavi N, Todehdehghan F and Shiravi A (2013). Effect of caffeine on motility and vitality of sperm and *in vitro* fertilisation of outbreed mouse in T6 and M16 media. Iranian Journal of Reproductive Medicine 11:741-746.

Nakai M, Nagai T, Tanihara F and Kikuchi K (2012). Image processing combined with chlortetracycline staining for assessment of boar sperm capacitation and fertility. Proceeding of Assisted Applied Animal Andrology Conference – Vancouver, Canada. pp 137-144.

Navarrete FA, Alvau A, Lee HC, Levin L, Buck J, Leon PM, Santi CM, Krapf D, Mager J, Fissore RA, Salicioni AM, Darszon A and Visconti PE (2016). Transient exposure to calcium ionophore enables *in vitro* fertilisation in sterile mouse models. 6, doi: https://doi.org/10.1038/srep33589.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gerami A, Gharahdaghi AA, Abarghani A and Ghanbari A (2007). Semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using SHOTOR diluent: effects of cooling rates and glycerol concentrations. Theriogenology 68: 618-625.

Niwa K and Ohgoda O (1988). Synergistic effect of caffeine and heparin on *in vitro* fertilisation of cattle oocytes matured in culture. Theriogenology 30:733-741.

Paulenz H, Söderquist L, Pérez-Pé R and Berg K (2002). Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology 57:823-836.

Pereira RJTA, Tuli RK, Wallenhorst S and Holtz W (2000). The effect of heparin, caffeine and calcium ionophore a 23187 on *in vitro* induction of the acrosome reaction in frozen-thawed bovine and caprine spermatozoa. Theriogenology 54:185-192.

Rathi R, Colenbrander B, Bevers MM and Gadella BM (2001). Evaluation of *in vitro* capacitation of stallion spermatozoa. Biology of Reproduction 65:462-470.

Saling PM and Storey BT (1979). Mouse gamete interactions during fertilisation *in vitro*: chlortetracycline as fluorescent probe for the mouse sperm acrosome reaction. Journal of Cell Biology 83:544-555.

Sebkova N, Cerna M, Ded L, Peknicova J and Dvorakova-Hortova K (2012). The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens. Reproduction 143:297-307.

Shorgan B (1984). Fertilisation of goat and ovine *in vitro* by ejaculated spermatozoa after treatment with ionophore A23187. Bull Nippon Veterinary Zoology College 33: 219-221.

Skidmore JA and Billah M (2006). Comparison of pregnancy status in dromedary camels (*Camelus dromedarius*) after deep intra-uterine *versus* cervical insemination. Theriogenology 66:292-296.

SPSS. Statistical Package for Social Science (2016). SPSS Inc, Chic, IL, USA Copyright© for Windows; version 24.0.

Tateno H, Krapf D, Hino T, Sánchez-Cárdenas C, Darszon A, Yanagimachi R and Visconti PE (2013). Ca^{2+} ionophore A23187 can make mouse spermatozoa capable of fertilising *in vitro* without activation of cAMP-dependent phosphorylation pathways. Proceeding of National Academic Science USA 110(46):18543-18548. doi: 10.1073/pnas.1317113110

Tibary A and Anouassi A (1997). "Management of reproduction in camelidae". In: Tibary A, ed, Theriogenology in Camelidae: Anatomy, Physiology, BSE, pathology and artificial breeding: actes editions. Institut Agronomique et Veterinaire Hassan II. pp 459-476.

Tibary A, Anouassi A, Sqhiri A and Khatir H (2007). Current knowledge and future challenges in camelid reproduction. Society of Reproduction and Fertility Supplement 64:297-313.

Tingari MD, Manna MM, Rahim AT, Ahmed AK and Hamad MH (1986). Studies on camel semen. I. Electroejaculation and some aspects of semen characteristics. Animal Reproduction Science 12:213-222.

Torner H, Heleil B, Alm H, Ghoneim IM, Srsen V, Kanitz W, Tuchscherer A and Fattouh EM (2003). Changes in cumulus-oocyte complexes of pregnant and non pregnant camels (*Camelus dromedarius*) during maturation *in vitro*. Theriogenology 60:977-987.

Tsien RY (1989). Fluorescent indicators of ion concentrations. In: Taylor DL, Wang YL, eds, Fluorescence Microscopy of Living Cells in Culture. Part 6B. Quantitative Fluorescence Microscopy—Imaging and Spectroscopy. Methods Cell Biol., New York: Academic Press; Vol 30, chapter 5. pp 127-156.

Tulsiani DRP and Abou-Haila A (2012). Biological Processes that Prepare Mammalian Spermatozoa to Interact with an Egg and Fertilise It. Scientifica (Cairo) doi: 10.6064/2012/607427

Varner DD, Ward CR, Storey BT and Kenney RM (1987). Induction and characterisation of acrosome reaction in equine spermatozoa. American Journal of Veterinary Research 48:1983-1989.

Waheed MM, Meligy AMA and Dhalam SA (2018). Determination of some trace elements in seminal

plasma and serum of camels (*Camelus dromedarius*) and their correlation to fertility. Reproduction in Domestic Animals 53:1367-1374.

Wang WH, Abeydeera LR, Fraser LR and Niwa K (1995). Functional analysis using chlortetracycline fluorescence and *in vitro* fertilisation of frozen-thawed ejaculated boar spermatozoa incubated in a protein-free chemically defined medium. Journal of Reproduction and Fertility 104(2):305-313.

Wani NA (2002). *In vitro* maturation and *in vitro* fertilisation of sheep oocytes: Review. Small Ruminant Research 44:89-95.

Wani NA (2009). *In vitro* embryo production in camel (*Camelus dromedarius*) from *in vitro* matured oocytes fertilised with epididymal spermatozoa stored at 4 degree C. Animal Reproduction Science 111:69-79.

Ward CR and Storey BT (1984). Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Developmental Biology 104:287-296.

Zhang JJ, Muzs LZ and Boyle MS (1991). Variations in structural and functional changes of stallion spermatozoa in response to calcium ionophore A23187. Journal of Reproduction and Fertility Supplement 44:199-205.