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CHARACTERIZATION OF RHEOTAXIS OF CAPACITATED AND NON-CAPACITATED BUFFALO BULLS SPERM USING MICROFLUIDICS

AMANY M. ABD EL-KAWY; HAITHAM A. MOFADAL; MOHAMED SAMY YOUSEF AND TAYMOUR M. EL-SHERRY

Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

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ABSTRACT

The present study aimed to determine the impact of sperm capacitation on sperm rheotaxis and sperm kinematics within microfluidics of buffalo cryopreserved semen. semen straws (n = 24) were obtained from 3 fertile buffalo bulls. Each straw was divided into three aliquots: control aliquot, caffeine aliquot and heparin aliquot. Sperm rheotaxis and all sperm kinematics were determined through a computer-assisted sperm analysis (CASA) system using the microfluidic platform with controlled flow velocity. The results showed that the positive rheotaxis % (PR%) of caffeine and heparin were significantly higher (PR% = 49.4±1 and 48.8±1, respectively) than control (45.1±1) ($p<0.0001$). Sperm kinematics show a significant variation among the three aliquots. Results show no significant differences in VCL of caffeine and heparin compared to control. While VCL of caffeine aliquot was significantly higher than that of heparin ($p=0.01$). The VAP of the caffeine aliquot was significantly higher than that of heparin and control ($p=0.05$). VSL and BCF of caffeine and heparin aliquots were significantly higher than control ($p<0.001$ and $p<0.0001$, respectively). In conclusion, this study confirms that PR% and most sperm kinematics in buffalo frozen semen improved during invitro capacitation using caffeine and heparin.

***Key words**:* Sperm rheotaxis, sperm kinematics, CASA, Bovine, microchannels.

INTRODUCTION

In the past few decades assisted reproductive technologies have been improved to produce a large number of embryos (Camargo*et al.*, 2018; Verhaak *et al.*, 2007). Mammalian sperm need two essential steps in order to achieve fertilization, the first one being epididymal maturation that occurs inside the male epididymis (Cooper, 1995). The second step is capacitation that takes place after ejaculation within the female reproductive tract (Boué *et al.*, 1996; Sullivan, and Saez, 2013; Töpfer-Petersen *et al.*, 2005). Sperm capacitation was first detected by Chang (Chang, 1951) and Austin (Austin, 1952). It was first observed in rabbits (Chang, 1959) and later in humans (Steptoe, and Edwards, 1978). Their studies were vital to the future improvement of the in vitro fertilization techniques. Sperm Capacitation refers to the

Corresponding author: Amany M. Abd El-Kawy
E-mail address: amanymoatamed34@gmail.com
Present address: Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

functional changes that render the sperm cell capable of fertilization. These adaptations include the ability of sperm to bind with zona pellucida and ovum extracellular matrix (Saling *et al.*, 1978; Si and Olds-Clarke, 1999; Topper, 1999) acrosomal reaction (Florman and First, 1988; Saling, 1979), start the hyperactivation, as whiplash flagellar movement is important for sperm to penetrate the ovum (Ho and Suarez 2001) and sperm ability to combine with the egg (Evans and Florman, 2002). At the molecular level, the capacitation process leads to the loss of sperm plasma membrane cholesterol resulting in increased membrane fluidity, changes in concentrations of intracellular ions (Visconti, 2011), hyperpolarization of the sperm plasma membrane (Hernández-González, 2006), increased protein kinase activity (Krapf *et al.*, 2010), and protein tyrosine phosphorylation (Arcelay *et al.*, 2008). Many reagents can be used for capacitation induction in vitro such as Heparin, superoxide anion, bicarbonate, adenosine, and caffeine (Breininger, *et al.*, 2010), pentoxifylline, and kallikrein (Barakat, *et al.*, 2015; Maxwell, *et al.*, 1995). The long-lasting riddle in natural reproduction is how sperm swims and guided inside the female genital tract. There are three mechanisms for guiding sperm towards the oocyte: thermotaxis, chemotaxis and rheotaxis (Gaffney *et al.*, 2011; El-Sherry *et al.*, 2014). Sperm rheotaxis, which refers to the affinity of sperm to orient and navigate with or against the flow of the surrounding media. Sperm rheotaxis become essential to understand the journey of the mammalian sperm inside the female genital tract to reach the ovum at the fertilization site (El-Sherry *et al.*, 2014; Kantsler *et al.*, 2014; Miki and Clapham, 2013). Oviductal secretion plays different roles in fertilization such as clearing debris from the oviduct, lowering the viscosity of the fluid in the reproductive tract and creating the fluid current that guides sperm cells towards the oocyte (Miki and Clapham 2013). Rheotaxis motion has been studied in capacitated and non-capacitated sperm in high and low viscosity media through quantifying sperm head rolling rate (Miki and Clapham, 2013; Woolley, 2003). CatSper channels and Ca^{2+} influx are very important for sperm rotation (Miki, and Clapham, 2013). Caffeine was found to increase sperm numbers attached to the zona pellucida when it was used as a capacitation agent (Vandevoort *et al.*, 1994). Although sperm capacitation has been extensively investigated, there are no studies assessing the impact of sperm capacitation on sperm rheotaxis. This study was designed to determine the sperm rheotaxis in invitro capacitated sperm cells using caffeine and heparin inside microfluidic platform.

MATERIALS AND METHODS

1. Chemicals and media

Unless otherwise specified, all chemicals used in this study were purchased from Elgomhoria Pharmaceuticals (Assiut, Egypt). Magnesium chloride hexahydrate ($MgCl_2$ ($6h_20$)) and calcium dichloride ($CaCl_2$) were obtained from Alpha Chemika (India), Hepes from gibico (USA), phenol red from ALPHA Chem (India), Na lactate from oxford (India), gentamycin sulfate from Epico (Egypt), Bovine serum albumin from Hi Media (India), Na pyruvate from SDFCL (S D fine-chem limited) (Mumbai, India) caffeine from AppliChem GmbH (Darmstadt, Germany) and Heparin from pharmaceuticals and chemical (Egypt).

2. Experimental design and semen preparation

Frozen semen straws (n=24) were purchased from the directorate of veterinary medicine in Assiut Governorate from 3 fertile buffalo bulls (8 straws for each bull obtained from 4 different ejaculates).Two straws of the same bull were thawed in a water bath at 37 ° C for 30 s and pooled in an epindorf tube then, divided into 3 equal parts, each part was diluted with either invitro fertilization medium (IVF) or capacitation medium (IVF medium + capacitation agent) in 1:3 ratio, respectively. IVF medium comprises (TL-HEPES Stock, BSA, Na Pyruvate and Gentamicin sulfate) (Tríbulo P. *et al.*, 2019).

Group	Treatment
Control	IVF medium only
Caffeine	Capacitation media (0.125 mM caffeine)
Heparin	Capacitation media (20 µl/ml heparin)

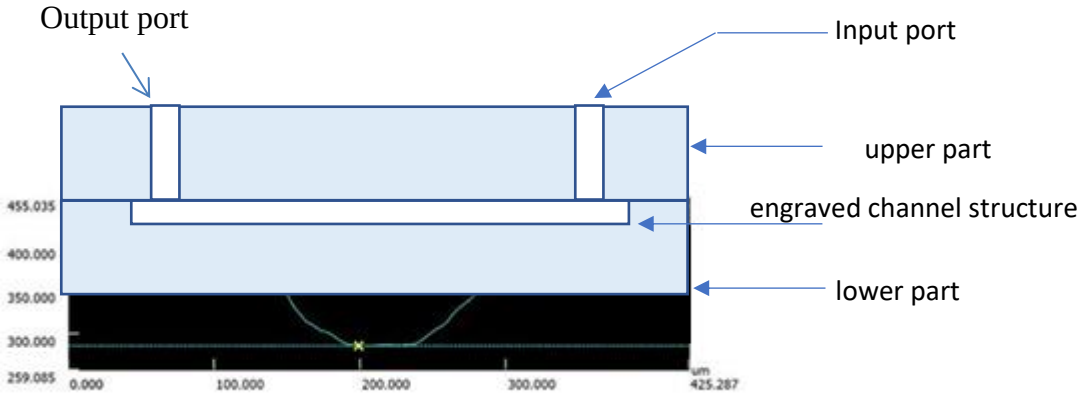
After mixing the 3 aliquots (control, caffeine, and heparin) were incubated in co_2 incubator at 38℃ and 5% co_2 for 45 minutes (Majeed A.F. *et al.*, 2019). All aliquots were examined for sperm rheotaxis% and sperm kinematics using CASA.

3 Microchannel fabrication

The chip is composed of 2 parts of Poly Methyl Methacrylate (PMMA), the lower part includes the engraved channel structure, and the upper part includes inlet ports, as shown in Fig1 (a). The created channel was made by direct write laser machining technique. VLS3.5 UNIVERSAL LASER SYSTEMS with a 30-watt CO_2 laser tube and 100µm laser beam diameter was used for channel fabrication. We found the best carving by adjusting the carving speed to 25 mm s^{-1} (10%) laser head translation speed and laser beam power to 5-watt (6%) laser beam power to produce more/less roughness at lowest available dimensions. The channel profile is Gaussian shape as shown in Fig1 (b). The fusion of the upper part and the lower part of the device was made by thermo-compression technique with acetic acid at 115℃ and 1 N for 7 min. By heating with acetic acid, better bonding at lower temperatures was achieved as well as bonding time (Nasser G. A. *et al.*, 2019).

(a)

(b)



4. Flow generation

The flow of liquid in the microchannel was induced by hydrostatic pressure. The level of liquid in the inlet of the microchannel was kept higher than that in the output reservoir by a height different Δh. Hydrostatic flow generation is of low-cost, simple and available method to produce flow inside microchannels and get rid of pulsating flow that is typical to syringe pumps (Moscovici M. *et al.*, 2010)

(1) $$Vav = \frac{(2\rho g Dh \Delta h)}{C\mu L}$$

where ρ is the liquid density, g is the gravitational acceleration, Dh is the channel hydraulic diameter, Δh is the height difference between reservoirs, C = friction factor (f) × Reynolds number (Re), µl is the liquid viscosity and L is the microchannel length (Munson, *et al.*, 2002). The velocity profile inside the channel was calculated using equation (2) for channels with an aspect ratio less than 0.5 (Shah, *et al.*, 1978)

(2) $$\frac{V}{V_{av}} = \left(\frac{m+1}{m}\right)\left(\frac{n+1}{n}\right)\left[1 - \left(\frac{y}{b}\right)^n\right]\left[1 - \left(\frac{z}{a}\right)^m\right]$$

where V is the liquid velocity at any location in channel a, and b is the channel width and height, respectively. y and z are the coordinates (measured from the centerline) of

any point in the channel where V is required, and *m* and *n* are numerical parameters dependent on the channel aspect ratio α = *b/a* according to equations (3) and (4)

$$m = 1.7 + 0.5\alpha^{-1.4} \quad (3)$$

$$n = 2 + 0.3(\alpha - 1/3) \begin{cases} \alpha < \frac{1}{3} \\ \alpha \geq \frac{1}{3} \end{cases} \quad (4)$$

n=2

The average liquid velocity in all experiments reported here was kept at 32 which mimics the natural fluid flow velocity inside the female genital tract (Tung C. *et al.*, 2014).

5. CASA image analysis

Sperm rheotaxis and sperm kinematics were assessed using a home-made computer-assisted sperm analysis (CASA) system (Department of Mechanical Engineering, Faculty of Engineering, Assiut University, Egypt; the plugin can be downloaded from the following URL:http://www.assiutmicrofluidics.com/research/casa) (Elsayed, *et al.*, 2015). Videos of sperm cells were taken with an Optika XDS-3 inverted microscope with phase contrast (also at 40 × objectives) coupled to a Tucsen ISH1000 camera at 30 frames per second. Recorded videos were processed using a home-developed CASA and the following parameters were determined: velocity parameters which include curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and average path velocity (VAP, µm/s). progression parameters such as linearity (LIN = VSL/VCL, %) and beat cross frequency (BCF, Hz).

6. Statistics

All data were analyzed by one-way analysis of variance (ANOVA), to detect the difference between the three groups (control, caffeine, and heparin). Data of positive rheotaxis sperm, VCL (µm/s), VAP(µm/s), VSL (µm/s), LIN (VSL/VCL) and BCF(HZ) are expressed as mean ± SEM.

RESULTS

1. Sperm positive rheotaxis

The results showed that positive rheotaxis percentage PR% was significantly higher in both caffeine and heparin-capacitated sperm samples (PR% = 49.4±1 and 48.8±1, respectively) than in control (45.1±1), Table 1 and Fig2 (a).

2. Kinematics of positive rheotactic sperm cells

Two categories of CASA parameters were used for sperm kinematics. The first one was velocity parameters that include VCL, VAP and VSL and the second category was progression parameters which include linearity and BCF. For velocity parameters: the results revealed that caffeine VCL was significantly higher than heparin while there was no significant difference in VCL between caffeine and heparin aliquots and control samples. On the other hand, the VAP of caffeine aliquot was significantly higher than both those of heparin and control. Also, VSL was significantly higher in caffeine and heparin-capacitated sperms in comparison with control as shown in Table 1, Fig2 (b, c, and d). For progression parameters, there was no significant difference in linearity between groups, while BCF was significantly higher in caffeine and heparin aliquots than control. as shown in Table 1, Fig 2 (e and f).

Table 1: Sperm number (N), the positive rheotaxis % (PR), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN=VSL/VCL × 100) and beat/cross-frequency (BCF) in capacitated sperms using caffeine and heparin VS control. Data represented in mean ± SEM. Different litters indicate significance at P < 0.05.

	N	PR%	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (VSL/VCL%)	BCF (Hz)
Control	5198	45.1±1[b]	21.5±0.2	21.3±0.2[b]	17.6±0.1[b]	0.8±0.01	1.6±0.02[b]
Caffeine	8852	49.4±1[a]	21.9±0.2	21.9±0.2[a]	18.1±0.1[a]	0.8±0.01	1.7±0.03[a]
Heparin	6172	48.8±1[a]	21.1±0.2	21.2±0.2[b]	18±0.1[a]	0.85±0.01	1.7±0.02[a]
P Value		<0.0001	0.01	0.6	<0.001)	0.2	<0.0001

-Super-scribed letters in columns are significantly different

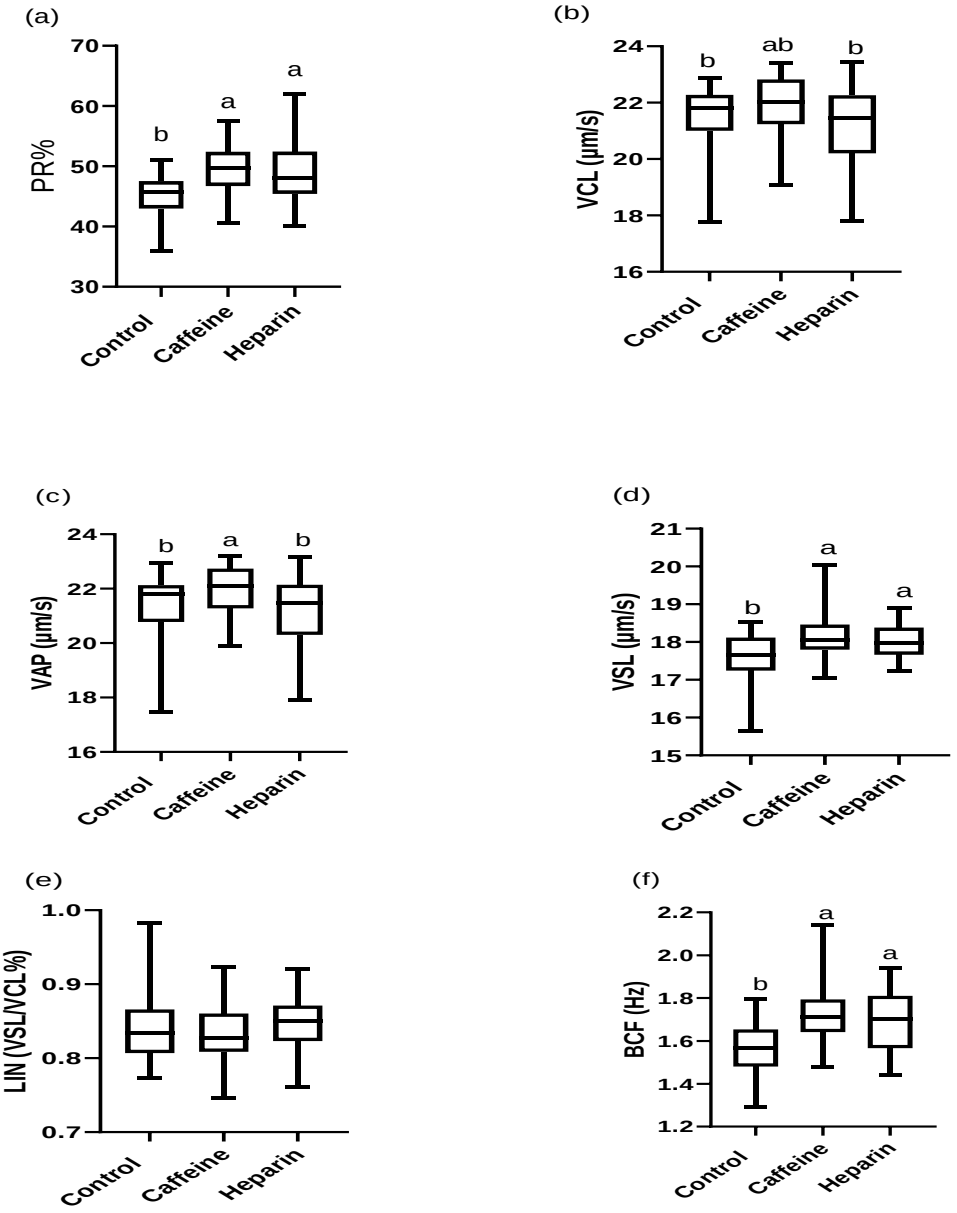


Fig 2: Sperm positive rheotaxis and sperm kinematics in capacitated and non-capacitated semen samples.
(a) Positive rheotaxis percentage (PR%), (b) curvilinear velocity (VCL), (c) average path velocity (VAP), (d) straight line velocity (VSL), (e) linearity (LIN) and (f) beat cross frequency (BCF). Data represented in mean ± SEM. Different litters indicate significance at P < 0.05.

DISCUSSION

The results showed that PR% with caffeine and heparin were significantly higher than control. In ram, a low concentration of caffeine was found to result in a significant improvement in sperm motility and hyperactivity with the highest motility after 1 hour of incubation (El-Shahat *et al.,* 2016). Sperm capacitation using heparin in ram semen leads to an increase in individual motility percentage (El-Shahat *et al.,* 2016). Bovine spermatozoa's total motility is enhanced after the addition of caffeine or pentoxifylline (Barakat *et al.,* 2015). The addition of caffeine was found to increase the intracellular calcium level that takes place 30 seconds after exposure and remains for a few minutes (Gualtieri *et al.,* 2005). This can explain the significant increase in PR% in caffeine group as Ca^{2+} and CatSper channels which are a family of voltagegated ion channels play an important role in sperm rheotaxis (Miki and Clapham, 2013). As it was reported that CatSper knockout sperm cells don't display rheotaxis instead it swims in circles regardless of the flow (Miki and Clapham, 2013). Furthermore, it was suggested that flow velocity regulating sperm rheotaxis as low flow velocity (which result in low shear stress) make Ca^{2+} mechanosensing ion channels (MSCs) opens, lead to increase sperm activity due to increase intracellular Ca^{2+} concentration (El-Sherry *et al*., 2014). In contrast, Boni *et al.* (2017) found no increase in intracellular calcium in caffeine-treated spermatozoa (Boni *et al.,* 2017). Furthermore, caffeine addition to the fertilization medium was reported to not enhance fertilization efficiency, and adding more than 2 mM of caffeine has an opposite effect on sperm motility (Momozawa K. and Fukuda, 2003). Capacitation makes some modifications to the sperm cells to make them fit for fertilization. These modifi-cations include ability of the sperm to bind with zona pellucida and extracellular matrix of the ovum (Saling *et al.,* 1978; Si and Olds-Clarke, 1999; Topper *et al.,* 1999) and later go through the acrosome reaction (Florman, and First, 1988; Saling *et al.,* 1979), the hyperactivation as the whiplash flagellar movement is important for sperm to penetrate the ovum (Ho and Suarez, 2001) and sperm ability to combine with the egg (Evans and Florman, 2002). It was mentioned earlier in the current study that caffeine and heparin increased the PR% and taking into consideration that sperms exhibited PR are of high quality with excellent potential for fertilization (De Martin *et al.,* 2017; Nagata *et al.,* 2019; Sarbandi *et al.,* 2021). We suggest that positive rheotaxis is an essential requirement for the sperm to reach and fertilize the oocyte.

Results of the present study showed no significant variation between the VCL of control and the VCL of capacitated sperms with caffeine or heparin. On the other hand, the caffeine VCL was significantly higher than heparin. Spermatozoa VCL of different capacitation agents such as caffeine, heparin and a mixture of caffeine and heparin were reported to show no significant variation with the control group, except with 2mM caffeine (Setiyono *et al.,* 2020). On the other hand, the progressive motility in frozen/thawed bovine spermatozoa treated with Caffeine exhibited a biphasic response, firstly a decrease in progressive and total motility, VSL, VAP, VCL and ALH was detected, and after that an increase in all kinetic parameters was observed (Boni *et al.,* 2017). Our results indicated that caffeine increased the VAP significantly in comparison to the heparin and control. While VSL was significantly higher in caffeine and heparin than control. On the contrary, the total motility and sperm velocity parameters VCL, VAP, and VSL were not affected by the presence or absence of caffeine in the Modena solution (Yamaguchi *et al.,* 2013). There were no significant differences in the linearity between the three groups in this study. The LIN of treated sperm with 5mM caffeine was found to be lower than other treatments till 30 minutes, after that the value was equal to the 2mM caffeine-treated sample (Setiyono *et al.,* 2020). After mixing frozen-thawed sperm

cells in Modena solution with caffeine, sperm cells showed improvements in straightness, progressive motility, and linearity (Yamaguchi *et al.,* 2013). In the current study, the BCF of caffeine and heparin aliquot was significantly higher than control. BCF was higher in capacitated sperms using Tyrode's HEPES-buffered medium with heparin (Chamberland *et al.,* 2001). BCF was reduced after the addition of 5 and 17 mM caffeine (Rogberg *et al.,* 1990). BCF was significantly affected by treatments with caffeine and the highest amount was observed in 0.5 mM/L caffeine treatment (Jenagrad *et al.,* 2018). An increase in progressive motility was found to occur in caffeine-treated bovine spermatozoa (Barakat *et al.,* 2015).

CONCLUSIONS

The present study indicates that positive rheotaxis is an important requirement for sperm cells in fertilization. Also, our study demonstrated that PR% increased by sperm capacitation using caffeine and heparin. Moreover, caffeine was found to be better than heparin in increasing PR%.

AUTHOR CONTRIBUTIONS

The present study was divided equally among the authors. Amany M. Abdel-Kawy and Taymour M. El-Sherry. including research study, statistical analysis, and writing of the paper. Haitham A. Mofadel worked in the practical part and helped in paper writing. Mohamed Samy Yousef shared in the research study. This research is part of Amany M. Abdel-Kawy master thesis and under the supervision of professors Taymour M. El-Sherry and Mohamed Samy Yousef.

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COMPETING INTERESTS

The authors declare no competing interests.

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خصائص الانجذاب التيارى على حيامن العجول الجاموس المفعلة والغير مفعلة للتلقيح باستخدام الموائع القليلة

أمانى معتمد عبد القوى ، هيثم عبد العليم مفضل ، محمد سامى يوسف ، تيمور محمد الشرى

E-mail: amanymoatamed34@gmail.com Assiut University web-site: www.aun.edu.eg

تهدف الدراسة الحالية إلى تحديد تأثير تأهيل الحيوانات المنوية على الانجذاب التياري وحركية الحيوانات المنوية داخل الموائع الدقيقة في السائل المنوي المجمد لطلائق الجاموس. تم الحصول على قصيبات السائل المنوي (عدد ٢٤ قصيبه) من ثلاث طلائق جاموس معلومين الخصوبة. تم تقسيم كل قصيبة إلى ثلاث مجموعات: المجموعة الضابطة ومجموعة الكافيين ومجموعة الهيبارين. تم تحديد الانجذاب التياري للحيوانات المنوية وجميع حركيات الحيوانات المنوية من خلال نظام تحليل الحيوانات المنوية بمساعدة الكمبيوتر باستخدام الموائع الدقيقة مع سرعة تدفق يمكن التحكم فيها. أظهرت النتائج أن نسبة الانجذاب التياري الإيجابي لمجموعتي الكافيين والهيبارين كان أعلى بكثير (=٤٩,٤ ±١ و٤٨,٨±١، على التوالي) من المجموعة الضابطة (٤٥,١±١). تظهر حركيات الحيوانات المنوية اختلافًا كبيرًا بين المجموعات الثلاث. أظهرت النتائج عدم وجود فروق ذات أهمية إحصائية في السرعة المنحنية للحيامن في مجموعتي الكافيين والهيبارين مقارنة بالمجموعة الضابطة. بينما كانت السرعة المنحنية لمجموعة الكافيين أعلى بكثير من مجموعة الهيبارين. وكان متوسط سرعة المسار للحيامن في مجموعة الكافيين أعلى بكثير من الهيبارين والمجموعة الضابطة. كانت سرعة الخط المستقيم وتردد ضربات الرأس للحيامن لمجموعتي الكافيين والهيبارين أعلى بكثير من المجموعة الضابطة. في الختام، تؤكد هذه الدراسة أن نسبة الانجذاب التياري الموجب ومعظم حركية الحيوانات المنوية في السائل المنوي المجمد للجاموس تحسنت خلال عملية تأهيل الحيوانات المنوية خارج الجسم باستخدام الكافيين والهيبارين.