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Sperm rheotaxis as a parameter for laboratory Evaluation of frozen semen in bull

Haitham A. Mofadel[1*], Hassan A. Hussein[1], Ahmed M.R. Fath El-Bab[2], Taymour M. El-Sherry[1]

[1]*Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt.*
[2]*Department of Mechatronics and Robotics, Egypt-Japan University of Science and Technology (E-JUST), Alexandria 21934, Egypt.*

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***Correspondence:**

Corresponding author: Haitham A. Mofadel
E-mail address: Haitham_Mofadel@aun.edu.eg

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ABSTRACT

Sperm rheotaxis is a sperm guidance mechanism that helps to guide and select high-quality sperm cells within the female reproductive tract. Semen is usually evaluated using either the traditional subjective method of evaluation or computer assisted sperm analysis (CASA). These methods provide information about sperm concentration, viability and motility parameters and ignoring sperm ability to travel inside the female reproductive tract. Our goal was to detect the individual variations between different sires through evaluation of post thawed frozen semen using sperm rheotaxis. We studied sperm rheotaxis and sperm kinematics inside microfluidic platforms together with subjective semen analysis (motility, viability, and sperm morphology) in post-thawed bull frozen semen in different sires. The results showed that the positive rheotaxis (PR) was higher in the post-thawed frozen semen of some bulls ($P < 0.05$) than in others. Our results also revealed that the bulls with the higher PR% have significantly higher straight-line velocity (VSL) and subjective analysis parameters (motility and viability) ($P < 0.05$) than bull with the lower PR. Furthermore, PR was positively correlated with VSL ($r = 0.67$, $P < 0.0001$) and with subjective motility and viability ($r = 0.57$, $P = 0.007$ and $r = 0.49$; $P = 0.02$, respectively). Thus, we concluded that sperm rheotaxis should be used as a new parameter in evaluation of post-thawed frozen semen quality in bull.

Introduction

Bull fertility is an important factor in cattle reproduction especially after introduction of artificial insemination (AI) technology, as one bull could be used to inseminate large number of cows. However, cryopreservation extends the storage of sperm, associated cryoinjury may cause loss of sperm motility and cause acrosomal damage, mitochondrial membrane depolarization, cryo-capacitation and change in plasma membrane permeability (Bailey *et al*., 2000). These factors make spermatozoa loss its ability to interact with the female reproductive tract and thus fertilization failure (Chen *et al*., 2015). Post thawing cryo-survival rate of spermatozoa is considered one of the most vital criteria in bull selection inside AI centers. As it was found that semen of some bulls is more sensitive to cryopreservation than others (Holt, 2000). Male fertility is mainly based on post-thawed semen assessment using conventional parameters such as sperm motility, morphology, viability, biochemical estimations of enzyme release, membrane, and acrosomal integrity. Amongst these parameters motility is believed to be the most important trait associated with spermatozoa fertilizing ability. Motility remains the parameter of choice to reveal the degree of sperm damage caused by the cryopreservation process. Sperm motility evaluation is commonly performed by visual assessment using a microscope equipped with phase contrast optics. Nevertheless, this technique still poor predictor for fertility potential because of its subjectivity (Du Plessis *et al*., 1988; Fitzpatrick *et al*., 2002; Holroyd *et al*., 2002). On the other hand, CASA gives a wider range of spermatozoa motility parameters which in turn offer valuable information about spermatozoa physiological status and therefore has the ability for more accurate prediction of fertility than the parameters evaluated by subjective microscopical semen examination (Christensen *et al*., 1999; Farrell *et al*., 1998). Unfortunately CASA parameters give no idea about the role of selection carried out by the female reproductive tract (Wang and Swerdloff, 2014). Sperm positive rheotaxis (PR) referred to the ability of the spermatozoa to swim against the flow (El-Sherry *et al*., 2014). Selected spermatozoa based on rheotaxis found to have a good quality parameters such as higher motility (Sarbandi *et al*., 2021), improved chromatin maturity (De Martin *et al*., 2017), improved morphology, viability and lower DNA fragmentation (Romero-Aguirregomezcorta *et al*., 2021). Moreover pre-freezing sperm selection based on rheotaxis showed improvement in cryo-survival rate, post thawed sperm lifespan and in-vivo fertility (Nagata *et al*., 2019b). in ram, It was reported that high PR resulted in a higher pregnancy rate and lower pregnancy loss and found to be positively correlated with fertility (Abdel-Ghani *et al*., 2020). Such evidence highlights the importance of sperm rheotaxis in the fertilization process. Thus, this study was designed to detect the individual variation between different cattle sires in post-thawed frozen semen quality using sperm rheotaxis along with CASA parameters for positive rheotactic sperm population and subjective semen analysis.

Materials and methods

Semen sample

Frozen semen from three fertile bulls (21 straws 7 for each). Straws were obtained from three different ejaculates for each bull. Straws were purchased from the Directorate of Veterinary Medicine of the Assiut governorate. For semen quality evaluation (PR%, CASA, and subjective semen analysis) semen straws were thawed in a water bath at 37°C for 30s and assisted directly without any other treatment or incubation except for dilution with sodium citrate.

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Post-thaw subjective evaluation of motility, viability, and morphology.

Sperm motility was performed using light microscope. Semen drop was placed on a warm dry clean slide, cover slide was placed over the drop and examined under high power x40. Only sperms with forward progressive motility towards the head are considered to be normal.

The viability of the sperm cells was assessed using live/dead stain as follows: One drop of semen was added to two drops of eosin stain 2% and four drops of Nigrosin stain 10%. Then thin smears are made from this mixture, dried in air and examined using light microscope under high power x40. The percentage of live sperm was calculated by counting two hundred sperm cells in different fields.

Sperm morphology was evaluated using alkaline methyl violet stain according to (Hackett and Macpherson, 1965)

Sperm rheotaxis and kinematics were analyzed using CASA.

The thawed semen straws (n=21) were diluted with sodium citrate in a 1: 3 ratios, respectively. As glycerol used in semen cryopreservation prevents flow generation inside microchannel. Then a straight microchannel with 200 µm width, 100 µm height was used to study sperm rheotaxis (PR) and sperm kinematics. The sperm concentration after dilution was around 15×10^6 sperm/ml

PR and kinetic parameters of sperm cells (n =75137 for all examined straws) were determined through 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). Sperm cell videos 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. The recorded videos were processed using a home-developed CASA and the following parameters were determined: velocity parameters which included straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s) and curvilinear velocity (VCL, µm/s) and progression parameters (linearity (LIN=VSL/VCL) and beat cross frequency (BCF, Hz).

Microfluidic Device fabrication

Lithography Chip fabrication

The chip involves 2 PMMA parts, the lower part had the engraved channel structure, and the upper part held inlet ports, as shown in Fig. 1a.

The used channel was made by direct write laser machining technique. VLS3.5 UNEVERSAL LASER SYSTEMS with a 30-Watt CO_2 laser tube and 100µm laser beam was used for channel fabrication. We got the best engraving by adjusting the engraving speed to 25mm s^{-1} (10%) laser head translation speed and laser beam power to 5-Watt (6%) laser beam power to get less roughness at the lowest available dimensions. The channel profile is Gaussian shape as shown in Fig. 1b.

Bonding of the upper and bottom parts (which contained the channel) was done by the thermo-compression method with acetic acid at 115°C and 1 N for 7 min. By heating with acetic acid, better bonding at lower temperature was achieved as well as bonding time. The final dimensions of the microchannel used in this study was 200×100 µm (Width x Height) (Nasser *et al*., 2019). Microfluidic device used in the present study was shown in Fig. 1c.

Flow generation

Hydrostatic pressure was used to induce liquid flow inside the microchannel by keeping the liquid level in the inlet reservoir higher than that in the output reservoir at a different height Δh. Hydrostatic flow generation is a simple and low-cost method of generating flow inside microchannels and does not suffer from the pulsating flow typical to syringe pumps (Moscovici *et al*., 2010) Average velocity inside the microchannel was calculated by using the Darcy–Weisbach equation (Munson *et al*., 2002).

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

where ρ is the density of the liquid, g is the gravitational acceleration, Dh is the hydraulic diameter, Δh is the height difference between the reservoirs, C = friction factor (f) × Reynolds number (Re), µ is the viscosity of the liquid 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 and AL, 1978).

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

Where V is the liquid velocity at any location in channel a, and b is the channel width and height, respectively. However, 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).

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

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

Statistics

Data from subjective sperm analyses, sperm rheotaxis, and all sperm kinematic parameters were expressed as mean ± SEM. All data were analyzed using one way analysis of variance (ANOVA) followed by Tukey multiple comparison test to determine the differences between the means of different groups. Linear Relationship has been used to analyze the correlation between VSL, subjective sperm motility and viability with PR. Statistical analysis was performed using Graph Pad Prism version 8.0.0 for Windows, Graph Pad Software, San Diego, California, USA, www.graphpad.com"

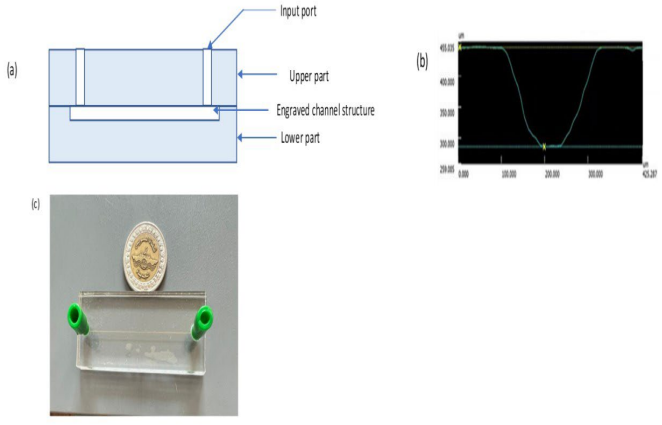

Fig. 1. (a) Chip schematic drawing. (b) Channel profile. (c) Picture of the microfluidic device used in the present study.

Results

Subjective semen analysis

The results showed that the individual motility and viability of bulls 1 and 3 were significantly higher than those of bull 2 ($p < 0.0001$; Table 1). On the other hand, Results revealed no significant difference among bulls

in sperm morphology (Table 1).

Table 1. Subjective analysis (individual motility, viability, and morphology %) in post-thawed frozen semen.

	Individual motility (%)	Viability (%)	Normal morphology (%)
Bull 1	54.5±1.2[a]	57.9±0.9[a]	93.00±0.3
Bull 2	40.0±2.0[b]	43.5±1.4[b]	93.50±.6.0
Bull 3	50.0±1.5[a]	54.6±1.0[a]	92.8±0.7
P value	<0.0001	<0.0001	0.7

Data represented in mean ± SEM. Values with different superscripts are significantly different in column (P < 0.05).

Sperm rheotaxis

Positive rheotaxis was significantly higher in bulls 1 and 3 than in bull 2 (p<0.0001;Table 2).

Sperm kinematics

Two sets of parameters were used. Velocity parameters, which include curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). The other set is progression parameters, which include linearity (LIN=VSL/VCL) and beat / cross frequency (BCF). The VSL was significantly higher in bulls 1 and 3 than in bull 2. The VCL, the VAP and linearity values of bull 1 and 3 were higher than those of bull 2 but without significant difference. The BCF was significantly higher in bull 1 and 2 than in bull 3 (Table 2).

Relationship between VSL, motility and viability with PR.

Our results showed a significant positive correlation between VSL, sperm subjective motility and viability with PR% (r = 0.67 P = <0.0001) (r = 0.57, P = 0.007) (r = 0.49, P = 0.02) (Fig. 2 a, b, c), respectively.

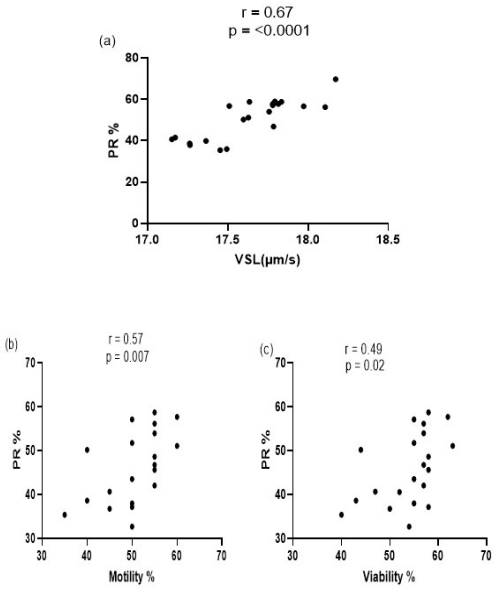

Fig. 2. Showing the relation between (a) VSL with positive rheotaxis (b) motility with positive rheotaxis (PR) (c) Viability with positive rheotaxis (PR). indicate Significant at p < 0.05.

Discussion

The results showed that subjective motility and viability were significantly higher in bulls 1 and 3 than in bull 2, while there was no significant difference in normal sperm morphology among these bulls. Motility still the most valuable parameter to detect cryo-damage of spermatozoa caused by freezing process (Kathiravan *et al*., 2011). Furthermore, a significant correlation has been found between spermatozoa motility percentage and pregnancy rates in cattle (Holroyd *et al*., 1993). A strong correlation was detected between post thaw semen viability and field fertility (56-day non return rate) (Januskauskas *et al*., 2000). The percent of normal morphology of sperm cells was constantly correlated with the calf output (Fitzpatrick *et al*., 2002). Also, normal sperm morphology and vitality percentage were significantly correlated with fertilization rates when used in-vitro (Du Plessis *et al*., 1988). On the other hand, standard semen analysis using microscope with phase contrast optics was reported to not represent an accurate fertility predictor because of the subjectivity of the technique (Du Plessis *et al*., 1988; Kathiravan *et al*., 2011). Furthermore, the relationship between subjectively evaluated motility and fertility was found to be contradictory: as some reports demonstrated the presence of a significant relationship between them (Correa *et al*., 1997; Kjoestad *et al*., 1993; Wood *et al*., 1986; Zhang *et al*., 1998) while others could not prove such a relationship (Andersson *et al*., 1992; Januskauskas *et al*., 1999; Söderquist *et al*., 1991).

In the present study, the PR was significantly higher in bulls 1 and 3 than in bull 2. In addition, we found a positive relationship between PR and VSL and subjective semen analysis parameters (motility and viability). Positive sperm rheotaxis was reported as a valuable parameter in the evaluation of ram semen to assess the reproductive performance. As a high percentage of positive rheotaxis >40% achieve higher pregnancy rates (Abdel-Ghani *et al*., 2020). Moreover, It was found that positive sperm rheotaxis percentage was strongly correlated with fertility in human and it was suggested that such parameter should be used as a fertility indicator in semen analysis and as a selection method in assisted reproductive technology (El-Sherry *et al*., 2022). The pre-freezing selection of bovine spermatozoa using rheotaxis had a positive effect on the cryo-survival and fertilization ability of sperm cells. These rheotaxis selected spermatozoa showed no change in structural integrity, viability, and biological function after freezing which related to fertilization capability. In addition, when these pre-freezing selected spermatozoa used in-vivo it results in improvement of pregnancy rat in Japanese Black heifers and Holstein repeat breeders (Nagata *et al*., 2019a). Selected sperms based on rheotaxis were found to have good quality parameters such as higher motility (Sarbandi *et al*., 2021), improved chromatin maturity (De Martin *et al*., 2017), improved morphology, viability, and lower DNA fragmentation (Romero-Aguirregomezcorta *et al*., 2021). All these proofs, in addition to our results that show strong relation between VSL, motility and viability with positive rheotaxis, confirms the existence of a positive relationship between sperm rheotaxis and high-quality semen samples.

Regarding sperm kinematics, the results revealed that VSL was significantly higher in bulls 1 and 3 than in bull 2. Even the rest of velocity parameters (VCL and VAP) in addition to linearity were insignificantly higher in bulls 1 and 3 than bull 2. The BCF was found to be higher in bulls 1 and 2 than in bull 3. Motion kinetics are very important factors in fertility. As these parameters play a vital role in sperm transportation towards the fertilization site and even when sperm come in contact with the oocyte (Rahman *et al*., 2013). Furthermore, a strong correlation between motility characteristics (VCL, VSL, LIN) and competitive fertility index had been observed in post-thawed frozen bull semen (AMANN, 1989). A high correlation has been reported between combined motion kinematics and bull fertility with good predictive value (r2 = 0.63–0.98) (Farrell *et al*., 1998). Oliveira *et al*. (2013) have reported a high correlation between CASA parameters combination and bull fertility. Furthermore, it was found that velocity parameters (VCL, VAP, and VSL) of the post-thawed bull semen have a significant correlation with the fertility of the bull (Nagy *et al*., 2015).

In the present study VSL (progressive velocity) was significantly lower in one bull than in the others. Even the rest of velocity parameters (VCL

Table 2. The positive rheotaxis % (PR) and sperm kinematics in post-thawed frozen semen.

	N	PR (%)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (VSL/VCL)	BCF (Hz)
Bull 1	31791	54 ± 1.8[a]	22.7± 0.14	22.6±0.1	17.7±0.04[a]	0.78 ±0.01	1.7±0.02[a]
Bull 2	34977	41 ± 1.8[b]	22.6± 0.09	22.5±0.1	17.4±0.1[b]	0.77 ±0.004	1.7±0.02[a]
Bull 3	8369	56 ± 3.9[a]	23± 0.2	23± 0.2	18±0.1[a]	0.79 ±0.01	1.6±0.04[b]
P value		<0.0001	0.4	0.1	<0.0001	0.3	0.01

Data are represented as mean ± SEM. Values with different superscripts are significantly different in column (P < 0.05). N: Sperm number; PR %: Positive rheotaxis %; VCL: Curvilinear velocity; VSL: Straight line velocity; VAP: Average path velocity; LIN=VSL/VCL: linearity and (BCF) beat/cross frequency.

and VAP) was lower in the same bull but without significance and This can be explained by the fact that some bull's semen is more sensitive to the freezing process (low freezability) than others (Holt, 2000). The reason behind the decreased motility during cryopreservation is due to the reduction in mitochondrial activity (Yoon *et al*., 2015). And motility is strongly related to mitochondrial activity (De Lamirande and GAGNON, 1992; Sarıözkan *et al*., 2009). Cryo-damage decreases sperm flagellar activity due to the degenerative process that causes impairment of mitochondrial function (Said *et al*., 2010). Among all sperm kinematics sperm progressive motility is believed to be vital for mammalian sperm cells to travel through the uterotubal junction and enter the fallopian tube, while non-progressive swimming seems to make it hard for sperm to reach the fertilization site (Gaddum-Rosse, 1981; Shalgi *et al*., 1992). Moreover, boar spermatozoa isolated from progressive subpopulation showed significant increase in velocity and linearity during in-vitro capacitation. While those who have non-progressive sperms did not practically change their motile parameters (Ramió *et al*., 2008). BCF represents the frequency with which the actual trajectory of the sperm crosses the average path trajectory. In a study between high and low fertility Holstein bulls there was a positive correlation between velocity parameters (VCL, VAP, and VSL) and non-return rat. While there was a negative correlation between BCF and non-return rat in post thawed frozen semen (Shojaei *et al*., 2012). On the other hand BCF was found to have almost no correlation with bull fertility in many studies (Kathiravan *et al*., 2011). It was reported that the best progression of bull sperms through fluid current take place with high velocity parameters (VCL, VSL and VAP) and low BCF (El-sherry *et al*., 2017).

From these results, we can conclude that bull 2 has the lowest PR%, subjective motility, and viability among the 3 bulls. Moreover, the sperms of bull 2 appears to swim in a more oscillatory and less progressive pattern than the sperm cells of bulls 1 and 3 as indicated by their lower VSL (progressive velocity). This variation in subjective analysis and sperm kinematics was probably caused by cryo-damage that appears to occur in higher degrees in some bulls that has low freeze ability (bull 2) than others (bull 1,3). Even more, this bull 1 with the low freeze ability had a lower PR % than other bulls.

Conclusion

Bulls with low PR during semen evaluation have low motility and viability and unsatisfactory sperm kinematics in semen evaluation. Therefore, we strongly recommend using sperm rheotaxis as an indicator for semen quality in post-thawed frozen semen in bull.

Conflict of interest

The authors declare that there is no conflict of interest.

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