Journal of King Saud University - Science 36 (2024) 103135

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Contents lists available at ScienceDirect

Journal of King Saud University - Science

journal homepage: www.sciencedirect.com

Full Length Article

The impact of adding calcium ionomycin on the sperm capacitation medium of frozen thawed bovine spermatozoa

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ARTICLE INFO

Keywords:
Bull semen
Calcium ionophore
Capacitation
Frozen-thawed

ABSTRACT

It is postulated that alterations in membranous lipids construction, surface characteristics, fluidity, Ca^{2+} permeability, and cholesterol levels collectively contribute to the process of capacitation induction. The change in sperm membrane calcium permeability following capacitation is the main signal for the acrosome reaction. The acrosome response is commonly induced in vitro using calcium ionophore. The study's objectives are to determine how the calcium ionophore (ionomycin) and the solvent dimethyl sulfoxide (DMSO) affect the motility, hyperactivity, and velocity of bovine frozen–thawed spermatozoa at varying concentrations and incubation times. The capacitation medium consisted of Bracket–Oliphant medium. Four capacitation media were prepared; two treated groups were supplemented with 25 nM and 50 nM of ionomycin, one group was without ionomycin, and the control group was supplemented with DMSO. Sperm motility decreased substantially with increasing time period in all four groups, according to the findings. The result found that there was some variance in the sperm parameters between the groups and treatment times. Overall, the group exposed to 25 nM had better semen and sperm parameters than the group exposed to 50 nM. In conclusion, the role of calcium ionophore had better semen and sperm parameters in the group exposed with a lower concentration than a higher concentration on sperm.

1. Introduction

The presence of mature oocytes and the occurrence of the acrosome response in spermatozoa are essential prerequisites for the initiation of fertilization, both in natural reproductive processes (in vivo) and laboratory-controlled settings (in vitro). During the fertilization process, these sperm can penetrate the oocyte's zona pellucida and fuse with its ooplasm (Yanagimachi, 2022).

Modifications to the membrane, including the alterations in membranous phospholipids (PL) construction, surface characteristics, fluidity, calcium permeability, and cholesterol (Ch) content, are believed to be the main causes of capacitation (El-Shahat et al., 2023). Most of these changes are related to the plasma membrane of the sperm, which confirms the theory that sperm capacitation is a series of significant actions leading to plasma membrane maturation (Morcillo et al., 2022).

A change in the sperm membrane's permeability to calcium is the primary signal that indicates the beginning of the acrosome response once capacitation has taken place (Benko et al., 2022). The cytoplasmic membranes of ejaculated spermatozoa are covered with proteins, cholesterol, and glycoprotein molecules that come from epididymal or seminal plasma. These elements make the sperm membrane stiffer, and more stable and stop the sperm from going through an early acrosome reaction (Maitan et al., 2022). The process of sperm capacitation includes removing inhibitors from its head, increasing the pH level, and reducing the (Ch/PL) ratio to make its membrane more permeable to $Ca2^{+}$ ions (Layek et al., 2022). The increasing of Ca^{2+} ions inside the spermatozoon activates phospholipase A2, which, in turn, catalyzes the formation and accumulation of lysophosphatidylcholine which triggers an acrosome reaction of the spermatozoon (Shan et al., 2021).

Sperm capacitation can be performed in the laboratory by subjecting the sperm to a washing procedure using a medium that contains

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https://doi.org/10.1016/j.jksus.2024.103135
Received 22 November 2023; Received in revised form 30 January 2024; Accepted 12 February 2024
Available online 15 February 2024

electrolytes at comparable concentrations, metabolic energy sources, and chemicals with the ability to modify sperm viability and motility, induce the acrosome reaction, and influence its velocity. The proline amino acids taurine and hypotaurine, as well as the catecholamines, such as adrenaline, which also causes hyperactivation and the acrosome response, are some of the chemicals that are responsible for sperm motility and vitality. Other chemicals responsible include the catecholamines (Haslem et al., 2022; Qamar et al., 2023). In recent years, calcium ionophore (also known as ionomycin or calcimycin) has emerged as the component that is utilized most frequently for the purpose of stimulating the sperm acrosome reaction in the capacitation media.

Amran et al. (2023) indicated that the calcium ionophore causes variations in the capacitation of bull sperm based on the concentration of those ions in the in vitro media. Additionally, the acrosome response was seen in sperm from many species upon exposure to the calcium ionophore (Balu et al., 2022).

Glutathione (GSH), Residing as an endogenous antioxidant in animals, the inclusion of this substance in the frozen diluent of semen has been seen to substantially enhance the quality of thawed sperm. Several studies have shown that the inclusion of a specific concentration of glutathione (GSH) during the cryopreservation process of cow, sheep, pig, and Chios ram semen may result in a notable enhancement in sperm fertilization capacity upon thawing (Ansari et al., 2012; Olfati Karaji et al., 2014; Wu et al., 2021). The adding calcium ionophores to capacitation medium of bull spermatozoa considering the incubation duration was not investigated. Therefore, this in vitro experiment aims to establish the suitable concentrations and incubation durations of calcium ionophores on motility, hyperactivity, and velocity of bovine frozen-thawed spermatozoa capacitated in vitro.

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all chemicals and in vitro media utilized in this undertaking were acquired from Sigma.

2.2. Capacitation medium

The thawed semen was capacitated in the Bracket–Oliphant medium (112 mM NaCl, 4 mM KCl, 2.25 mM CaCl2.2H2O, 0.5 mM MgCl2.6H2O, 37 mM NaHCO3, 0.83 mM NaH2PO4.2H2O, and 0.05 mM phenol red) supplemented with 10 μg/ml heparin sodium salt (C26H41NO34S4), 3mg/ml BSA, 1.25mM sodium pyruvate (C3H3NaO3), and 0.5 μg/ml gentamycin sulfate (C21H45N5O11S). The pH of in vitro capacitation media was adjusted (7.2–7.4), filtered (0.22 μm filter), and incubated for two hours in a humidified, 5%CO2 and 39 °C for equilibration.

2.3. Ionomycin solution

A 10 mM stock solution (1 mg/133.85 μL) of ionomycin was prepared in DMSO, divided in Eppendorf tubes, and preserved at −20 °C.

2.4. Frozen semen

Frozen semen of Holstein Friesian cattle was obtained from the Center for Asset Conservation and Genetic Improvement in Al-Kharj city, which is affiliated with the Saudi Ministry of Environment, Water, and Agriculture.

2.5. Semen thawing and dilution

In each experimental trial, a total of three frozen straws were subjected to a thawing process in a water bath (37 °C) for a duration of 45 s. Subsequently, these thawed straws were combined and placed together in a conical centrifuge tube (15 ml) pre-filled with five milliliters of capacitation medium. This step was undertaken to minimize any potential discrepancies or inconsistencies that may arise between individual straws. The collected semen was subjected to two rounds of washing with the capacitation medium using centrifugation at a force of 500*g* for a duration of 5 min. The pellets obtained were subjected to dilution to achieve 4×10^6/mL of sperm concentration. Subsequently, the diluted pellets were divided into four distinct experimental groups.

2.6. Experimental groups

The sperm samples were partitioned into four distinct cohorts for the purpose of assessment at four different time intervals: immediately (0 h), after one hour (1 h), after two hours (2 h), and after three hours (3 h) from the start of capacitation. The volume and structure of capacitation media, and sperm concentration (4×10^6/mL) were unification for all study groups. No ionomycin was added to the control group, while the second capacitated medium was supplemented with DMSO. The thawed sperm in group three and four were capacitated in the presence of 25 nM and 50 nM of ionomycin, respectively. The process of capacitation was carried out in each experimental group for a duration of three hours, under controlled conditions of 39 °C temperature, 5% CO2 concentration, and a humidified environment. The concentration of sperm cells and other characteristics related to their motility were assessed on four occasions using computer-assisted sperm analysis.

2.7. Sperm motility assessment by CASA

At 0, 1, 2, and 3 h, the spermatozoa's overall and increasing motility was measured during capacitation using the Hamilton Thorne CASA system version 12 TOX IVOS software (USA). The standard manufacturing parameters for bull sperm were used, and 70% straight spermatozoa (STR) and 50 μm/s of path velocity (VAP) were considered as a progressive motile. The same capacitation media were used for sample diluting. Three microliters of diluted sperm was added to a counting chamber that had been preheated to 37 °C and analyzed with regard to the kinetic properties of the spermatozoa. At a frame rate of 60 Hz, 30 frames from each of 10 different microscopic fields were studied. The total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/second), straight-line velocity (VSL, μm/second), curvilinear velocity (VCL, μm/second), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %) of the spermatozoa were evaluated.

2.8. Statistical analyses

A completely randomized design was used for data analysis using the GLM procedure of SAS (SAS Institute, 9.4), except for the effect treatments, which were analyzed using the PROC MIXED procedure for repeated measurements at 0, 1, 2, and 3 h. The sperm kinetics data obtained from CASA were statistically analyzed and a $P \leq 0.05$ was considered significant.

3. Results

The result of the impact of ionomycin supplementation (25 and 50 nM) and DMSO treatment of sperm on the TM and PM of bull sperm during a period of 3 h compared to the control groups is illustrated in (Table 1). The findings demonstrated that TM decreased as the treatment time increased across all groups. Although the control and DMSO groups exhibited superior sperm TM compared to the treatment group at the lower duration of exposure, this difference was not observed at the 3-h mark. Interestingly, the group treated with a concentration of 25 nM demonstrated higher total motility compared to the other experimental groups. There was no statistically significant variation observed in the PM at the initial time among the various groups. However, as the

Table 1
Effect of ionomycin supplementation (25 and 50 nM) and DMSO on the total motility and progressive motility of frozen–thawed bovine sperm over 3 h compared to the control.

Parameters	Treatment	Time				±SEM	*P*-value
		0	1	2	3		
Total motilityTM (%)	Control	62.22^{Aa}	41.89^{Ab}	20.56^{Ac}	10.22^{ABc}	±4.73	0.0002
	DMSO	62.44^{Aa}	35.00^{Ab}	25.33^{Ab}	7.67^{BCc}	±3.99	<0.0001
	Iono25	49.56^{Aba}	33.89^{Ab}	17.78^{Ac}	14.00^{Ac}	±3.73	0.0005
	Iono50	41.61^{Ba}	19.78^{Bb}	6.00^{Bc}	3.61^{Cc}	±2.80	<0.0001
±SEM		±5.46	±3.86	±3.44	±1.85		
P-value		0.07	0.02	0.02	0.02		
Progressive motility PM (%)	Control	26.11^{a}	30.56^{Aa}	11.67^{Ab}	5.00^{Bb}	±2.61	0.0004
	DMSO	27.22^{a}	27.00^{Aa}	15.67^{Ab}	5.00^{Bc}	±2.84	0.001
	Iono25	28.89^{a}	22.78^{Ab}	11.67^{Ac}	11.66^{Ac}	±1.71	0.0002
	Iono50	21.44^{a}	13.00^{Bb}	3.83^{Bc}	1.78^{Bc}	±1.37	0.0001
±SEM		±2.94	±2.30	±2.05	±1.23		
P-value		0.38	0.003	0.02	0.003		

Note: A, B and C = significantly different within the same row or treatment, / a, b, and c = significantly difference within the same column different group of treatments.

treatment duration increased, the group that received the higher treatment had a statistically significant decrease in PM. In comparing the PM within the same group at the different treatment times, all the groups (rows) showed a highly significant decrease in the PM within each group (rows); see Table 1. Table 2 illustrates the results of the impact of the ionomycin supplementation (25 and 50nM) and DMSO on the APV, SLV, CLV, and ALH displacement of the frozen–thawed bovine sperm during the 3 h compared to those in the untreated medium. The data of the APV indicator of capacitated spermatozoa did not display any statistical significance among the four groups at the ionomycin concentration or at incubation times. Regarding the VSL, the treated groups (25 and 50 nM) showed significantly higher rates (58.37 and 50.66, respectively) compared to the DMSO and control groups (45.27 and 48.50 respectively). The VCL indicated significant differences in each group of treatment at the different times from 0 to 3 h but did not indicate any significant difference between the different treatments (Table 2). For the amplitude of ALH, the control and the low treated group (25 nM) illustrated a significant difference in each group of sperm at 0, 1, 2, and 3 h (row), but the DMSO and the high treated (50nM) group were not significantly different (Table 2). Comparing the different groups (column), only the high treated group (50uM) showed a lower value than the other groups (Table 2). The result in (Table 3) illustrates the impact of DMSO and ionomycin supplementation on the BCF, STR, and LIN of frozen–thawed bovine sperm over 3 h. compared to the control group. Regarding the BCF of sperm, there was no significant difference within the groups over the 3 h, except for the DMSO group, which was

Table 2
The effect of ionomycin supplementation (25 and 50 nM) and DMSO on the average path velocity, straight line velocity, curvilinear velocity, and amplitude of lateral head displacement of frozen–thawed bovine sperm over 3 h compared to the control group.

Parameters	Treatment	Time				±SEM	*P*-value
		0	1	2	3		
Average path velocity VAP (μm/s)	Control	65.50	70.83	68.79	59.26	±3.98	0.26
	DMSO	67.23	75.48	67.12	65.08	±4.89	0.49
	Iono25	70.69^{ab}	76.93^{a}	59.54^{b}	71.35^{ab}	±3.51	0.04
	Iono50	65.71^{a}	70.72^{a}	65.11^{a}	53.07^{b}	±3.48	0.04
±SEM		±1.63	±3.11	±3.70	±6.19		
P-value		1.17	0.42	0.37	0.26		
Straight line velocity VSL (μm/s)	Control	45.27^{Bb}	62.66^{a}	57.89^{a}	52.00^{ab}	±3.36	0.03
	DMSO	48.50^{Bb}	66.94^{a}	60.90^{ab}	58.84^{ab}	±3.75	0.05
	Iono25	58.37^{A}	65.93	50.18	57.93	±4.55	0.19
	Iono50	50.66^{Bab}	61.57^{a}	54.11^{ab}	42.33^{b}	±3.90	0.05
±SEM		±1.66	±2.94	±3.45	±6.16		
P-value		0.003	0.55	0.22	0.28		
Curvilinear velocity VCL (μm/s)	Control	151.01^{Aa}	123.22^{b}	102.43^{Bcb}	88.40^{Bc}	±7.33	0.002
	DMSO	140.97^{Aba}	122.82^{a}	96.39^{Bb}	88.16^{Bb}	±7.73	0.005
	Iono25	133.68^{Ba}	128.20^{a}	97.42^{Bb}	124.82^{Aa}	±5.76	0.009
	Iono50	137.71^{Aba}	111.21^{b}	118.50^{Ab}	95.95^{Bc}	±4.25	0.0008
±SEM		±4.46	±6.94	±4.73	±8.62		
P-value		0.10	0.41	0.04	0.05		
Amplitude of lateral head displacement ALH (μm)	Control	6.34^{a}	5.13^{Ba}	3.90^{b}	3.74^{Bb}	±0.37	0.004
	DMSO	6.21^{a}	4.81^{Bab}	3.90^{b}	6.37^{Aa}	±0.64	0.07
	Iono25	6.57^{a}	6.08^{Aa}	5.37^{b}	6.21^{Aa}	±0.16	0.004
	Iono50	5.94	4.43^{B}	5.62	5.17^{AB}	±0.46	0.19
±SEM		±0.20	±0.21	±0.53	±0.65		
P-value		0.25	0.003	0.09	0.07		

Note: A, B and C = significantly different within the same row, / a, b, and c = significantly difference within the same column.

Table 3
Effect of DMSO and ionomycin supplementation on the beat cross frequency, straightness, and linearity of frozen–thawed bovine sperm over 3 h compared to the control group.

Parameters	Treatment	Time				±SEM	*P*-value
		0	1	2	3		
Beat cross frequency	Control	29.42^{AB}	29.46	24.60^{B}	29.26^{AB}	±1.72	0.20
BCF (Hz)	DMSO	27.99^{Bb}	28.72^{b}	29.51^{ABb}	36.00^{Aa}	±1.98	0.07
	Iono25	33.84^{A}	33.68	37.03^{A}	35.53^{A}	±1.05	0.16
	Iono50	29.44^{AB}	30.31	30.46^{AB}	25.46^{B}	±2.94	0.61
±SEM		±1.38	±1.51	±2.46	±2.53		
P-value		0.07	0.18	0.04	0.05		
Straightness	Control	70.91^{Cc}	86.88^{a}	83.33^{b}	86.76^{a}	±0.86	<0.0001
STR (%)	DMSO	71.76^{Cb}	87.42^{a}	88.54^{a}	89.90^{a}	±1.79	0.0003
	Iono25	87.21^{A}	91.11	88.33	82.57	±2.91	0.29
	Iono50	77.43^{B}	86.57	82.91	79.95	±2.78	0.19
±SEM		±0.81	±1.33	±2.03	±3.69		
P-value		<0.0001	0.13	0.15	0.31		
Linearity	Control	34.64^{Cc}	55.32^{b}	61.56^{ABa}	65.78^{Aa}	±1.67	<0.0001
LIN (%)	DMSO	37.79^{Cc}	57.78^{b}	66.79^{Aa}	68.53^{Aa}	±2.12	<0.0001
	Iono25	52.54^{A}	62.57	63.68^{A}	51.50^{B}	±3.78	0.10
	Iono50	42.54^{Bb}	59.53^{a}	54.78^{Ba}	50.28^{Bab}	±3.05	0.02
SEM		±1.46	±3.13	±2.58	±3.51		
P-value		0.0001	0.46	0.05	0.01		

Note: A, B and C = significantly different within the same row, / a, b, and c = significantly difference within the same column.

significantly different at 3 h. In comparing the STR of sperm, the control and the DMSO group did show a significant change over time compared to the low and high treated groups (25 and 50 nM) that did not change over time. The LIN showed differences between the different groups at 0, 2, and 3 h at P values of $0 < 001$, $0 < 0.05$, and $0 < 0.01$ respectively; the treated groups showed a higher rate at time 0 but a lower rate after 3 h, compared to the control and DMSO groups (Table 3). The results which are shown, summarize the impact of ionomycin on the sperm kinetics and motility parameters over time, as well as the interaction of the bovine sperm over the 3 h compared to the control. The result showed that the high ionomycin treatment showed a significantly lower total and progressive sperm motility with the increase in the treatment time compared to all other groups. Also, in all other sperm parameters, values for the high (50 nM) treated group were lower than those for the other groups (Table 4).

4. Discussion

The CASA is an excellent tool to use to provide a good profile of the fertility potential of semen and sperm analysis, through the measurement of several parameters of semen and sperm quality as illustrated and used by several investigators (Najjar et al., 2013; Galmessa et al., 2014; Simonik et al., 2015; Malama et al., 2017; Patel and Dhami, 2016; Kumar et al., 2017; Islam et al., 2017; Maylem et al., 2018), whose results agreed with the current study. In addition, this study utilized CASA to evaluate the impact of adding the Ca ionomycin at two different concentrations (25 and 50 nM) on the thawed semen and a sperm analysis of capacitation compared to a control and DMSO. The result showed that the effect of the lower concentration (25 nM) was better than that of the higher one (50 nM) on the semen and sperm parameters. This could be due to the side effects of increasing the Ca ionomycin concentration on the integrity of the cell membrane of the sperm during the capacitation process; as stated by Tasdemir et al. (2013), sperm cells are extremely sensitive to changes in the osmolality of the surrounding fluid, and such changes have an effect not only on sperm motility but also on all other semen and sperm characteristics. Also, as indicated by Zou et al. (2021), adding 2 mmol/l glutathione to the semen diluent improved the quality of frozen Guanzhong dairy goat sperm, which was better than 3 and 4 mmol/l. After thawing, the rates of sperm viability, plasma membrane intactness, and acrosome intactness were found to be at their maximum. This study found that major sperm parameters, including VAP, VCL, VSL, ALH, BCF, and LIN, decreased considerably with the treatment duration from 0 to 3 h, making motility the most important determinant in fertility (Cancel et al., 2000). The measures of VAP, VCL, ALH, and BCF are commonly linked to hyperactivated motility and capacitation. According to Maylem et al. (2018), an

Table 4
Effect of DMSO and ionomycin on the sperm kinetics, motility parameters, time, and interaction of frozen–thawed bovine sperm over 3 h compared to the control.

Parameters	Treatment				±SEM	*P*-value		
	Control	DMSO	Ionomycin 25 nM	Ionomycin 50 nM		Treatment	Time	Treatment *Time
TM (%)	33.72^{a}	32.61^{a}	28.81^{a}	17.75^{b}	±1.94	<0.0001	<0.0001	0.22
PM (%)	18.33^{a}	18.72^{a}	18.75^{a}	10.01^{b}	±1.11	<0.0001	<0.0001	0.03
VAP (μm/s)	66.09^{ab}	68.73^{ab}	69.63^{a}	63.65^{b}	±2.01	0.02	0.003	0.26
VSL (μm/s)	54.45^{ab}	58.80^{a}	58.10^{a}	52.17^{b}	±1.96	0.001	0.0001	0.13
VCL (μm/s)	116.27^{b}	112.08^{b}	121.03^{a}	115.84^{b}	±3.21	<0.0001	<0.0001	0.003
ALH (μm)	4.78^{b}	5.32^{b}	6.06^{a}	5.29^{b}	±0.22	<0.0001	0.0002	0.01
BCF (Hz)	28.18^{b}	30.56^{b}	35.02^{a}	28.92^{b}	±1.02	0.003	0.78	0.06
STR (%)	81.97^{b}	84.41^{ab}	87.31^{a}	81.71^{b}	±1.12	0.004	<0.0001	0.001
LIN (%)	54.33^{ab}	57.72^{a}	57.57^{a}	51.78^{b}	±1.39	0.01	<0.0001	<0.0001

Note: TM, Total motility; PM, Progressive motility; VAP, Average path velocity; VSL, Straight line velocity; VCL, Curvilinear velocity; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency; STR, Straightness; LIN, linearity. a, b, and c = significantly difference between the different group.

increase in VCL values is observed in sperm cells that have been incubated under capacitating conditions. This elevation is suggestive of the cells undergoing a robust hyperactivated pattern of activity, which is defined by a large amplitude of flagella binding. The study conducted by Chatiza et al. (2012) reported that the velocity of capacitated sperm cells ranged from 105 to 143 μm/sec, whereas hyperactivated sperm cells exhibited a velocity range of 114 to 195 μm/sec. The elevated value reported by ALH, resulting from hyperactivation during the in vivo capacitation process, was found to be linked to fertility and reproduction. The observed substantial rise in the ALH value of buffalo sperm cells can be mostly attributed to the adverse impact of cell cryopreservation (Maylem et al., 2018).

5. Conclusion

Through this study, the significant role of calcium ionophore on sperm motility parameters was clear during capacitation media, especially at 25 μM. Furthermore, the CASA is an excellent tool to use to provide a good profile of the fertility potential of semen and sperm analysis, through the measurement of several parameters of semen and sperm quality, as illustrated by several investigators whose results agreed with those of the current study.

6. Founding statement

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research through the project number IFP-IMSIU-2023034. The authors also appreciate the Deanship of Scientific Research at Imam Mohammad Ibn Saud Islamic University (IMSIU) for supporting and supervising this project.

CRediT authorship contribution statement

Mohammed Aleissa: Conceptualization, Funding acquisition. **Ahmad Alhimaidi:** Writing – original draft, Writing – review & editing, Supervision. **Ramzi Amran:** Data curation, Writing – original draft. **Aiman Ammari:** Formal analysis, Writing – original draft. **Muath Al-Ghadi:** Conceptualization, Methodology. **Mohammed Mubarak:** Visualization. **Nasir Ibrahim:** Investigation. **Mohammed Al-Zharani:** Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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