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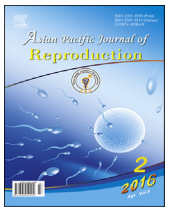

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Effect of heparin, caffeine and calcium ionophore A23187 on *in vitro* induction of the acrosome reaction of fresh ram spermatozoa

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ABSTRACT

Objective: To determine the effect of different concentrations of heparin, caffeine or calcium ionophore and incubation time on motility, hyperactivity (HA) and acrosome reaction (AR) of ram sperm *in vitro*.

Methods: Semen samples were collected by artificial vagina from three mature ram. Split fractions (0.1 mL) of the pooled semen were layered under 1 mL of S-TALP medium supplemented with either heparin (10, 25, 50, 75 and 100 μg/mL), caffeine (0.5, 0.75, 1, 2 mg/mL), or calcium ionophore A23187 (0.5, 0.75, 1, 1.55 mM/mL). Individual motility, hyperactivity percentage and acrosomal status were recorded at 0, 1, 2, 3 and 4 h post-incubation for all treatments and control. Moreover, they were examined for ability to fertilize sheep oocyte *in vitro*.

Results: Heparin, caffeine and calcium ionophore A23187 at a concentration of 75 μg/mL, 1 mg/mL, and 1.55 mM/mL respectively can be used as a protocol to provide the best results for *in vitro* cap citation and acrosome reaction in ram. The penetration rates of rate of oocytes inseminated with spermatozoa treated with calcium ionophore A23187, and heparin were higher as compared with caffeine. Moreover, heparin achieved higher fertilization rates but without significant difference with others.

Conclusion: The best concentration of heparin, caffeine and ionophore A23187 are 75 μg/mL, 1 mg/mL, 1.55 mM/mL for 3, 1, 4 h incubation respectively and can be used for *in vitro* fertilization in sheep.

1. Introduction

Development of successful *in vitro* fertilization (IVF) techniques is essential for the study of the basic aspects of fertilization process. Freshly ejaculated mammalian spermatozoa are not immediately capable of achieving fertilization [1]. During residence in the female tract, the sperm cell undergoes a complex and poorly understood set of modifications which confer fertilization competence, a process collectively called capacitation [2–5]. Capacitation is believed primarily to involve membrane modifications, including changes in lipid composition, surface properties, fluidity, permeability to calcium and lowered concentration of cholesterol in membranes [6]. Most of these alterations are related to changes in the plasma membrane of spermatozoa and have led to the contention that capacitation is a process of membrane maturation [7,8]. An unregulated capacitation process causes sperm to undergo a spontaneous acrosome reaction and resulting in loss of sperm fertilization capacity [5]. Fresh ram spermatozoa will spontaneously undergo the acrosome reaction when incubated at 39 °C over a period of 4 h in the absence of any inducing agent [9]. The acrosome reaction occurs in response to natural inducers, *i.e.*, the zona pellucida and oviductal fluids, but it can also be artificially induced by a variety of substances such as Ca^{2+} ionophore A23187 [10], heparin [11], caffeine [12]. Many researches are concerned with method of recovery and maturation of sheep oocytes, however, few studies are concerned with the process of IVF, particularly, preparation of the ram spermatozoa prior to insemination of oocytes. The effects of many capacitating

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factors have been studied in separate researches, and under different conditions. Very few data is available about comparing the effect of different capacitating active compounds on capacitation and acrosome reaction of ram spermatozoa. Therefore, the present study was aimed to investigate the effect of different concentrations and incubation times of heparin, caffeine or calcium ionophore A23187 on the motility, hyperactivity, and acrosome reaction of ram spermatozoa and ability of them on *in vitro* fertilization of ram oocytes.

2. Materials and methods

2.1. Semen collection

Semen was collected by artificial vagina from 3 ram of proven fertility, aged 2–3 years and housed indoors at farm of Animal Reproduction Research Institute. All semen sampled were evaluated immediately for percentage of individual motility (IM%). Samples with IM% not less than 60% were pooled.

2.2. Sperm capacitation

Split fractions (0.1 mL) of the pooled semen were layered under 1 mL of S-TALP medium, according the method described by [13], supplemented with either heparin (10, 25, 50, 75 and 100 μg/mL), caffeine (0.5, 0.75, 1, 2 mg/mL) or calcium ionophore A23187 (0.5, 0.75, 1, 1.55 mM/mL) according to experimental procedure. Semen diluted in S-TALP medium without treatment was used as a control. This technique (swim up technique) was performed in 15 mL centrifuge tubes, held at an angle 45° and incubated in an atmosphere of 5% CO_2 incubator at 39 °C. Individual motility percentage (IM%), hyperactivity percentage (HA%) and acrosomal status were recorded at 0, 1, 2, 3 and 4 h post-incubation for all treatments.

2.3. Evaluation of sperm individual motility

Percentage of individual motility (IM%) was subjectively estimated in a small drop of the sperm suspension from the most supernatant and covered by a cover slip, and examined under phase contrast microscope (400×) equipped with a heated (37 °C). Only progressive forward motility was considered among different treatments and control.

2.4. Evaluation of sperm hyperactivity

Percentage of hyperactivated motility (HA%) were determined by recording the percentage of sperm cells with flagellar beating vigor and circular movement [14,15]. Hyperactivated motility percentage was considered from the percentage of IM% and expressed by "pluses" [15], where (+) means HA% <20%, ++ means HA% 20%–40%, +++ means HA% 40%–60%, ++++ means HA% 60%–80% and +++++ means HA% >80%.

2.5. Evaluation of sperm acrosomal status

Percentage of incomplete and complete acrosome reacted spermatozoa were determined by silver nitrate staining technique according to [16]. A total of 100 spermatozoa were checked in randomly selected fields under oil immersion lens (1 000×) of phase contrast microscope. The spermatozoa were classified into three groups: (1). Spermatozoa with an intact plasma and outer acrosomal membrane; (2). Spermatozoa with incomplete AR showing fenestrations, vesiculation and loosening between plasma membrane and outer acrosomal membrane; (3). Spermatozoa with complete AR showing complete loss of the outer acrosomal membrane leaving cup-shaped appearance. Both incomplete and complete AR percentages were considered collectively as total AR.

2.6. Evaluation of the fertilizing capacity of the treated ram spermatozoa

2.6.1. Collection of ovaries

The ovaries of native breed of sheep of unknown reproductive history (1–4 years old) were collected from El-Moneib abattoir. The ovaries were separated shortly after slaughter of animals, dissecting away from the surrounding tissues and maintained in a thermo box containing a pre-warmed sterile saline (30 °C) supplemented with antibiotic (100 IU/mL penicillin and 100 μg/mL streptomycin). The average transport time of the ovaries to the laboratory was 2 h. At the laboratory, the ovaries were then washed with warm sterile saline to remove adhering blood [17].

2.6.2. Oocytes recovery and selection

Oocytes were harvested from the ovaries by aspiration technique using 21 G needle fitted to a 10 mL disposable syringe containing a small volume of maturation medium. Follicular fluids from all visible surface follicles ranged from 2 to 5 mm in diameter were aspirated. The contents were pooled in a sterile 15 mL centrifuge tubes and allowed to settle for 5 min in laminar flow hood. The clear fluid was then discarded while the bottom portion was poured in Petri dish containing hormone free Ham's F-10 media examined under stereomicroscope under low magnification (10×–20×) for the presence of oocytes [18].Oocytes were evaluated on the bases of the characteristics of surrounding cumulus investment, homogeneity of ooplasm mass and regularity of the peripheral shape. Oocytes with a complete corona layered and at least two compact cumulus cell layers and with a homogenous granulated ooplasm were used in this study. Oocytes with no cumulus cell layers (completely denuded) or those dark scattered or irregular ooplasm were discarded. Selected cumulus oocyte complexes (COCs) were washed three times with maturation medium [19].

2.6.3. In vitro oocyte maturation and fertilization

After preparation of Ham's F-10, minidrops of 50 μL were tittered in a polystyrene culture dish (35 × 10 mm) and covered with equilibrated sterile. Millipore filtered (0.45 μM) light-weight paraffin oil and incubated in CO_2 incubator in 5% CO_2, 39 °C and at maximum humidity for at least 1 h before culturing. Group of 10–15 selected oocytes were transported to each minidrop then the culture dish was incubated statically for 24 h in the previously mentioned incubation conditions. Then the oocytes were assessed for maturation signs. The oocytes showing expanded cumulus cells, perivitelline space or extruded first polar body were selected for IVF [20]. Selected *in vitro* matured oocytes were washed three times in S-TALP medium. The surrounding cumulus cells were partially removed by gentle pipetting. Five to 10 oocytes were then allocated to each minidrop, containing F-TALP medium, in

polystyrene culture dish covered with warm sterile paraffin oil and incubated for 1 h until spermatozoa were added. Pretreated semen with optimal concentration of the different capacitating compound and optimal time of incubation (determined on the bases of the best motility, hyperactivity, percentage of incomplete AR with high tendency to undergo complete AR, in addition to total AR) was used for insemination. Sperm cell concentration of semen was estimated after swim up and a total of 2×10^6 sperm cell were added to the minidrops so that the final volume of each minidrop is 50 µL. Oocytes and spermatozoa were incubated together for 6 h at 39 °C under 5% CO_2 in air and at maximum humidity. At the end of the incubation period, oocytes/zygotes were examined for the evidence of fertilization. Oocytes with male and female pronuclei and/or secondary polar bodies were considered to be normally fertilized. The penetration and fertilization rates were recorded [21].

2.7. Statistical analysis

The obtained data for motility, hyperactivity and acrosome reaction were analyzed statistically using Costat computer program; version 3.03, Copyright Cottort Software. Data from 10 replicates were subjected to analysis of variance (two way ANOVA) to clarify the effect of different capacitating agents concentrations and incubation times. Moreover, unpaired *t* test was used to evaluate the differences between means. On the other hand *in vitro* penetration and fertilization rates were analyzed by using Chi-square analysis (χ^2).

3. Results

3.1. Effect of different concentrations of heparin on ram sperm functions

3.1.1. Effect on sperm motility (IM)

The effect of different heparin concentrations and different incubation times on motility of ram sperm is shown in Table 1. Irrespective of incubation time, there was a highly significant ($P < 0.05$) increase in motility percentage of spermatozoa treated with 10, 25, 50, 75 and 100 µg/mL heparin as compared to control one. Immediately after dilution sperm motility was significantly higher in semen sample with 100 mg/mL heparin than concentrations of 10, 25, 50 µg/mL. However, there were no significant differences between the treatments for motility after one hour of incubation and all over the incubation period. Meanwhile a significant decrease in motility started from the second hour of incubation period in semen samples diluted with 75 and 100 mg/mL heparin.

3.1.2. Effect on sperm hyperactivity (HA)

The first hour after treatment, the highest HA% was observed at 100 µg/mL heparin (2.80 ± 0.32) followed by 50 µg/mL (2.60 ± 0.80) and 75 µg/mL (1.80 ± 0.24) but showing no significant differences among the three concentrations (Table 2). Irrespective of heparin concentration, incubation of ram spermatozoa for 4 h resulted in a significant ($P < 0.05$) increase in HA% as compared to 0 time.

3.1.3. Effect on total acrosome reaction percentage (AR %)

A significant ($P < 0.05$) increase in total AR% was concomitant to the increase in incubation time, and a highly significant ($P < 0.05$) increase was achieved after 3 and 4 h incubation as shown in Table 3. It was clear that 50 µg/mL and 75 µg/mL heparin achieved a highly significant ($P < 0.01$) increase in total AR% (89.00 ± 1.97 and 88.00 ± 2.00, respectively) as compared to control (0.00 ± 0.00).

3.2. Effect of different concentrations of caffeine on ram sperm functions

3.2.1. Effect on sperm motility

Table 4 shows the influence of different concentrations of caffeine and increasing incubation time on individual motility percentage of ram spermatozoa. Higher concentration (2 mg/mL) resulted in a significant ($P < 0.05$) decrease in IM% immediately after dilution and during 1st and 2nd hours of incubation. However, low concentrations of caffeine (0.5, 0.75 and 1 mg/mL) increased IM% without any significant differences between them and control. Maximum improvement in IM% was noticed after 1 h incubation and maintained up to 2 h followed by a significant ($P < 0.05$) decrease when incubation time was prolonged.

3.2.2. Effect on sperm hyperactivity

The effect of caffeine and incubation time on HA of ram spermatozoa are presented in Table 5. A highly significant ($P < 0.05$) increase in HA% was achieved by treatment of ram spermatozoa with different concentrations of caffeine (0.5, 0.75, 1 and 2 mg/mL) as compared to untreated control one. Irrespective to caffeine concentration, maximum increase in HA% was noticed after 4 h incubation in control semen samples. Meanwhile a highly significant ($P < 0.01$) increase in HA% was observed in all caffeine treated samples after 2 h of incubation.

Table 1
Effect of different concentrations of heparin (µg/mL) and incubation time (hours) on motility of ram spermatozoa.

Concentration	Incubation time (hours)				
	0	1	2	3	4
Control	77.5 ± 0.83^{Ba}	68.50 ± 2.47^{Bb}	64.0 ± 3.05^{Bbc}	59.50 ± 2.52^{Bc}	40.50 ± 3.11^{Bd}
10	78.0 ± 1.29^{Ba}	78.0 ± 1.24^{Aa}	78.50 ± 1.30^{Aa}	$76.50\ 1.83^{Aa}$	62.0 ± 2.13^{Ab}
25	78.5 ± 0.76^{Ba}	78.0 ± 1.69^{Aa}	78.00 ± 1.85^{Aa}	$75.50\ 1.38^{Aa}$	59.00 ± 2.76^{Ab}
50	78.0 ± 1.52^{Ba}	78.5 ± 1.50^{Aa}	79.00 ± 1.63^{Aa}	73.50 ± 2.58^{Aa}	59.50 ± 3.28^{Ab}
75	80.5 ± 1.16^{ABa}	80.5 ± 1.38^{Aa}	79.00 ± 1.94^{Ab}	73.0 ± 1.69^{Ab}	62.0 ± 1.10^{Ac}
100	82.5 ± 0.83^{Aa}	81.5 ± 0.76^{Aa}	77.0 ± 2.00^{Aab}	70.50 ± 3.53^{Ab}	55.50 ± 3.53^{Ac}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B) and different rows (a, b, c, d) are significant at least at $P < 0.05$.

Table 2
Effect of different heparin concentrations (μg/mL) and incubation time (hours) on hyperactivity of ram spermatozoa.

Concentration	Incubation time (hours)				
	0	1	2	3	4
Control	0.00 ± 0.0^{Ab}	0.00 ± 0.00^{Cb}	0.20 ± 0.13^{Cb}	1.20 ± 0.13^{Ba}	1.40 ± 0.16^{Ca}
10	0.00 ± 0.00^{Ab}	0.20 ± 0.13^{Cb}	2.40 ± 0.33^{Ba}	3.20 ± 0.24^{Aa}	3.00 ± 0.29^{Ba}
25	0.00 ± 0.00^{Ac}	1.00 ± 0.20^{BCb}	2.60 ± 0.26^{Ba}	3.20 ± 0.24^{Aa}	3.00 ± 0.51^{Ba}
50	0.00 ± 0.00^{Ab}	2.60 ± 0.80^{Aa}	3.60 ± 0.80^{ABa}	3.80 ± 0.24^{Aa}	3.80 ± 0.32^{Aba}
75	0.00 ± 0.00^{Ac}	1.80 ± 0.24^{ABb}	3.80 ± 0.61^{ABa}	3.60 ± 0.33^{Aa}	4.00 ± 0.36^{Aba}
100	0.00 ± 0.00^{Ac}	2.80 ± 0.32^{Ab}	5.0 ± 0.55^{Aa}	4.20 ± 0.32^{Aa}	5.00 ± 0.36^{Aa}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C) and different rows (a, b, c) are significant at least at $P < 0.05$.

Table 3
Effect of different heparin concentrations (μg/mL) and incubation time (hours) on total acrosome reaction of ram sperm.

Concentration	Incubation time				
	0	1	2	3	4
Control	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ca}	0.00 ± 0.00^{Ea}	0.00 ± 0.00^{Ea}	0.00 ± 0.00^{Ea}
10	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Cc}	0.00 ± 0.00^{Ec}	10.80 ± 1.78^{Db}	14.40 ± 1.49^{Da}
25	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Cc}	11.70 ± 1.51^{Db}	62.40 ± 2.78^{Ca}	64.30 ± 3.12^{Ca}
50	0.00 ± 0.00^{Ae}	8.10 ± 1.12^{Bd}	26.90 ± 1.59^{Cc}	79.00 ± 1.90^{Bb}	89.00 ± 1.97^{Aa}
75	0.00 ± 0.00^{Ad}	11.20 ± 1.27^{Ac}	54.80 ± 2.43^{Ab}	86.00 ± 2.24^{Aa}	88.70 ± 2.00^{Aa}
100	0.00 ± 0.00^{Ad}	13.20 ± 1.70^{Ac}	41.30 ± 1.97^{Bb}	81.10 ± 2.14^{ABa}	80.40 ± 2.50^{Ba}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C, D, E) and different rows (a, b, c, d, e) are significant at least at $P < 0.05$.

3.2.3. Effect on total acrosome reaction

Data regarding the effect of caffeine and incubation time on TAR% of ram spermatozoa are presented in Table 6. Irrespective to incubation time, when ram spermatozoa were treated with 1 mg/mL caffeine, a highly significant ($P < 0.01$) increase in total AR% of (19.90 ± 2.11) was noticed after 1 h of incubation. After 2 h of incubation, the total AR% was significantly higher ($P < 0.01$) in semen samples treated with 0.75, 1 and 2 mg/mL of caffeine than 0.5 mg/mL and control. The highest effect of treatment of caffeine on total AR% was observed during the last two hours of incubation period with 1 and 2 mg of caffeine concentrations. On the other hand, time dependent improvement in total AR% was detected. A maximum total AR% was noticed after 3 and 4 h incubation.

Table 4
Effect of different caffeine concentrations (mg/mL) and incubation time (hours) on motility of ram spermatozoa.

Concentration	Incubation time				
	0	1	2	3	4
Control	78.00 ± 1.69^{Aa}	76.00 ± 1.24^{Aa}	70.50 ± 1.57^{Aa}	62.00 ± 4.54^{Ab}	39.00 ± 3.48^{Ac}
0.5	77.50 ± 0.83^{Aa}	77.00 ± 1.69^{Aa}	73.00 ± 0.81^{Aa}	64.50 ± 3.76^{Ab}	36.50 ± 3.87^{Ac}
0.75	81.50 ± 0.76^{Aa}	77.00 ± 1.69^{Aab}	72.50 ± 0.83^{Ab}	62.50 ± 3.00^{Ac}	42.00 ± 3.09^{Ad}
1	73.00 ± 0.81^{Ba}	75.50 ± 1.38^{Aa}	69.00 ± 1.24^{Aa}	58.00 ± 4.42^{Ab}	41.00 ± 3.48^{Ac}
2	64.00 ± 2.21^{Ca}	66.00 ± 2.76^{Ba}	55.00 ± 3.57^{Bab}	50.00 ± 4.94^{Ab}	32.00 ± 3.74^{Ac}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C) and different rows (a, b, c) are significant at least at $P < 0.05$.

Table 5
Effect of different caffeine concentrations (mg/mL) and incubation time (hours) on hyperactivity of ram spermatozoa.

Concentration l	Incubation time				
	0	1	2	3	4
Control	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Dc}	0.00 ± 0.00 ^{Cc}	0.80 ± 0.24^{Db}	1.40 ± 0.16^{Ca}
0.5	0.00 ± 0.00^{Ab}	1.20 ± 0.32^{Cb}	2.80 ± 0.67^{Ba}	3.20 ± 0.53^{Ca}	3.80 ± 0.53^{Ba}
0.75	0.00 ± 0.00^{Ac}	2.40 ± 0.16^{Bb}	4.80 ± 0.71^{Aa}	5.80 ± 0.43^{Aa}	5.60 ± 0.16^{Aa}
1	0.00 ± 0.00^{Ac}	3.20 ± 0.48^{Ab}	4.40 ± 0.44^{ABa}	4.80 ± 0.32^{ABa}	5.20 ± 0.38^{Aa}
2	0.00 ± 0.00^{Ac}	1.20 ± 0.13^{Cb}	3.60 ± 0.44^{ABa}	3.80 ± 0.38^{BCa}	3.80 ± 0.24^{Ba}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C, D) and different rows (a, b, c) are significant at least at $P < 0.05$.

Table 6
Effect of different caffeine concentrations (mg/mL) and incubation time (hours) on total acrosome reaction of ram spermatozoa.

Concentration	Incubation time (hours)				
	0	1	2	3	4
Control	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Cc}	0.00 ± 0.00^{Cc}	13.40 ± 1.34^{Db}	24.70 ± 2.69^{Da}
0.5	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Cd}	11.40 ± 1.34^{Bc}	21.40 ± 1.52^{Cb}	44.90 ± 1.88^{Ca}
0.75	0.00 ± 0.00^{Ae}	10.70 ± 1.78^{Bd}	40.00 ± 2.77^{Ac}	57.40 ± 1.32^{Bb}	70.00 ± 2.27^{Ba}
1	0.00 ± 0.00^{Ad}	19.90 ± 2.11^{Ac}	42.00 ± 1.59^{Ab}	83.40 ± 1.37^{Aa}	79.40 ± 2.81^{Aa}
2	0.00 ± 0.00	12.60 ± 1.80	4.80 ± 3.70	9.60 ± 4.30	74.60 ± 5.70

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C, D) and different rows (a, b, c, d) are significant at least at $P < 0.05$.

3.3. Effect of different concentrations of calcium ionophore A23187 on ram sperm functions

3.3.1. Effect on sperm motility

Immediately after dilution different concentrations of calcium ionophore A23187 (0.5, 0.75, 1, 15.5 mM/mL) showed a significant ($P < 0.05$) increase in IM when compared to control (Table 7). There was no significant differences could be detected in IM between control sample and different concentrations of calcium ionophore A23187 treated samples during different hours of incubation period. After 3 h of incubation, IM% was significantly ($P < 0.05$) higher in semen samples treated with 1.55 mM/mL calcium ionophore than control. With respect to time factor, there was a significant ($P < 0.05$) decrease in motility simultaneous to the increase in incubation time.

3.3.2. Effect on sperm hyperactivity

Table 8 presents the effect of different concentrations of calcium ionophore A23187 and different incubation times on HA% of ram spermatozoa. There was significant increase in HA

Table 7
Effect of different Ca^{2+} inophoreA23187 concentrations (mM/mL) and incubation time (hours) on motility of ram spermatozoa.

Concentration	Incubation time				
	0	1	2	3	4
Control	76.50 ± 1.50^{Ba}	76.50 ± 1.70^{Aa}	76.00 ± 1.10^{Aa}	67.00 ± 1.52^{Bb}	62.00 ± 2.25^{Ac}
0.5	81.50 ± 1.83^{Aa}	81.50 ± 1.30^{Aa}	75.50 ± 1.38^{Ab}	73.00 ± 1.69^{ABb}	65.50 ± 1.74^{Ac}
0.75	80.50 ± 1.57^{Aba}	81.50 ± 1.30^{Aa}	79.00 ± 1.45^{Aa}	72.00 ± 2.70^{ABb}	63.00 ± 2.37^{Ac}
1	82.50 ± 0.83^{Aa}	80.50 ± 1.38^{Aab}	75.50 ± 1.89^{Abc}	72.00 ± 2.37^{ABc}	64.00 ± 2.44^{Ad}
1.55	84.00 ± 0.66^{Aa}	80.50 ± 1.38^{Aab}	75.00 ± 1.29^{Ab}	75.50 ± 1.74^{Ab}	62.00 ± 3.09^{Ac}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B) and different rows (a, b, c, d) are significant at least at $P < 0.05$.

Table 8
Effect of different Ca^{2+} ionophoreA23187concentrations (mM/mL) and incubation time (hours) on hyperactivity of ram spermatozoa.

Concentration	Incubation time				
	0	1	2	3	4
Control	0.00 ± 0.00^{Aa}	0.60 ± 0.39^{Ca}	1.20 ± 0.79^{Ba}	0.6 ± 0.26^{Ba}	0.60 ± 0.26^{Ba}
0.5	0.00 ± 0.00^{Ab}	2.80 ± 0.24^{ABa}	2.80 ± 0.48^{ABa}	4.00 ± 0.81^{Aa}	2.60 ± 0.44^{ABa}
0.75	0.00 ± 0.00^{Ab}	3.60 ± 0.77^{Aa}	3.00 ± 0.66^{ABa}	1.60 ± 0.65^{ABab}	2.20 ± 0.67^{ABa}
1	0.00 ± 0.00^{Ab}	1.80 ± 0.48^{BCa}	2.60 ± 0.44^{Aba}	2.40 ± 0.68^{ABa}	3.40 ± 0.88^{Aa}
1.55	0.00 ± 0.00^{Ac}	1.60 ± 0.44^{BCb}	3.00 ± 0.00^{Aab}	3.60 ± 0.74^{Aa}	3.00 ± 0.72^{ABab}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C) and different rows (a, b, c) are significant at least at $P < 0.05$.

Table 9
Effect of different Ca^{2+} ionophore A23187 concentrations (mM/mL) and incubation time (hours)on total acrosome reaction of ram spermatozoa.

Concentration	Incubation time				
	0	1	2	3	4
Control	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Cc}	0.00 ± 0.00^{Dc}	17.70 ± 1.24^{Db}	30.00 ± 1.29^{Ca}
0.5	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Cd}	21.20 ± 1.94^{Cc}	58.40 ± 2.28^{Cb}	72.30 ± 3.20^{Ba}
0.75	0.00 ± 0.00^{Ae}	12.50 ± 1.54^{Bd}	61.50 ± 2.02^{Ac}	81.10 ± 2.16^{Bb}	81.20 ± 2.43^{Aa}
1	0.00 ± 0.00^{Ae}	10.50 ± 1.03^{Bd}	49.30 ± 3.15^{Bc}	92.10 ± 1.38^{Aa}	84.90 ± 3.38^{Ab}
1.55	0.00 ± 0.00^{Ae}	18.20 ± 0.86^{Ad}	60.50 ± 2.79^{Ac}	97.30 ± 2.31^{Aa}	85.90 ± 1.68^{Ab}

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B, C, D) and different rows (a, b, c, d, e) are significant at least at $P < 0.05$.

Table 10
Effect of best concentration of different capacitating active compounds on in. vitro fertilization of ovine oocytes.

Treatment	Number of matured oocytes	Penetration rate (%)	Fertilization rate (%)	Abnormal fertilization (%)
Caffeine	28	13/28(46.43)[a]	12/28(42.86)[abc]	1/28(3.57)[c]
Heparin	34	19/34(55.88)[a]	18/34(52.94)[a]	1/34(2.94)[c]
Ca. ionophore	46	27/46(58.69)[a]	21/46(45.65)[abc]	6/46(13.04)[b]

Data were expressed as Means ± SE, different alphabetical superscripts in the same columns (A, B) and different rows (a, b, c) are significant at least at $P < 0.05$.

% for different concentrations of calcium ionophore A23187 as compared to control one without any significant difference between them. Irrespective to concentration, maximum HA% was observed in semen samples after 2 h incubation.

3.3.3. Effect on total acrosome reaction percentage

Table 9 describes the effect of calcium ionophore A23187 and incubation time on percentage of total AR% of ram spermatozoa. A significant ($P < 0.05$) increase of total AR% was noticed in all calcium ionophore A23187 treated spermatozoa. The maximum effect was detected in samples treated with 1.55 mM/mL as compared to control. Likewise, a significant ($P < 0.05$) increase in total AR% was noticed throughout the incubation period with maximum effect after 3 and 4 h.

3.4. Effect of best concentration of heparin, caffeine or calcium ionophore A23187 on in vitro fertilization of ewe oocytes

Table 10 showed that penetration rates of rate of oocytes inseminated with spermatozoa treated with calcium ionophore A23187, and heparin were higher as compared with caffeine. Moreover, heparin achieved higher fertilization rates but without significant difference with others.

4. Discussion

The *in vivo* sperm capacitation occurs during migration in the reproductive tract of the female, whereas *in vitro* capacitation requires the exposition of fresh or freeze semen to specific capacitating agents [22]. Capacitation process is a prerequisite step for sperm to bind to the zona pellucida, it tested acrosomic reaction in response to natural agonistics and express hypermotility, a special movement that allows spermatozoa to move in the viscose fluid of the oviduct and get into the zona pellucida [23].

In the present study, different concentrations of heparin were used. Regarding the effect of heparin on motility, treatment of ram spermatozoa with 10 and 75 μg/mL resulted in improved individual motility percentage. In addition, 100 μg/mL heparin achieved best HA% after 4 h incubation. In this respect, [24] indicated that heparin at dose 10 μg/mL potentially induces the capacitation of ram fresh ejaculated and frozen-thawed spermatozoa *in vitro*. However, some other authors utilized 100 μg heparin with 1 h of incubation in ram [25] or 2 h of incubation to promote sperm capacitation [26] in bull semen. Furthermore, the capacitation of epididymal ram spermatozoa after incubation in TALP media with 100 μg/mL heparin, resulted in the best result for sperm maturation and capacitating *in vitro* [27]. Heparin is a potent glycosaminoglycan required for bovine sperm capacitation [28] that has been shown to improve the fertilizing ability of goat and ram spermatozoa when used in addition to ESS [29]. This increase in motility and HA% is due to the ability of heparin to induce increased calcium uptake, increased intracellular free calcium and decreased calmodulin concentration in spermatozoa [30–32].The present finding revealed that, ram spermatozoa treated with 100 μg/mL heparin showed maximum increase in the incomplete AR% after 2 and 3 h incubation. In addition, the tendency of spermatozoa treated with 50 or 75 μg/mL heparin to show complete AR after longer incubation time was very high if compared to other concentrations. Moreover, 50 and 75 μg/mL resulted in a maximum total AR%. The data obtained by us are better than 9.6% and16.5% results obtained by [33,34], respectively in sheep. This is difference may be attributed to use different concentration of heparin (2.5 μg/mL) and short incubation period (2 h).

With respect to the effect of caffeine on ram sperm functions, the current study showed that, supplementation of ram semen with low concentration (up to 1 mg/mL) of caffeine during swim up revealed a significant ($P < 0.05$) improvement in sperm motility and hyperactivity in a dose dependent manner, with maximum motility after 1 h incubation. Similar tendency was observed for hyperactivity after 2 h. Higher caffeine concentration or longer incubation time resulted in a reverse effect in sperm motility. Similar finding was obtained by [35,36]. The reduction of the motility of *in vitro* capacitated spermatozoa treated with heparin or caffeine may be due to reduction of the glycosylable substrates like glucose and/or fructose, and also to a reduction in the availability of pyruvate and lactate, substrates that during the cell metabolism are primordial to produce ATPs [37]. Caffeine was reported to induce an increase in intracellular calcium and an immediate hyperactivation of ram sperm [38]. The essential role of calcium on sperm capacitation has been proved in several mammalian species, and an increase in intracellular Ca^{2+} concentration has been shown in hyperactivated flagella. Thus, under our experimental conditions treatment of ram spermatozoa with 1 mg/mL of caffeine for 4 h was considered the best concentration of caffeine to be used for IVF. However, other study [39] indicates that concentration of 4 mmol has beneficial effects on ram sperm motility. They observed positive impact of this concentration on sperm motility at all time points. This concentration of caffeine may be a threshold value for stimulating ram sperm.

Regarding the effect of the calcium ionophore A23187, the current work suggested that significant effect could be detected in all treated spermatozoa with different concentrations as compared to control one. On the other hand, higher concentrations (1.55 mM/mL) resulted in a significant ($P < 0.01$) increase in motility percent if compared to control after 2 h of incubation. With respect to time factor, there was a significant ($P < 0.05$) decrease in motility simultaneous to the increase in incubation time. The reverse trend in the percent of individual motility of ram spermatozoa was also noticed in by [40,41] who reported that maximum response was achieved at an ionophore concentration of 1 μM or above, whereas during two hours' incubation ionophore concentrations of 0.01 μM or less had no

observable. They added that, calcium ionophore treatment had a dramatic effect on sperm motility. In the presence of Ca^{2+}, levels of A23187 at or above 0.1 μM abolished motility. The decline of motility simultaneous to the increase in incubation time may be due to reduction in cellular ATPases, which fail to maintain normal intracellular calcium concentrations not only in head region, but also in midpiece where it affects mitochondrial function [42]. Hyperactivated motility in the current study was significantly ($P < 0.01$) increased in samples treated with different concentrations of calcium ionophore A23187 as compared to control one without any significant difference between them. Irrespective to concentration, maximum HA% was detected in spermatozoa after 2 h incubation. Calcium ionophore A23187 induced an immediate calcium uptake into both fresh and frozen-thawed bull spermatozoa [43].

Regarding the effect on acrosome reaction, our results revealed that incomplete AR% was significantly increased corresponding to different concentrations of calcium ionophore A23187 with higher tendency to undergo complete and total AR % in spermatozoa treated with 1.55 mM/mL (over 90%) after 3 h of incubation at 37 °C. These results are however, less comparable with the findings of [9,44] who reported that the proportion of the population which had undergone a spontaneous acrosome reaction increased over 3–8 h, indicating a measure of asynchrony, whereas over 90% of the cells undergoing a reaction within 30 min of exposure to calcium ionophore. On other hand, there was a marked interaction between concentration of A23187 and incubation time upon the number of spermatozoa having undergone AR. As the concentration of A23187 increased, the percentage of spermatozoa with intact (non-reacted) acrosomes decreased over the incubation time [41]. In conclusion, under our experimental conditions, the best concentration of heparin, caffeine and ionophore A23187 are 75 μg/mL, 1 mg/mL, 1.55 mM/mL for 3, 1, 4 h incubation respectively and can be used for *in vitro* fertilization in sheep.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. *Physiology of reproduction*. New York: Raven Press; 1994, p. 189-317.

[2] Jaiswal BS, Cohen-Dayag A, Tur-Kaspa I, Eisenbach M. Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction. *FEBS Lett* 1998; **427**: 309-313.

[3] Bedu-Addo K, Lefièvre L, Moseley FLC, Barratt CLR, Publicover SJ. Bicarbonate and bovine serum albumin reversibly 'switch' capacitation-induced events in human spermatozoa. *Mol Hum Reprod* 2005; **11**: 683-691.

[4] Huang YH, Chen YH, Lin CM, Ciou YY, Kuo SP, Chen CT, et al. Suppression effect of seminal vesicle autoantigen on platelet-activating factor-induced mouse sperm capacitation. *J Cell Biochem* 2007; **100**: 941-951.

[5] Kheradmand A1, Sookhtezary A1, Moosavi SM, Joorabi S. The effect of platelet activating factor on the motility and acrosome reaction of ram spermatozoa. *Iran J Vet Res* 2009; **10**: 315-322.

[6] Davis BK. Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. *Proc Natl Acad Sci USA* 1981; **78**: 75-80.

[7] Jones RE. Synthesis of ether lipids and phosphatidylethanolamine by ejaculated human spermatozoa. *Arch Androl* 1997; **38**: 181-189.

[8] Sukardi S, Elliott RM, Withers JO, Fontaine U, Millar JD, Curry MR, et al. Calcium-binding proteins from the outer acrosomal membrane of ram spermatozoa: potential candidates for involvement in the acrosome reaction. *Reproduction* 2001; **122**: 939-946.

[9] Watson PF, Jones PS, Plummer JM. A quantitative comparison of the spontaneous and ionophore-induced acrosome reaction in ejaculated ram spermatozoa: the effects of temperature, time and individual. *Anim Reprod Sci* 1991; **24**: 93-108.

[10] Shamiah Sh M, Saifelnasr EOH, El-Sayed Eetidal H, Abdel-Khalek AE, Ashmawy TM, El-Gendy ME. *In vitro* induction of the acrosome reaction in local Egyptian rams spermatozoa by calcium ionophore (A23187). *Egypt J Anim Prod* 2015; **52**(2): 129-134.

[11] Kim EY, Noh EH, Ji Noh E, Park MJ, Park HY, Lee DS, et al. Effect of glycosaminoglycans on *In vitro* fertilizing ability and *In vitro* developmental potential of bovine embryos. *Asian Australas J Anim Sci* 2013; **26**: 178-188.

[12] Barakat IAH, Danfour MA, Galewan FAM, Dkhil MA. Effect of various concentrations of caffeine, pentoxifylline, and kallikrein on hyperactivation of frozen bovine semen. *Bio Med Res Int* 2015: 7948575. http://dx.doi.org/10.1155/2015/948575.

[13] El-Shahat KH. *Studies on in vitro fertilization of small ruminant oocytes*. PhD. Thesis submitted to Faculty of Vet Med Cairo University. 2001.

[14] Kawakami E, Arai T, Nakamura U. Effects of medium containing heparin and theophylline on capacitation and metabolic enzyme activities of ejaculated spermatozoa from dogs with asthenozoospermia. *Anim Reprod Sci* 1999; **54**: 251-259.

[15] Darwish GM. Evaluation of fertilizing efficiency of buffalo spermatozoa *in vitro* subsequent using some capacitating agents. *J Egypt Vet Med Assoc* 2004; **64**(4): 323-336.

[16] El-Amrawi GA, Nemetallah BR. Study on capacitation of buffalo spermatozoa. In: *3rd Annual Congress of Egypt Society of Animal Reproduction Fertility, Cairo*; 1992, p. 214-222.

[17] El-Shahat KH. Effect of addition of follicular fluid, oviductal or granulose cell co-culture on cleavage and embryonic development of buffalo oocytes. *Vet Med J Giza* 2011; **59**(4): 33-38.

[18] Marie WF. *The effect of various capacitating active compounds and capacitation time on the in vitro fertilization of buffalo sperm*. Ph.D. Thesis submitted to Faculty of Vet Med Cairo University. 2005.

[19] Amer HA, Hegab AO, Zaabal SM. Effects of ovarian morphology on oocytes quantity and quality, granulose cells, *in vitro*. *Anim Reprod* 2008; **5**: 55-62.

[20] Abeydeera LR, Wei-Hua W, Prather RS, Day BN. Maturation *in vitro* of pig oocytes in protein-free culture media: fertilization and subsequent embryo development *in vitro*. *Biol Reprod* 1998; **58**: 1316-1320.

[21] Dell' Aquila ME, Cho YS, Minoia P, Traina V, Fuscos S, Lacalandra GM, et al. Intracytoplasmic sperm injection (ICSP) versus conventional IVF on abattoir- derived and *in vitro*-matured equine oocytes. *Theriologenology* 1997; **47**: 1139-1156.

[22] Liu Y, Wang DK, Chen LM. The physiology of bicarbonate transporters in mammalian reproduction. *Biol Reprod* 2012; **86**(4): 33-37.

[23] Hiromi K, Joachim B, Josef H, Werner L, Akira I. Effects of heparin on the time course of sperm penetration during *in vitro* fertilization in sheep. *Mem Res Inst Kinki Univ Jpn* 2000; **3**: 19-26.

[24] Willaims RM, Graham JK, Hammerstedt RH. Determination of the capacity of ram epididymal and ejaculated sperm to undergo the acrosome reaction and penetrate ova. *Biol Reprod* 1991; **44**(6): 1080-1091.

[25] Schellander K, Brackett BG, Keefer CL, Fayrer-Hossken AR. Testing capacitation of bull sperm with zona-free hamster ova. *Anim Reprod Sci* 1989; **18**: 95-104.

[26] Majeed AF, Al-Jumaily MMT. *In vitro* capacitation of ram spermatozoa. *Al-Anbar J Vet Sci* 2011; **4**: 94-96.

[27] Parrish JJ. Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. *Theriogenology* 2014; **81**: 67-73.

[28] Cox JF, Alfaro V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. *Reprod Dom Anim* 2007; **42**: 83-87.
[29] Handrow RR, First NI, Parrish JJ. Calcium requirement and increased association with bovine sperm during its capacitation by heparin. *J Exp Zool* 1989; **252**: 174-182.
[30] Leclerc P, Sirard MA, Chafouleas JG, Lambert RD. Decrease in calmodulin concentrations during heparin-induced capacitation in bovine spermatozoa. *J Reprod Fertl* 1992; **94**: 23-32.
[31] Leclerc P, de Lamirande E, Gagnon C. Cyclic adenosine 3′,5′-monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* 1996; **55**: 684-692.
[32] Thompson JCE, Cummins JM. The effects of washing and protein supplernentation on the acrosome reaction of ram spermatozoa *in vitro*. *Anim Reprod Sci* 1985; **9**(1): 75-86.
[33] Ferrari S, Barnabe YH, Zuge RM, Zogno MA. Effect of two ram sperm capacitating media on acrosome reaction and zona free hamster oocyte penetration test. *Braz J Vet Res Anim Sci São Paulo* 2000; **37**(3): 229-233.
[34] Cohen-Dayag A, Tur-Kaspa I, Dor I, Mashiach S, Eisenbach M. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. *Proc Natl Acad Sci USA* 1995; **92**: 11039-11043.
[35] El-Gaafary MN. *The characteristics of semen from Welsh Mountain and Cambridge rams*. Ph.D. thesis submitted to UCNW, Bangor, Me, U S A. 2010.
[36] Fernández RF, Gutiérrez GY, Gómez NNE, Hernández PJE. Motilidad y Sobrevivencia Espermática *in vitro* Con La Utilización De Pirofosfato De Tiamina En Semen Caprino. *Rev Salud Anim* 2003; **25**(1): 34-38.
[37] Colás C, Cebrian-Perez JA, Muino-Blanco T. Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. *Int J Androl* 2010; **33**: 187-197.
[38] HO HC, Suarez SS. Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction* 2001; **122**: 519-526.
[39] Špalekova E, Makarevich AV, Pivko J. Effect of caffeine on parameters of ram sperm motility. *Slovak J Anim Sci* 2011; **44**(2): 78-83.
[40] Shams-Borhan G, Harrison R. Production, characterization, and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete Res* 1981; **4**: 407-432.
[41] Ucar Ö, Parkinson TJ. *In vitro* induction of the acrosome reaction in ovine spermatozoa by calcium ionophore A23187. *Acta Vet Hung* 2003; **51**(1): 103-109.
[42] Purohit SB, Laloraya M, Kumar GP. Role of ions and ion channels in capacitation and acrosome reaction of spermatozoa. *Asian J Androl* 1999; **1**: 95-107.
[43] Landim-Alvarenga FC, Graham JK, Alvarenga MA, Squires EL. Calcium influx into equine and bovine spermatozoa during *in vitro* capacitation. *Anim Reprod* 2004; **1**(1): 96-105.
[44] Flechon JE, Harrison RA, Flechon B, Escaig J. Membrane fusion events in the Ca^{2+} ionophore-induced acrosome reaction of ram spermatozoa. *J Cell Sci* 1986; **81**: 43-63.