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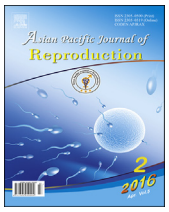

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Effect of heparin, caffeine and calcium ionophore A23187 on *in vitro* induction of the acrosome reaction of fresh ram spermatozoa

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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