Effect of Royal Jelly on the Fertilizing Ability of Buffalo Spermatozoa *In Vitro*

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Abstract: The aim of the present study was to assess the effect of addition of Royal jelly in presence of heparin on buffalo (Bubalus Bubalis) sperm motility, acrosome reaction and *in vitro* fertilization (IVF) of buffalo oocytes. Frozen buffalo spermatozoa from five bulls were thawed and motile fraction was obtained by swim up technique. The spermatozoa were washed, treated with100 µg/ml heparin, and then exposed to 0.4% Royal Jelly (RJ) for 3 h. Sperm motility, acrosomal integrity and fertilization rate of matured oocytes were assessed at 1, 2 and 3 h. The percentages of sperm motility, intact acrosome and fertilization rate of matured oocytes were higher ($P<0.05$) in 0.4% RJ compared to that in the control. After 2 h of incubation the percentage of motility, intact acrosome of spermatozoa and fertilization rate of matured oocytes, respectively, were 93.6 %, 77.6% and 72.6% in 0.4% RJ. These results suggest that treating buffalo sperm with 0.4% RJ in combination with heparin is effective not only to induce sperm acrosome reaction but also is effective for *in vitro* fertilizing capacity of the cryopreserved buffalo spermatozoa.

Keywords: Buffalo, Capacitation, Spermatozoa, IVF, Royal Jelly.

INTRODUCTION

The enhancement of artificial insemination is a valuable tool in genetic improvement programs [1]. However, the biggest problem to exploiting the cryopreserved buffalo semen is damage of sperm membrane structure during freezing and thawing which lead to a fewer viable and motile cell post-thawing [2] Therefore, An important factor in the efficiency of extender is supplementation with royal jelly to enhance the viability and longevity of post-thawed spermatozoa [3]. *In vitro* fertilization rate of *in vitro* matured oocytes using frozen thawed spermatozoa was low due to low fertilizing ability of buffalo spermatozoa [4]. Therefore, there is an urgent need to study the possibilities of improving fertilizing ability of buffalo spermatozoa.

Royal jelly is secreted by the hypopharyngeal glands of worker bees to feed young larvae and the adult queen bee. On dry matter basis, royal jelly contains considerable amounts of proteins, lipid, sugars and amino acids. Royal jelly also contains vitamin A, B (pantothenic acid, has antioxidant effect), C, D and E, mineral salts are in descending order: K, Ca, Na, Zn, Fe, Cu, and Mn, enzymes antibiotic components. It also has an abundance of nucleic acid-DNA and RNA. Also it contains sterols, phosphorous compounds and acetylcholine, which is needed to transmit nerve messages from cell to cell [5, 6].

Mammalian sperm must undergo a series of controlled molecular processes called capacitation before they are capable of penetrating and fertilizing the oocyte. Although capacitation naturally occurs in the female reproductive tract [7], it can be also induced *in vitro* using specific media and physical conditions [8]. Capacitation is a complex molecular process that results in changes of calcium concentration, protein phosphorylation, acrosomal matrix and membrane rearrangement.

Parrish *et al*. [9] reported that increased fertilization rates *in vitro* were obtained when frozen-thawed spermatozoa were treated with heparin which is known as the most potent glycosaminoglycan for inducing capacitation and acrosome reactions

Vijayaraghavan *et al*. [10] reported that the various adenosine analogues contains motility stimulants such as the adenosine monophosphate ((AMP) N (1)-oxide), are already known to enhance the motility of sperm by an inhibiting phosphodiesterase activity, thus enhancing cAMP at the level of the sperm tail. The same author found that AMP N (1)-oxide stimulated the phosphorylation of not only mitogen-activated protein kinase (MAPK) but also that of cAMP/calcium-response element-binding protein (CREB) [10]. In mouse spermatozoa, adenosine modulates the adenylyl cyclase/cAMP pathway [11] and induces sperm response *via* adenosine A_2 receptors in uncapacitated cells and through adenosine A_1 receptors in capacitated cells [12].

Fertility is equally related to the quality of the spermatozoa and oocytes apart from appropriate conditions for fertilization and embryo development. In recent years, only one study has been focused on

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improving the quality of buffalo spermatozoa by addition of RJ *in vitro* [3]. There fore an important goal for the present study was to determine the effect of addition of royal jelly on buffalo spermatozoa through investigation the effect of royal jelly on spermatozoa motility, acrosome reaction and *in vitro* fertilizing potentials of frozen-thawed buffalo spermatozoa.

MATERIALS AND METHODS

Chemicals

All materials were purchased from Sigma Chemical Company (St Louis, Missouri) unless otherwise indicated.

Royal Jelly

Pure royal jelly capsules were used in this study (Pharco Pharmaceuticals Co., Egypt). Each capsule (1000 mg) was dissolved in 10 ml double distilled water to get concentration of 100 mg ml^{-1}.

Semen Collection and Processing

Frozen semen samples were utilized from four buffalo (Bubalus Bubalis) bulls of proven fertility aged between 2 and 6 years, kept at the Artificial Insemination Center, Beni-Suef Governorate, Egypt. All the samples were collected within a month. The bulls were consistently giving ejaculates with a concentration of > 500 million cells/ml, pre-freeze motility of > 60%, and total abnormality of < 20%. The post-thaw motility of the semen was also consistently >40%. The animals were reported to have good fertility after artificial insemination.

Three straws from each treatments were thawed in water bath at 38ºC for 30 sec and motile spermatozoa were separated by swim up technique [8] in the fertilization medium, modified Tyroid's Albumine Lactate-Pyruvate (TALP) containing bovine serum albumin (BSA) for I h as recorded by Parrish *et al.* [9]. Sperm suspension was incubated for various periods between 1 and 3 h at 37ºC under 5% CO_2 in air. The upper most layer of the medium containing the most spermatozoa was collected and washed twice by centrifugation at 2000 rpm for 10 minutes. The sperm pellet was resuspended in the fertilization medium containing 10 µg/ml heparin and 0.4% RJ. The concentration of RJ was chosen according to the results previously obtained in this laboratory [3]. Sperm drops (50 µl) were made on the petri-dishes and covered with paraffin oil and were incubated for the relevant time (1, 2, 3 h) under paraffin oil at 37°C, 5% CO_2. During incubation, sperm motility and acrosome reaction were assessed at hourly interval for 3 h.

Assessment of Sperm Motility

A 50-ul droplet of the spermatozoa and Tris-RJ extender mixture was placed on a pre-warmed, clean microscopic slide (37ºC) and covered with a pre-warmed (35°C), clean cover slip (18 × 18 mm). A video recording was made using light microscope (× 400) with an attached video camera, a video monitor and a video machine. A minimum of 500 spermatozoa from at least two different drops was analyzed for each sample [4, 13].

Assessment of Acrosomal Reaction

Dual staining procedure initially developed by Sidhu *et al.* (14), which was used with some modification to identify the clear acrosome structure of buffalo spermatozoa. One hundred microliters of semen were mixed with 0.2 percent trypan blue (in TALP medium without BSA) and incubated for 10 min on a clean glass slide at 37°C. After the incubation period, smears of the semen were prepared gently on the glass slides and allowed to dry for 15 min at room temperature. A 0.72% (W/V) Giemsa stock solution was prepared by dissolving 1 g of Giemsa dye in a glycerol-methanol mixture (54:84). One gram of Giemsa was diluted five times with distilled water (final concentration of Giemsa working solution was approximately 0.15%). The smears of spermatozoa previously stained with trypan blue were then stained with Giemsa for 1 h at room temperature to evaluate the acrosomal status of the spermatozoa. Smears were dried between the folds of filter paper and stored. The dried smears were studied at 1000X under a light microscope using oil immersion without cover-glass. The slides (200) were used for measurement within a week of preparation.

Evaluation of *In Vitro* Fertilizing Potential of the Treated Semen

Ovaries from sexually matured buffaloes were obtained from slaughter house within one hour of slaughter and transported to the laboratory at 25-30ºC in phosphate buffer saline supplemented with antibiotics. Ovaries were washed two or to three times in PBS. Oocytes were collected by aspiration technique with washing medium (Tissue culture medium-199, TCM-199). Oocytes with compact and dense cumulus cell layers were washed thrice and oocytes (8-10) were introduced into 50 µl of maturation medium (TCM-199

supplemented with 10% fetal calf serum) and cultured for 24 h under sterile paraffin oil at 38ºC, 5% CO_2 and 95% humidity. Oocytes maturation was assessed by cumulus expansion and release of first polar body [15].

At the end of 1, 2, 3 h sperm incubation periods, 50 µl sperm suspension was added to the fertilization drops, containing the *in vitro* matured buffalo oocytes and incubated under sterile mineral oil for 6 h. After that, the inseminated oocytes were freed from extra cumuls cells and attached spermatozoa by gentle pipetting then cultured in TCM-199 supplemented with 10% fetal calf serum for 2 days. The proportion of cleaved oocytes was recorded 48 h after insemination.

Statistical Analysis

Values expressed in the figures and tables are Means± SE from a minimum of 5 experiments. Statistical analysis was carried out by Student t test [16].

RESULTS

Sperm Motility

More than 90% of spermatozoa were found motile throughout the preincubation period up to 3 h in RJ and most of the motile sperm were showing hyperactivated movement. After insemination, sperm looked more vigorous in the medium with RJ than in control by subjective observation. The sperm motility (percent) was significantly higher ($p < 0.05$) in the RJ group compared with that in the control group at 1 h, 2 h and 3 h incubation (Table **1**).

Acrosomal Reaction

The percentages of sperm exhibiting an acrosome reaction in the presence of 0.4% RJ are presented in Table **1**. The acrosomal reacted spermatozoa (percent) was significantly higher ($p < 0.05$) in the RJ group compared with that in to the control group at 1 h (80.1±3.02 vs. 70.4±3.95), 2h (77.6±3.06 vs. 61.7±3.95), and 3h (71.3±3.14 vs. 55.6±5.06) of incubation.

In Vitro Fertilization

When sperm were incubated in the TALP medium with RJ, there was an overall increase in fertilization rate in all pre-incubation times periods (Table **1**). Highest IVF rates (72.6-75.1%) were obtained when spermatozoa were incubated for 1-2 h.

DISCUSSION

Cryopreservation induces sublethal damage to the spermatozoa, thereby reduce their fertile life. The present study revealed that addition of 0.4% RJ in presence of heparin to the capacitation medium improves buffalo semen characteristics in a dose dependant manner. These results were in consistent with the finding of our report [3]. This may be attributed to the effect of RJ containing motility stimulants such as adenosine and adenosine monophosphate ((AMP) N (1)-oxide), which are already known to enhance the motility of sperm by an inhibiting phosphodiesterase activity, thus enhancing cAMP at the level of the sperm tail and stimulated the phosphorylation of not only mitogen-activated protein kinase (MAPK) but also that of cAMP/calcium - response element-binding protein [8, 10, 11]. Additionally, RJ contains calcium which in presence of heparin enhances of capacitation process.

The highest fertilization rates were obtained with spermatozoa preincubated in 0.4% RJ for 1-2 h (Table **1**). The sperm preincubation time was extended to 3 h in order to examine if the longer preincubation would improve the fertilization of matured oocytes. The results presented in Table **1** show that this was the case, at least partly, suggesting that the pre-incubation time beyond the standard 3 h, which has been shown to be

Table 1: Effect of Capacitaion Medium Fortification with Royal Jelly on the Buffalo Semen Characteristics and the *In Vitro* Fertilization Rate

Incubation Period (h)	Sperm motility		Acrosomal Reaction		Fertilization rate	
	Control	0.4% RJ	Control	0.4% RJ	Control	0.4% RJ
0	92.3±3.02	92.3±3.02	75.3±3.02	75.3±3.02	48.3±3.02^{a}	48.3±3.02^{a}
1	90.4±3.95^{a}	95.1±3.02^{b}	70.4±3.95^{a}	80.1±3.02^{b}	35.4±3.95^{b}	75.1±3.02^{c}
2	80.7±3.95^{a}	93.6±3.06^{b}	61.7±3.95^{a}	77.6±3.06^{b}	30.7±3.95^{c}	72.6±3.06^{b}
3	70.6±5.06^{a}	90.3±3.14^{b}	55.6±5.06^{a}	71.3±3.14^{b}	26.6±5.06^{d}	65.3±3.14^{e}

^{a,b}Values with different superscript letters between groups for a particular attribute differ significantly ($p>0.05$).

sufficient for fertilization of matured oocytes, may be required for full attainment of capacitation to interact with matured oocytes.

CONCLUSIONS

The results of present study indicted that, the addition of royal jelly with heparin in capacitation medium enhanced the motility, acrosomal reaction of buffalo spermatozoa and the *in vitro* fertilizing capacity of the cryopreserved buffalo spermatozoa.

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