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Ameliorating effect of royal jelly on viability and longevity of frozen-thawed buffalo spermatozoa

Wpływ dodatku mleczka pszczelego na żywotność i przeżywalność zamrożonych-rozmrożonych plemników bawołu

Summary. Cryopreserved buffalo semen is generally acknowledged to have an impaired fertility by comparison with fresh semen. The reduction arises from both a lower viability post-thaw and sublethal dysfunction in a proportion of the surviving subpopulation. Thus, the present study was conducted to observe the effect of addition of royal jelly on subsequent survival and acrosomal integrity of buffalo spermatozoa during post-thawing incubation. Frozen-thawed semen samples were washed in Tris extender containing different concentrations of royal jelly (RJ): 0.0 control, 0.1, 0.2, 0.3, 0.4, 0.5%. Spermatozoa motility and viability in addition acrosomal integrity of thawed samples were assessed after thawing and at 30 min intervals during incubation at 37°C for 120 min. Results of experiment showed that the percentages of sperm function parameters were improved with an increase of royal jelly in Tris extender from 0.0 to 0.4% ($P < 0.05$). Statistical analysis for sperm motility and viability in addition acrosomal integrity (Tables 1, 2, 3) showed that best results (significant higher $P < 0.05$) occurred with RJ doses of 0.4 and 0.5% especially at 30–60 min incubation periods than that control. Therefore, the addition of 0.4% of royal jelly in Tris extender is recommended for dilution of thawed semen. These results indicated that addition of 0.4% RJ to Tris-buffer enhance and maintain the viability and longevity of buffalo spermatozoa for a long period and this additive can be used for increasing the possibility of collision between spermatozoa and ova during insemination.

Key words: royal jelly, buffalo, sperm, longevity, viability

INTRODUCTION

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, amino acids including 8 essential amino acids particularly rich in cystine, lysine and arginine, hormone rich substance (testosterone and insulin like growth factor-1), lipid, and sugars, 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 [Hove *et al*. 1985, Kodai *et al*. 2007]. The duration of motility and other sperm characteristics during post-thawing incubation is an indication of the usability of the semen [Saacke and White 1972]. The maintenance of higher motility in sperm during post-thawing incubation reflects a greater likelihood of their survival in the female genital tract to undergo capacitation and fertilize ova [Fiser *et al*. 1991].

The reasons for the loss of fertility are various such as factors affecting the proportion of survivors (e.g., cold shock susceptibility, cooling rate, diluent composition and osmotic stress) and factors influence functional status of survivors of sperm cells (e.g., membrane stability, oxidative damage, membrane receptor integrity, nuclear structure) [Aitken 1994].

Spermatozoa, unlike other cells, are unique in structure, function, and very susceptible to damage by ROS [Alvarez and Storey 1989]. It is worth mentioning that increased amount of ROS during freeze-thawed semen can be responsible for mitochondrial dysfunction, DNA, RNA and protein damage and lipid peroxidation [Alvarez and Storey 1989]. In effect, the membrane of mitochondria is highly susceptible to lipid peroxidative damage which may therefore result in impaired control of intracellular Ca^{2+} (as the mitochondria has a high Ca^{2+} sequestering ability), inhibition of respiration and depletion of ATP [Guerin 2001]. Lipid peroxidation has also been shown to induce irreversible loss of sperm motility, leakage of intracellular enzymes, damage to the chromatin and decreased capacity of sperm-oocyte plasma membrane fusion.

Functionally active spermatozoa membrane and the mitochondrial activity are required for spermatozoa metabolism [De Lamirande and Gagnon 1992]. However, spermatozoa motility reflects their mitochondrial function indirectly. The spermatozoa motility, viability and acrosomal integrity of sperm cells correlate positively with fertility of bovine bulls [Garner *et al*. 1997].

No report is available on elucidation of the role of RJ on spermatozoa function parameters in buffaloes. Thus, the aim of the present study was to observe the effect of addition of different concentrations of RJ on motion characteristics and acrosomal integrity of buffalo spermatozoa during post-thawing incubation.

MATERIALS AND METHODS

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 samples

Frozen semen straws from buffalo bull thawed by immersion in 37°C for 45 s and the semen was pooled and were mixed well in Tris buffer (0.25 M Tris, 80 mM citric acid, 69 mM fructose and antibiotics) and RJ in different concentrations (0.0, 0.1, 0.2, 0.3, 0.4, 0.5%.) in a coded 5 ml screw-caped vial for each concentration of RJ held at 37°C for 0, 30, 60, 90 and 120 min of incubation, duplicate 10 µl sub-samples were form each vial and evaluated independently. Treatment with 0.0% RJ was considered as control.

Assessment of spermatozoa functional parameters

The progressive motility, viability and acrosomal integrity of thawed spermatozoa after washed in Tris-extender plus RJ was assessed 4 times every 30 min interval over 2 h of incubation period.

Individual motility

A 50-ul droplet of the spermatozoa and Tris-RJ extender mixture was placed on a pre-warmed, clean microscopic slide (37°C) 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 [Zemjanis 1977].

Viability

Eosin-nigrosin staining has been used as a routine staining in order to evaluate sperm viability [Rodríguez-Martínez 2000], briefly, after washing sperm samples in saline solution (154 mM NaCl) at 37°C, one drop of the suspension containing $35 \cdot 10^6$ sperm/mL was placed on a tempered glass slide, which was mixed with one drop of EoNig solution (0.2 g of eosin and 2 g of nigrosin were dissolved in a buffered saline solution [153 mM NaCl and 9.65 mM NaH_2PO_4, pH 7.4], mixed for 2 h at room temperature and filtered to obtain the staining media). The mixture was smeared on the glass slide and let air dried. The samples were observed under a light microscope. Eosin penetrates in non viable cells which appeared red color. Nigrosin offers a dark background facilitating the detection of viable, non stained cells. Slides were observed under a light microscope at 100 × and assessed for the number of sperm that were alive at the time of fixation. A total of 100 cells were counted from each smear, having 4 smears analyzed from each frozen-thawed semen samples.

Acrosomal integrity

Frozen semen straws diluted with RJ of different concentrations were washed twice by centrifugation 600 g using sodium citrate buffer. The supernatant fluid was discarded at every time and the pellet was covered by a suitable amount of buffer. Dry smears were made from one drop of the washed diluted semen with one drop of Wells and Awa stain [1970]. The slides were observed under a light microscope at 100 × and assessed for the number of sperm that had undergone acrosome reaction. A total of 100 cells were counted from each smear, having 4 smears analyzed from frozen-thawed semen samples. The degree of change in the acrosomes was estimated by means of light microscopy after the specimens had been stained, using the following scoring system: 0 – normal acrosomem, 1 and 2 – stages of acrosomal damage, 3 – acrosome entirely lost [Watson and Matrin 1972].

Statistical analysis

Statistical analysis was performed by using ANOVA with Turkey test to shows the differences between the treatments over time. All values were expressed as mean ±SEM. A probability of $P < 0.05$ was set as significance level.

RESULTS

The results of the present study showed that royal jelly can enhance and maintain buffalo sperm function parameters in a dose and incubation time's dependent manner (Tab. 1, 2, 3).

Table 1. Progressive motility of buffalo spermatozoa thawed and held in Tris-RJ extender (0.0–0.5%) over 120 min incubation period (mean ±SEM)

Duration	RJ concentrations, %					
min	0.0	0.1	0.2	0.3	0.4	0.5
0	44.2 ±0.24^{e}	44.3 ± 0.2^{e}	44.1 ±0.27^{e}	44.2 ±0.24^{e}	44.4 ±0.27^{e}	44.1 ±0.3^{e}
30	35.00 ±0.3^{a}	36.2 ±2.4^{b}	38.2 ±0.24^{c}	40.2 ±0.27^{d}	43.6 ±0.26^{f}	43.7 ±0.26^{f}
60	29.8 ±0.2^{b}	34.0 ± 0.25^{a}	36.3 ±0.2^{d}	38.4 ±0.22^{c}	42.4 ±0.22^{f}	42.5 ± 0.26^{f}
90	23.5 ±0.22^{c}	30.4 ± 0.22^{d}	33.7 ±0.2^{a}	32.9 ±0.27^{f}	36.2 ±0.2^{b}	36.0 ±0.2^{b}
120	17.2 ±0.2^{f}	20.2 ±0.2^{c}	24.6 ±0.16^{b}	28.1 ±0.23^{a}	30.3 ±0.15^{d}	30.1 ±0.18^{d}

$^{a, b, c, d, e, f}$ Values with the different superscript in the same row or column were significantly different at $P > 0.05$

Results of experiment showed that the percentages of sperm function parameters were improved with an increase of royal jelly in Tris extender from 0.0 to 0.4% ($P < 0.05$). Applying analysis for sperm motility and viability (Tab. 1, 2) showed that the best result (significant higher $P < 0.05$) occurred with a RJ doses of 0.4 and 0.5% especially at 30 and 60 min incubation periods than that of control although the values in both concentrations of RJ (0.4, 0.5%) were nearly similar.

From the Tables (1, 2 and 3) it is clear that sperm motility, viability and acrosomal integrity decreased significantly after 90 min incubation in washed samples (Tab. 1) in control samples. RJ of different concentrations had a significant effect on the progressive forward motility ($P < 0.05$) during the 120 min study period. Treatment with RJ (0.4%) maintain ($P < 0.05$) progressive forward motility was higher at all time points, it was significantly ($P < 0.05$) higher at 30 and 60 min of incubation than the control (Tab. 1).

RJ had a significant effect on the sperm viability ($P < 0.05$) during the 120 min study period. Treatment with 5% RJ maintain sperm viability was higher at all time points ($P < 0.05$), it was significantly ($P < 0.05$) higher at 30 and 60 min of incubation than the control (Tab. 2).

Table 2. Viability of buffalo spermatozoa thawed and held in Tris-RJ extender (0.0–0.5%) over 120 min incubation period (mean ±SEM)

Duration min	RJ concentrations, %					
	0.0	0.1	0.2	0.3	0.4	0.5
0	48.3 ±0.2^{e}	48.5 ±0.2^{e}	48.4 ±0.2^{e}	48.3 ±0.2^{e}	48.5 ±0.2^{e}	48.4 ±0.2^{e}
30	35.4 ±0.16^{a}	41.3 ±0.15^{b}	43.7 ±0.15^{c}	45.6 ±0.16^{d}	47.3 ±0.15^{f}	47.4 ±0.26^{f}
60	29.7 ±0.15^{b}	38.6 ±0.16^{a}	40.3 ±0.26^{d}	42.3 ±0.15^{c}	45.2 ±0.2^{f}	45 ±0.2^{f}
90	21.6 ±0.16^{c}	32.14^{d}	36.2 ±0.25^{a}	38.1 ±0.16^{f}	41 ±0.16^{b}	41 ±0.2^{b}
120	18.5 ±0.16^{f}	24.5 ±0.16^{c}	30.9 ±0.18^{b}	32.5 ±0.16^{a}	37.4 ±0.16^{d}	37.4 ±0.16^{d}

$^{a, b, c, d, e, f}$ Values with the different superscript in the same row or column were significantly different at $P > 0.0001$

Table 3. Acrosomal defects of buffalo spermatozoa thawed and held in Tris-RJ extender (0.0–0.5%) over 120 min incubation period (mean ±SEM)

Duration Min	RJ concentrations, %					
	0.0	0.1	0.2	0.3	0.4	0.5
0	18.6 ±0.16^{e}	18.8 ±0.13^{e}	18.9 ±0.1^{e}	19.2 ±0.24^{e}	19.2 ±0.24^{e}	19.2 ±0.24^{e}
30	25.7 ±0.26^{a}	22 ±0.16^{b}	21.2 ±0.21^{c}	20.4 ±0.16^{d}	19.9 ±0.18^{f}	19.9 ±0.18^{f}
60	30.6 ±0.16^{b}	24.7 ±0.15^{a}	25.0 ±0.21^{d}	23.0 ±0.21^{c}	20.5 ±0.16^{f}	20.5 ±0.16^{f}
90	34.5 ±0.2^{c}	28.7 ±0.15^{d}	27.1 ±0.18^{a}	26.3 ±0.15^{f}	22.7 ±0.15^{a}	22.7 ±0.15^{a}
120	40.6 ±0.16^{f}	31.6 ±0.16^{c}	32.4 ±0.16^{b}	28.7 ±0.15^{a}	24.6 ±0.16^{d}	24.5 ±0.16^{d}

$^{a, b, c, d, e, f}$ Values with the different superscript in the same row or column were significantly different at $P > 0.0001$

RJ had significant ($P < 0.05$) effect on acrosomal integrity during the 120 min study period. In addition, acrosomal integrity were significantly ($P < 0.05$) higher in 0.4% RJ group at 30 and 60 min of incubation than control (Tab. 3).

DISCUSSION

Results of experiment showed that the percentages of sperm function parameters were improved with an increase of royal jelly in Tris extender from 0.0 to 0.4%. The sperm function parameters were significantly ($P < 0.05$) higher at 0.4% RJ as compared to the control. Over a period of 120 min of incubation, up to one-half of sperm in each sample were motile, live and intact on using Tris 0.4% RJ extender. This may be attributed to RJ may have reduced the exposure of spermatozoa to oxidative stress at or just after the thawing, by removing excess extracellular ROS present in the semen medium as its composition is highly containing pantothonic acid which have antioxidant effect [Hove *et al*. 1985]. It was suggested that RJ might enhance motility through partial capacitation of sperm cells as its composition is highly containing calcium ions [Kodai *et al*. 2007]. It was suggested that RJ might maintain the acrosomal integrity as its composition is highly variable containing hormones which decrease oxygen radicals and therefore reducing the number of molecules needed to be scavenged by the superoxide dismutase enzyme which were present normal in seminal plasma to counteract the effects of ROS on lipid peroxidation and prevent spermatozoal damage [Gancarczyk *et al*. 2006].

Fructose uptake is not consistent during the period of incubation. Fructose uptake may be increased within 30–60 min of incubation. Then the fructose uptake may be slowed between 90 and 120 min might be to compensate for the high intake during the first 30–60 min. Motility of spermatozoa was related to the rate of sugar utilization in the seminal plasma [Biswas *et al*. 1987].

The buffalo spermatozoal motility characteristics decline with incubation time. In the present study, the percentage of motile spermatozoa declined significantly during incubation. This may be due to the inability of spermatozoa to generate ATP through mitochondrial respiration as a consequence of mitochondrial aging [Cummins *et al*. 1994, Viswanath and Shannon 1997] or toxic effect of dead sperm associated with the liberation of amino oxidase activity [Shannon and Curson 1972]. Krzyzosiak *et al*. [1999] observed that under aerobic condition during incubation, spermatozoal velocities did not change. In the present study, the aerobic conditions were maintained as sample was withdrawn at an hourly interval during post-thawing incubation. The present finding was in agreement to these reports as the percentage of ram spermatozoa with normal acrosome decreased significantly during incubation [Pontbriand *et al*. 1989].

This effect could be attributed to the royal jelly contain vitamin C, vitamin E and arginine. Vitamin E and C is a well-documented antioxidant and has been shown to inhibit free-radical induced damage to sensitive cell membranes of the testis and reduced lipid peroxidation in tissue estimation by malodialdehyde, so vitamin E and C significantly decreased MDA, and increased in glutathione level [Ebisch *et al*. 2006]. A positive effect of IGF-I on functional membrane integrity further suggests the role of RJ on spermatozoa membrane stability.

In summary, the study indicated that addition of RJ had significant effect on spermatozoa survivability and acrosomal integrity during post-thawing incubation. The survivability and acrosomal integrity of spermatozoa were better when thawed semen were washed with Tris 0.4% RJ extender and this additive can be used for increasing the possibility of collision between spermatozoa and ova during insemination.

This study suggested that RJ could act as a stimulator of buffalo sperm hyperactivation. Evidence presented above support the concept that RJ plays an important role in regulating sperm functions. However, many questions about its mechanism of action remain to be clarified

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Streszczenie. Powszechnie uznaje się, że nasienie przechowywane w stanie zamrożonym ma osłabioną zdolność zapładniania w porównaniu z nasieniem świeżym. To ograniczenie wynika zarówno z mniejszej żywotności po rozmrożeniu oraz dysfunkcji subletalnej w stosunku do przeżywającej subpopulacji. Niniejsze badanie przeprowadzono w celu określenia wpływu dodatku mleczka pszczelego na przeżycie i akrosomlaną integralność plemników bawołu podczas inkubacji po rozmrożeniu. Zamrożone-rozmrożone próbki nasienia opłukano w Tris zawierającym różne stężenia mleczka pszczelego: 0,0 (kontrola), 0,1, 0,2, 0,3, 0,4, 0,5%. Ruchliwość i żywotność plemników oraz akrosomalną integralność rozmrożonych próbek oceniono po rozmrożeniu oraz w 30-minutowych przerwach podczas inkubacji trwającej 120 min, w temp. 37°C. Wyniki doświadczenia pokazały, że parametry funkcji plemników poprawiały się wraz ze wzrostem stężenia mleczka pszczelego w Tris z 0,0 do 0,4% ($P < 0,05$). Analiza statystyczna ruchliwości i żywotności plemników oraz integralności akrosomalnej (tab. 1, 2, 3) wykazała, że najlepsze rezultaty (istotnie wyższe $P < 0,05$) w porównaniu z kontrolą osiągnięto przy dawkach mleczka pszczelego 0,4 i 0,5%, zwłaszcza przy okresach inkubacji 30–60 min. Dodatek 0,4% do Tris jest więc zalecany do rozcieńczenia rozmrożonego nasienia. Wzmaga on i zachowuje żywotność oraz przeżywalność plemników przez długi okres. Dodatek ten zwiększa możliwość zderzenia plemników z jajeczkami podczas inseminacji.

Słowa kluczowe: mleczko pszczele, bawół, nasienie, przeżywalność, żywotność