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Effect of Royal Jelly on Viability and *in vitro* Maturation of Egyptian Sheep Oocytes in Serum Supplemented Medium

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Abstract: The present study was conducted to investigate the effects of addition of different concentrations (0.1, 0.2, 0.2, 0.4 and 0.5%, respectively) of Royal Jelly on the viability and maturation of the Egyptian sheep oocytes during *in vitro* culture of oocytes in serum supplemented tissue culture medium. Sheep ovaries were collected form local slaughterhouse and then COCs were recovered from visible antral follicles (2-6 mm) by aspiration method and randomly divided into 6 groups. Each group of oocytes was incubated in tissue culture media -199 (TCM-199) with 10% Fetal Calf Serum (FCS) with different doses of royal jelly (RJ). The treatments were: Control 0%, RJ 0.1%,RJ 0.2%, RJ 0.3%, RJ 0.4 ,RJ 0.5%, for 24-28 h at 39 ºC under 5% CO_2 in air and 95% humidity. Following the culture period, COCs were evaluated for morphology of cumulus expansion and viability by trypan blue exclusion test to investigate the effect of RJ on viability, cumulus expansion and *in vitro* maturation of sheep oocytes. The results indicated that the oocytes showing morphologically cumulus full expanded and lived increased significantly with RJ supplementation in a dose-dependent manner up to 0.4%. The proportion of oocytes showing morphologically cumulus full expanded and lived oocytes was higher for all the supplements compared to control media. Following 24-28 h of oocyte culture in 10% FCS with TCM-199 containing various concentrations of RJ [0.1% (75%), 0.2% (80%), 0.3% (85%), 0.4% (94%) and 0.5% RJ (94%] resulted in higher maturation rate ($p<0.05$) rate than the control (60%). Therefore, the present study demonstrated that the addition of RJ to culture media could significantly improve the viability and *in vitro* maturation of sheep oocytes.

Key word: Maturation, oocytes, ovine, royal jelly, viability

INTRODUCTION

Mammalian oocytes collected from antral follicles can complete meiotic maturation in media *in vitro*. However, subsequent developments of sheep oocytes matured and embryos cultured *in vitro* are quite lower than those matured and developed in vivo. This is mainly due to media that can not provide optimal conditions for the oocytes. In vivo conditions cannot be mimicked totally under *in vitro* situations, but developmental capabilities of oocytes can be improved by supplementation of various hormones, growth factors, serum, cells, follicular fluid, and other substances added to the maturation media and various culture media (Romero-Arredondo and Seidel, 1996; Choi *et al*., 2001).

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 (Hove *et al*., 1985; Kodai *et al*., 2007).

The importance of RJ may be due to its contents of hormones, trace nutrients and proteins such as globulin and albumin. Among the latter, Estradiol has been found to improve the completion of maturational changes and also to support the synthesis of presumed male pronuclear growth factor (Moor and Warnes, 1978; Fukui and Ono, 1989).

Buccione *et al.* (1990) explained that the presence of gonadotropins in the maturation media increases the level of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system, and induced cumulus expansion in intact complexes.

TCM-199 supplemented with fetal calf serum (FCS) is the most widely used culture medium for *in vitro* maturation of mammalian oocytes (Sanbuissho and Threlfall, 1989; Arunakumari *et al*., 2007).

Moor and Seamark (1986) and Mori *et al.* (2000) suggested that the coupling of cumulus cells and oocyte

is important for the uptake of nutrients by oocytes during maturation in culture medium and to facilitate the transport of signals into and out of the oocyte. Furthermore, Cox *et al.* (1993), Chauhan *et al.* (1997) and Ali and Sirard (2002) reported that the presence of cumulus cells during IVM of animal oocytes may provide an energy source or may produce factor(s) or hormones capable of regulating maturation.

Trypan blue stain has been used previously to detect oocyte viability (Freshney, 2000) and Dead oocytes displayed a dark blue ooplasm with translucent cumulus cells. Trypan blue exclusion test provides an assessment of cell membrane integrity as those cells with damaged or non-intact cell membranes permit the passage of the trypan blue toward the nucleus of oocyte. Abd-Allah *et al.* (2008) reported that the developmental ability of camel oocytes was not affected by trypan blue staining.

The present study was designed to evaluate the effect of addition of RJ to culture media (TCM-199) on viability, cumulus expansion and *in vitro* maturation of Egyptian sheep oocytes.

MATERIALS AND METHODS

Chemicals: All materials were purchased from Sigma Chemical Company (St. Louis, MO, USA) 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.

Oocyte recovery: This study was conducted at the *In vitro* Fertilization Laboratory, Faculty of Veterinary Medicine, Beni-Suef University, Egypt. From January to March 2011, sheep ovaries (n = 180) were collected at the moment of slaughter at a local abattoir in Cairo, stripped of fat tissue and ligaments, and rinsed in saline solution (37ºC). Sheep oocytes were obtained by aspiration of 2-6 mm follicles from ovaries collected from a slaughterhouse. Only oocytes having a dense cumulus cell mass and homogeneous cytoplasm were selected for the study. Oocytes were washed 3 times with Dulbecco's phosphate-buffered saline. For all culture experiments, maturation of oocytes took place in TCM-199+10% FCS+50 μg/mL gentamycine sulpahate.

***In vitro* Maturation (IVM):** Oocytes with a homogenous and evenly granulated cytoplasm and three or more layers of cumulus cells were placed into drops (100 μL) of maturation medium under paraffin oil, and cultured in 35 mM petri-dishes at 39ºC under an atmosphere of 5% CO_2 in air, 95% humidity, for 28 h. The oocytes were matured in TCM-199+10% FCS+50 μg/mL gentamycine sulpahate. The treatment included different concentrations of RJ with addition of 10% FCS. TCM-199 with serum supplementation formed the control. The treatments were:

- Control
- RJ 0.1%
- RJ 0.2%
- RJ 0.3%
- RJ 0.4%
- RJ 0.5%

For all experiments, 10 oocytes were transferred separately into a 50 uL drop of TCM-199, covered with sterile mineral oil in a polystyrene culture dish (3.5 mm × 10 mm) which had been previously kept for about 2 h in a CO_2 incubator before the oocytes were added. The oocytes were cultured for 24-28 h at 39ºC in an atmosphere of 5% CO_2 in air with 95% humidity.

Morphological evaluation of cumulus cells expansion: Following the culture period, the COCs cultured using different concentrations of RJ with serum supplementation were evaluated morphologically for cumulus expansion using procedures described previously for bovine (Lorenzo *et al*., 1994). The matured oocytes were scored for granulose-oocyte cell expansion as previously described for bovine by Lorenzo *et al*. (1994) with minor modification: CE+ for granulosa-full expanded oocytes, CE+/- for granulose- partially expanded oocytes, (whenever there were few expansion of cumulus layers or cumulus cells were non-homogeneously spread and clustered cells were still observed or moderate expansion of cumulus layers) and CE- for granulosa-non expanded oocytes.

Viability evaluation of cumulus cell expansion using the trypan blue exclusion test: Following the culture period, the COCs cultured using different concentrations of RJ with serum supplementation were evaluated the viability of all grades of cumulus expanded oocytes using procedures described previously for camel oocytes (Abd-Allah *et al*., 2008). All classes of oocytes were examined for viability using the trypan blue exclusion test. Cumulus expanded oocytes were categorized on the basis of the degree of dye exclusion. Unstained oocytes were classified as live and fully stained oocytes as dead.

Statistical analysis: Data were analyzed using Chi-square analysis (Snedecor and Cochran, 1980).

RESULTS

Cumulus cell expansion, viability and *in vitro* maturation rate of ovine oocytes were found in the present

Table 1:Effect of different concentrations of royal jelly on cumulus expansion, viability and maturation rate of sheep oocytes in TCM-199

		Morphological observation Cumulus expansion			Trypan blue exclusion test Live oocytes			
Treatment	No. of COCs	CE+	CE+/-	CE-	CE+	CE+/-	CE-	Maturation rate
TCM-199 (control)	100	30%[a] (30/100)	20%[a] (20/100)	50%[a] (50/100)	76.6.3%[a] (23/30)	75%[a] (12/20)	50%[a] (25/50)	60%[a] (60/100)
TCM+RJ 0.1%	100	40%[b] (40/100)	30%[b] (30/100)	30%[b] (30/100)	87.5%[b] (35/40)	83.3%[b] (25/30)	50%[b] (15/30)	75%[b] (75/100)
TCM+RJ 0.2%	100	45%[b] (45/100)	35%[b] (35/100)	20%[b] (20/100)	88.8%[b] (40/45)	82.8%[b] (29/35)	55%[b] (11/20)	80%[b] (80/100)
TCM+RJ 0.3%	100	50%[b] (50/100)	40% (40/100)	10%[b] (10/100)	90%[b] (45/50)	85%[b] (34/40)	60%[b] (6/10)	85%[b] (85/100)
TCM+RJ 0.4%	100	55%[b] (55/100)	45%[b] (45/100)	5%[b] (5/100)	92.7%[b] (51/55)	88.8%[b] (40/45)	60%[b] (3/5)	94%[b] (94/100)
TCM+RJ 0.5%	100	55%[b] (55/100)	45%[b] (45/100)	5%[b] (5/100)	92.7%[b] (51/55)	88.8%[b] (40/45)	60%[b] (3/5)	94%[b] (94/100)

Values within column with different superscript were significantly different at $p<0.05$; CE+: Cumulus-full expanded oocytes; CE+/-: Cumulus-partially expanded oocytes (whenever there were few expansion of cumulus layers or cumulus cells were non-homogeneously spread and clustered cells were still observed or moderate expansion of cumulus layers); CE-: Cumulus-non expanded oocytes

study to be activated by the addition of RJ plus FCS to the culture medium (TCM-199).

A total of 600 cultured grade oocytes were recovered from 180 ovaries (3.3 per ovary). At the end of experiment, the proportion of COCs showing different grades of cumulus expansion, viability of oocytes and *in vitro* maturation of oocytes with different treatment groups of RJ are presented in Table 1.

Compared to control media, supplementation of RJ at all concentrations showed a significantly higher ($p<0.05$) full cumulus expansion of CE+ grade. The proportion of oocytes showing full expanded cumulus cells and viable (CE+) increased with RJ supplementation in a dose-dependent manner up to 0.4% (which also showed the highest CE+ cumulus expansion). However, at 0.5% RJ further increase was not seen in addition, and there were no differences between different doses of RJ supplementation.

The proportion of viable ovine oocytes was higher for all the supplements dependent effect of RJ supplementation (Table 1).

Following 24-28 h of oocyte culture in 10% FCS with TCM 199 containing various concentrations of RJ [0.1% (75%), 0.2% (80%), 0.3% (85%), 0.4% (94%) and 0.5% RJ (94%] resulted in higher maturation rate ($p<0.05$) rate than the control (60%).

DISCUSSION

In this report, RJ at different concentrations was employed in TCM-199 media, to analyze the effects exerted by RJ on *in vitro* maturation of sheep oocytes using a 5% CO_2 in air environment.

The use of serum as a supplement in the *in vitro* maturation media for oocytes seems to be controversial because of the variety of substances that the sera obtained from different sources may contain which may have beneficial or harmful effects. The present study demonstrated that RJ at different concentrations when combined with serum supplements has a beneficial effect on the *in vitro* maturation.

The proportion of oocytes showing CE+ cumulus expansion, viability and *in vitro* maturation of ovine oocytes was higher for all the supplements dependent effect of RJ supplementation. Therefore, the higher maturation rate *in vitro* of sheep oocytes in the present work might be attributed to some factors in RJ composition such as gonadotropins and essential amino acids that stimulate DNA and RNA synthesis and enhance cell division in addition, increases the level of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system, and induced cumulus expansion in intact complexes.

The results of the present study indicate that cumulus expansion of sheep oocytes are significantly higher when they are cultured in a medium (10% FCS with TCM 199) supplemented with various doses (0.1, 0.2, 0.3, 0.4, 0.5%, respectively) than without RJ. However, there were no differences between different doses of RJ supplementation.

The cumulus expansion of the sheep oocytes obtained in the present study is similar to previous studies of Farag *et al.* (2009) on sheep and Abd-Allah (2011) on buffaloes.

CONCLUSION

The study showed that supplementation of RJ in maturation media positively affected cumulus expansion and maturation so it could be concluded that RJ could be used as a beneficial additive to synthetic culture media used for *in vitro* maturation in sheep oocytes.

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