Effects Of Royal Jelly on Nuclear Maturation, Fertilization and Culture of Bovine Oocytes

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SUMMARY

The present study investigates the effects of royal jelly, which could be an alternative to serum replacement, on bovine oocyte maturation and embryo culture. Two experiments were designed for the effects of royal jelly on oocyte maturation (Experiment 1) and blastocyst development (Experiment 2). Ovaries, collected at a local abattoir, were transported to the laboratory in a flask. The selected oocytes were matured in bicarbonate-buffered Medium 199 containing Earle's salts supplemented with 10 % (v/v) foetal bovine serum or honeybee royal jelly (RJ; 1.25% w/v, RJ (1.25) and 0.625% w/v, RJ (0.625)) and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin sulphate) under a humidified atmosphere of 5 % CO2 at 38 °C for 22-24 hours in Experiment 1. After maturation, oocytes were fertilised in fertilisation medium (TALP) supplemented with 6 mg/mL fatty acid-free BSA and 10 µg/mL heparin and following fertilization, presumptive zygotes were cultured in synthetic oviduct fluid (SOF) supplemented with %10 FBS for 8 days under a humidified atmosphere of 5% CO_2 5% O_2 90% N_2 at 38 °C in Experiment 2.

Royal jelly supplementation significantly ($P< 0.05$) reduced the number of third-class cumulus cells (FBS; 72.6%, RJ (1.25); 59.6%, and RJ (0.625); 63.8% of the group) compared with the serum used in Experiment 1. However, the results showed that supplementation of different concentrations of royal jelly in the maturation media did not affect the number of (MII) Metaphase II (FBS;77.6%, RJ (0.625); 71.5% and RJ (1.25); 64.3%) at the stage of nuclear maturation reached by the oocytes at the end of the maturation period in Experiment 1. The result also showed supplementation of royal jelly during the maturation period had no significant effect on the cleavage rates (FBS: 65.2% and RJ (0.625); 61.6 %) in Experiment 2. Royal jelly supplementation to the maturation medium had also no significant effect ($P=0.09$) on the morula stage (FBS; 29.5 % and RJ (0.625); 26.7 %) and ($P > 0.05$) on the blastocyst stage of the embryos (FBS; 20.6% and RJ (0.625); 18.7%). However, royal jelly supplementation to the maturation medium resulted in a decrease in blastocyst diameter and total cell counts.

As conclusion, the supplementation of 1.25% w/v and 0.625% w/v royal jelly to bovine oocyte maturation media reduced cumulus cells expansion but did not have negative effects on bovine oocyte maturation, fertilization, and blastocyst development. Therefore, the addition of royal jelly has some potential to be used as an alternative to serum for bovine oocytes during maturation.

KEY WORDS

Royal jelly, serum, bovine, oocyte maturation, embryo culture.

INTRODUCTION

Numerous studies have been conducted regarding in vitro maturation (IVM) and fertilization (IVF) processes with the aim of comprehending the underlying mechanisms governing the maturation of bovine and ovine oocytes. Studies have documented that the composition of the maturation medium for oocytes can bring about modifications in the subsequent development and quality of embryos, including their progression to the blastocyst stage, even during the latter culture period[1,2]. Also, the efficiency of Assisted Reproductive Technologies is highly dependent on the production of good-quality embryos. Serum, potentially including energy substrates, amino acids, growth factors, and vitamins, has been involved in oocyte maturation systems for many years[5,6]. Media containing serum, gonadotropins, oestradiol, growth hormones, and growth factors have been shown to have beneficial effects on IVM, IVF, and blastocyst formation in several species[1,2,3,4,5,6,7,8]. While the utilization of serum in oocyte and embryo culture systems has proven successful over an extended period, concerns have also arisen regarding its potential negative impact on embryo development. These concerns have attributed the ambiguous composition of serum, which lacks well-defined parameters and has been associated with some adverse effects [5,6,8]. It has also been reported that the use of serum in embryo culture systems resulted in abnormally large lambs[7,9]. The composition of *in vitro* culture systems may also affect post-thaw survival rates of embryos. It has been reported that supplementation of *in vitro* culture systems with serum containing lipids reduces the survival rate of bovine embryos after cryopreservation and complete removal of and fatty acids from culture resulted in high survival rates after cryopreservation[10]. Therefore, defined culture systems have been developed to overcome the adverse effects of serum by replacing it with Bovine Serum Albumin

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(BSA), Poly Vinyl Alcohol (PVA), or amino acids[8,11,12].

Royal Jelly is a creamy, milky-white substance secreted by the secretory glands of worker bees *(Apis mellifera)* for stimulation of growth and development by providing a balance of nutrients to the larvae "bee-embryo". The main ingredient of royal jelly is protein; two thirds albumin and one third globulin. These proportions reflect those of the human body. Royal jelly also contains essential amino acids, vitamins (B1, B2, B12), pantothenic acid, folic acid, nicotinic acid, inositol, microelements (Cu, Fe, Mg, Al, Co etc.) and many kinds of enzymes and insulin like material[13]. Royal jelly effectively protects female and male gametes and has been reported as a strong antioxidant and a potent free radical scavenger, which is secreted from both the hypopharyngeal and mandibular glands of young worker bees[13,14]. In recent studies, the addition of royal jelly to the media has been shown to be beneficial *in vitro* maturation and fertilization of sheep and goat oocytes[15,16,17].

Therefore, royal jelly could be a good candidate to replace serum in culture systems. The aim of the present study was to investigate whether honeybee royal jelly could be an alternative source to serum when included in *in vitro* maturation of bovine oocytes.

MATERIALS AND METHODS

All reagents and media were from Sigma Chemical Co (USA) unless otherwise stated.

Royal Jelly Preparation

The utilized royal jelly for both Experiment 1 and Experiment 2 was sourced from bee colonies at the Faculty of Cukurova University in Turkey. A quantity of 10 grams of royal jelly was dissolved within 10 milliliters of Tissue Culture Medium-199 (TCM 199) and subsequently preserved in 1 milliliter aliquots at a temperature of -20°C. A 1 ml aliquot (containing 1 g of royal jelly per ml) was subjected to gradual logarithmic dilution encompassing descending concentrations: 10% w/v royal jelly (%10w/v RJ), 5% w/v royal jelly (%5 w/v RJ), 2.5% w/v royal jelly (%2.5 w/v RJ), 1.25% w/v royal jelly (%1.25 w/v RJ), and 0.625% w/v royal jelly (%0.625 RJ). The investigation into varied levels of royal jelly concentration, as conducted by Onal[20], was aimed at identifying the optimal dosage for facilitating bovine oocyte maturation. In accordance with these findings, Experiment 1 employed the concentrations of 1.25% w/v and 0.625% w/v royal jelly, while Experiment 2 only employed the concentration of 0.625% w/v royal jelly.

Experiment 1

Bovine ovaries were collected from a local slaughterhouse and transferred to the laboratory at approximately +35 °C in PBS (Dulbecco's Phosphate Buffered Saline) supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin sulphate (Penicillin-Streptomycin, USA). Cumulus oocyte complexes (COCs) were aspirated using an 18-g needle and a 10-ml syringe to obtain follicles 2 to 8 mm in diameter. The COCs were then placed in 1 to 2 ml of Hepes-buffered Medium 199 containing Earle's salts supplemented with 10 % v/v foetal bovine serum (FBS) (Gibco, USA) and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin sulphate). A total 651 COCs contained compact, non-atretic cumulus investments and uniformly granulated cytoplasm were selected for maturation. All COCs were washed twice in Hepes-buffered Medium 199 (Gibco, USA) and then once in maturation medium. The control group involved a TCM 199 containing Earle's salts, supplemented with 10% v/v fetal bovine serum, antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin sulfate), sodium pyruvate (22 µg/mL), follicle-stimulating hormone (FSH) at a concentration of 5 µg/mL, and luteinizing hormone (LH) at a concentration of 5 µg/mL. While 10% v/v FBS concentration of was replaced with two different concentrations of royal jelly, namely 1.25% w/v RJ (1.25) and 0.625% w/v RJ (0.625), to assess the expansion of cumulus cells (categorized as full, moderate, and slightly expanded), as per the description by Gordon. I, 2003 [18] and the nuclear maturation in Experiment 1.

Experiment 2

A total of 390 COCs matured with the procedures outlined in Experiment 1. However, in Experiment 2, only a %0.625 w/v RJ (0.625) substitution was employed in conjunction with a 10% v/v fetal bovine serum to evaluate the early embryonic development of bovine oocytes.

In experiment 2, matured oocytes were fertilised with frozen semen that was thawed at +38°C in a flask from a tested bull. Following maturation, most of the cumulus cells were removed by vortexing, leaving three to five layers around the oocyte, before washing twice in Hepes-buffered TALP supplemented with 3 mg/mL BSA (Fraction V), 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate. Thawed semen was layered (100 µL) under 1 mL of a modified calcium-free Tyrode's Albumin Lactate Pyruvate (TALP) capacitation medium containing 6 mg/mL BSA fraction V, 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate (pH 7.4) in dolphin nose eppendorf tubes to facilitate capacitation using the swim-up procedure[19]. After incubation (30 min) at +38°C in a humidified atmosphere of 5% CO_2 in air, the upper 0.7 mL of medium was collected and centrifuged (300xg) for 10 min before the supernatant was removed, leaving live sperm in the pellet in approximately 100 µL in capacitation medium. The pellet was then resuspended, and motile sperm were counted to give a final concentration of $1x10^6$/ml of motile sperm. Then, 4 µL of capacitation medium containing motile sperm was added to 46 µL of fertilisation medium drops. The fertilisation medium was modified TALP supplemented with 0.2 µmol/L penicillamine, 0.1 µmol/L hypotaurine, 0.02 µmol/L epinephrine, 6 mg/mL fatty acid-free BSA, 30 µg/mL heparin, 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate (pH 7.8). Matured oocytes (10 oocytes per well) were placed in the fertilisation drop where oocytes and spermatozoa were co-incubated together for 10 hours under mineral oil at +38 °C in a humidified atmosphere of 5 % CO2 in air. Following fertilization, all remaining cumulus cells were removed by vorteking and presumptive zygotes were washed twice in Hepes-buffered TALP. Finally, the presumptive zygotes matured in %10 FBS or %0.625 RJ (0.625) royal jelly were further cultured in Synthetic Oviductal Fluid (SOF) supplemented with %10 FBS and 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate (pH 7.4) in Experiment 2.

Fluorescent Staining

A total of 42 blastocysts were used to determine the number of live-dead cells in two experiments. For cell counting, 2 ng/ml fluorescent bis-benzimide dye (Hoechst H33258) was prepared in PBS and each blastocyst was taken into a solution containing 15 µl dye and left in the incubator for 10 minutes. At the end of the incubation period, the blastocysts were washed 3

times in 50 l drops of dye-free PBS. Then, they were taken in 10 l drops in a petri dish and examined with an inverted fluorescent microscope.

Statistical Analysis

Data were analysed by using a one-way analysis of variance (ANOVA) performed with MINITAB 10, following appropriate transformation where necessary (proportion of cleaved zygotes and blastocyst yields, arcsine-transformation; blastocyst cell numbers an, log10 transformation; blastocyst diameter, no transformation). The expansion of cumulus cells was evaluated using the Chi-Square test (²). Pairwise comparisons of treatment mean (expressed as means ± standard error of the mean, s.e.m.) were conducted using Tukey's honest significant difference (HSD) test. The results are presented as untransformed means along with their respective standard errors (± s.e.m.).

RESULTS

In experiment 1, the expansion of cumulus cells of maturing bovine oocytes was detailed within Table 1. The inclusion of royal jelly within the maturation medium yielded a significant impact ($P < 0.05$) on the fully expended cumulus cells, leading to a reduction in the expansion rate (FBS: 72.6%, RJ (0.625): 63.8%, and RJ (1.25): 59.6% of the respective groups) in comparison to the employment of serum in Experiment 1.

In addition, experiment 1 showed that while replacement of %1.25 royal jelly significantly reduced the number of oocytes, %0.625 royal jelly substitution to replace of %10 FBS serum did not reduce the number of oocytes reaching metaphase II (Table 2), which is known to be the critical stage of nuclear maturation in bovine oocytes (FBS; 77.6% vs. RJ (0.625); 71.57% and RJ (1.25); 64.38% group). Similarly, there were no significant differences in the number of oocytes (FBS; 6.8% vs. RJ (0.625); 8.2% and RJ (1.25); 8.0% group) reaching metaphase I (Table 2). Results also indicated that there were more oocytes at Germinal Vesicle Breakdown (GVBD) and Anaphase (A)- Telophase (T) stage in royal jelly (RJ) supplemented groups compared to that of serum (FBS).

The results of experiment 2, the final culture phase of the present study, are shown in Table 3. The results showed that the fertilisation rates (FBS; 65.2% and RJ (0.625); 61.6%) were similar in the control group and the royal jelly supplemented group ($P>0.05$). Royal jelly supplementation did not significantly change fertilization rates between groups. Following fertilization, the presumptive zygotes obtained from royal jelly and serum supplemented maturation media are cultured to the morula and the blastocyst stages. The results revealed that there are no significant ($P>0.05$) differences in the number of zygotes reaching the morula (FBS; 29.5% ± 0.98 vs. RJ (0.625); 26.7% ± 1.23) and the blastocyst (FBS; 20.6% ± 0.88 vs. RJ (0.625); 18.7% ± 1.08) stage, respectively. However, it was found that the number of zygotes reaching the 8-16 cell stage was increased in the royal jelly supplemented group (RJ (0.625); 54.8 ± 1.74%) compared to the addition of serum (FBS; 49.8 ± 0.59%) ($P < 0.02$).

The results of experiment 2 also show that the blastocyst diameters (μm) (FBS; 211 ± 5.2 and RJ (0.625); 197 ± 4.3) and total cell count (FBS; 117 ± 3.61 and RJ (0.625); 101 ± 2.39) of the blastocysts, which is an important criterion for a healthy blastocyst, were statistically different (Table 3).

DISCUSSION

The use of serum and gonadotropins during maturation of bovine and ovine oocytes is a generally accepted practise and is used by many laboratories. In this study, royal jelly was supplemented to TCM-199 media, at various concentrations to evaluate the effects exerted on *in vitro* bovine oocyte maturation and blastocyst development. Serum in oocyte and embryo culture media has important advantages, but many scientific articles also indicate that the cause of many problems in oocyte and embryo culture is due to undefined serum content[7,9,21,22]. In addition, some of the energy sources (glucose) used in culture media may not be utilised by the embryo in the early stages

Table 1 - The effects of serum and royal jelly on the expansion of cumulus cells surrounding the bovine oocytes.

Classes	FBS	RJ (0.625)	RJ (1.25)	P
% Slight expansion	14.3±3.20	13.7±3.08	23.8±4.01	NS
% Moderate expansion	13.1±2.34	22.5±3.65	16.5±3.25	NS
% Fully expansion	72.6±1.83	63.8±1.55	59.6±1.78	0.05

P>0.05 (Qhi Square)

Table 2 - The effects of serum and royal jelly on the nuclear maturation of bovine oocytes.

	FBS	RJ (0.625)	RJ (1.25)
Number of Oocyte	203	212	236
% Germinal Vesicle Breakdown (GVBD)	4.0±1.06^{a}	6.5±1.19^{ab}	11.5±1.59^{b}
% Metaphase I (MI)	6.8±0.90^{a}	8.2±1.32^{a}	8.0±1.31^{a}
% Anaphase (A) - Telophase (T)	10.3±1.44^{a}	12.9±1.41^{ab}	15.0±2.05^{b}
% Metaphase II (MII)	77.6±2.39^{a}	71.5±2.20^{a}	64.3±2.72^{b}

a, b: Different superscripts on the same line denote significant difference ($P < 0.05$).

Table 3 - The effects of %10 FBS and %0,625 royal jelly on the early bovine embryo development .

	FSB	RJ (0.625)
Number of Oocyte	201	189
% Cleavage (Day 2**)	65.2±2.72	61.6±4.06
%8-16 Cell Stage (Day 3**)	49.8±0.59	54.8±1.74 *
% Morula (Day 5**)	29.5±0.98	26.7±1.23
% Blastocyts (Day 8**)	20.6±0.88	18.7±1.08
Blastocyts Diamention (μm)	211 ±5.2	197 ±4.30 *
Total Cell Count	117 ±3.61	101 ±2.39 *

* Different superscripts on the same line indicates significant difference (P < 0.05).
** Fertilization (Day 0)

and may impair development[6]. For this reason, many scientific studies are being conducted on culture media to determine optimal conditions for oocytes and embryos. In our early study, different concentrations of royal jelly were tested to determine the optimal concentration of royal jelly for bovine oocyte maturation. It was found that the 1.25% (w/v) and 0.625% (w/v) royal jelly supplemented conditions were suitable for oocyte maturation[20]. The results of experiment 1 in present study showed that the percentage of bovine oocytes reaching the stage of nuclear maturation (metaphase II) was higher in the serum and RJ (0.625) supplemented group compared to RJ (1.25) group. Also, RJ (0.625) supplementation resulted in similar maturation rate to serum supplemented group (P > 0.05). Similarly, royal jelly reported to improve the percentage of bovine oocytes matured *in vitro* and their viability after maturation at a level of 10 mg/ml and 5 mg/ml, being superior to 10 mg/ml[23]. However, as the use of royal jelly percentage (%1.25) increased in this study, the number of oocytes reaching MII stage significantly reduced. This result in experiment 1 indicates that higher royal jelly supplementation in culture medium has negative effects on maturation of bovine oocytes. In another study, contrary to the results in experiment 1, royal jelly in maturation media had a positive effect on cumulus expansion and maturation, leading to the conclusion that royal jelly could be used as a beneficial additive to synthetic culture media for in vitro maturation of ovine oocytes[24]. Experiment 1 showed that cumulus expansion rates, which is one of the visual criteria for *in vitro* maturation, of bovine oocytes in serum was significantly lower in RJ (1.25) compared with serum supplementation in the control. Although the supplementation of royal jelly had a slightly positive effect on cumulus cell expansion in some studies[17,23,24], our results are not in complete agreement with these in terms of cumulus cell expansion. These differences may be due to the use of royal jelly in the presence of serum or concentration. It has been reported that the addition of oestrus bovine serum (ECS) or foetal bovine serum (FBS) to the media used for maturation of bovine oocytes significantly increases maturation rates and also significantly affects the rate of blastocyst formation, especially after fertilisation[25,26]. The positive effect of serum to improve maturation was also present in our study, but on the other hand high royal jelly (RJ (1.25)) supplementation did significantly reduce oocyte maturation.

The most critical stages in embryo culture studies are fertilisation and the number of presumptive zygotes reaching the blastocyst stage after fertilisation. The fertilisation, cleavage, morula and blastocyst rates of oocytes matured in media containing 10% FBS and 0.625% royal jelly were studied in experiment 2. The results showed that the fertilization rate of oocytes supplemented with royal jelly was similar to that of oocytes supplemented with serum (FBS: 65% and RJ (0.625): 61%). This similarity suggests that various nutrients such as proteins, amino acids, vitamins, and fatty acids in royal jelly can be utilised by the oocytes. In addition, oocytes maturing in media supplemented with serum and royal jelly did not differ between groups in terms of development rates to the morula stage (FBS: %29.5±0.98 and RJ (0.625): %26.7±1.23) and to blastocyst stage (FBS: %20.6±0.88 and RJ (0.625): %18.7±1.08) after fertilisation. Oocytes matured in media supplemented with royal jelly demonstrated the capability to reach the blastocyst stage following fertilization. However, these oocytes were adversely impacted (P>0.05) in terms of both blastocyst diameter and cell count. These observed disparities in blastocyst development in groups with royal jelly merit deeper exploration, particularly through an examination of their metabolic rates and the presence of oil droplets they contain. It has been reported that the addition of serum to culture media also reduces the number of cells and causes degeneration of mitochondria[27]. Zygotes resulting from fertilisation of oocytes developed in a medium containing royal jelly was divided more rapidly than those in a medium containing serum. However, rapidly dividing zygotes could not develop to the blastocyst stage at the same rate.

CONCLUSION

In conclusion, the results of this study indicated that the supplementation of royal jelly to bovine oocyte maturation media as an alternative to replace serum has the potential to be used during maturation, since royal jelly did not also have negative effects on the further cleavage and blastocyst developmental stages.

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