

How to Cite:

Al-Shammari, R. F. H., & Almeeni, I. M. (2022). Efficiency of royal Jelly on in vitro fertilization in local Iraqi ewes. *International Journal of Health Sciences*, 6(S5), 6960–6969.
<https://doi.org/10.53730/ijhs.v6nS5.10216>

Efficiency of royal Jelly on *in vitro* fertilization in local Iraqi ewes

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Abstract--This study was designed to investigate the effect of addition several concentrations of royal jelly to the media used in the in vitro maturation process of Iraqi sheep. The ovary was brought from the Al-Shulla slaughterhouse and transferred to the obstetric laboratory at the College of Veterinary Medicine at the University of Baghdad by a thermal insulating box containing the physiological solution with the antibiotic penicillin at a temperature of 30-38°C. In the laboratory, the ovaries were washed with a neutral buffer solution and then the oocytes were collected by two different methods slicing ovary or follicle aspiration. The oocytes were classification into four groups depending on the layers of the cumulus cells surrounding the oocytes, as well as the homogenous of the cytoplasm. Oocytes that are completely or partially surrounded by cumulus cells and homogeneous cytoplasm considered normal oocytes but the oocytes denuded from the cumulus cells and heterogeneous cytoplasm considered abnormal oocytes was discarded. Normal oocytes were divided randomly into four groups at maturation; the first group was matured by TCM media alone and was considered as a control group. As for the three groups supplement with royal jelly to the media used in three concentration of (5, 10, 15) mg/ml, respectively, as they were considered as therapeutic groups. After 24 hours, the rate of maturation was determined by the protrusion the first polar body in perivitelline space or the extension of the cumulus cells. The next step the mature oocytes is to incubate with the ram sperms collected from the ram testicles that was brought from the slaughterhouse for a period of 24 hours to determine the fertilization percentage in the four groups and to identify the effect of royal jelly on in vitro maturation and fertilization rate. The result of the current study that both methods of oocytes collection slicing and aspiration was practical

method that can be used in vitro fertilization technique. Also supplement of the media used in vitro maturation with royal jelly had a improve effect on in vitro maturation and fertilization rate, but the low concentration 5 mg/ml was not a significant effect than the control group, but when increase the concentration to 10 mg/ml there was a significant effect in maturation and fertilization rate compared to the control group. Also the concentration 10mg/ml was not a significant effect with the third concentration 15 mg/ml in the rate of in vitro maturation and fertilization. Conclusion the addition of royal jelly to the media used in vitro maturation had a positive effect to improve the rate of maturation and fertilization, also, the concentration of 10 mg/ml was an ideal concentration so recommend its use in future research.

Keywords---Sheep oocytes, royal jelly, *in vitro* fertilization (IVF).

Introduction

Sheep are important farm animals for milk, wool and meat production, lamb output is insufficient to meet the increased the human being, demand for meat because sheep are seasonal animals (Al-Shaeli et al., 2020; Kaabi *et al.*, 2020). The assisted reproductive technologies (ARTs), such as in vitro fertilization (IVF) are used to improve reproduction (Barakat and Al-Himaidi, 2013). In vitro maturation (IVM) is one of the most essential phases of IVF and depends on the success of the subsequent stages of IVF (Barakat *et al.*, 2018). Enhanced the IVM leads to improved early embryonic development and implantation rate in various mammalian species (Albuz *et al.*, 2010 ; Veshkini *et al.*, 2018). In vitro maturation efficiency can be increased by adding hormones, proteins, antioxidants and serum to the maturation medium (Zhou *et al.*, 2016; Soto-Heras *et al.*, 2018). The TCM-199 is the standard medium for oocyte maturation and it can be supplemented with a number of protein sources such as bovine serum albumin (BSA), sheep serum, fetal cattle serum and follicular fluid (Zhu *et al.*, 2018).

The honey bee is a sweet and sticky liquid made by bees that contains a huge number material possibly over 200 of substances such as proteins, sugars, carbohydrates, enzymes, vitamins, amino acids, mineral salts, flavonoids and antioxidants (Prazina and Mahmutovic, 2017). Different research has shown the royal jelly protects rats from toxicity and used supplement to improve the fecundity rate in mammals (Abd- Allah, 2012). Also the product used most widely for embryo production in vitro and the royal jelly it has been studied in various research the antioxidant mediated protective effect during ovine IVM and embryonic growth following IVF (Veshkini *et al.*, 2018). When royal jelly was added to the maturation medium suggest led to an increased the rate of oocyte maturation, blastocyst development, glutathione concentration and apoptosis reduced (Eshtiyaghi *et al.*, 2016). In Iraq Imad M. AL-Maeni, (2012) used plant extracts as alternatives to antioxidants to improve *in vitro* fertilization, but there was no study that used royal jelly as supplement tissue culture media to improve the maturation and fertilization, so the current study was designed to study the

effect and compare different concentration of royal jelly as additive to the maturation media on oocytes maturation and fertilization rate in local Iraq ewes.

Material and Methods

Male and female genital collection

The male genitalia (testes with attached epididymis) and female genitalia will collect from alshulla slaughterhouse and belong to adult animals (3-6) years according to the dental formula (Ruggeri *et al.*, 2015). The samples were collected and transported to a veterinary medicine laboratory in a veterinary medicine College at Baghdad University for 1-3 hrs. in a thermal box containing normal saline (0.9%) supplemented with 100 IU/ml penicillin and 1 mg/ml streptomycin. The Royal jelly used in the study was purchased from a medical pharmacy.

Preparation of culture media

The media in used present study is tissue culture media 199 the product from GmbH Germany company, which was prepared for direct use, where 100 ml taken from this tissue culture media and placed in valgon capacity 200 ml under the hood (laminar airflow) supplement with (PMSG 10 IU /ml, HCG 10 IU /ml. estradiol 1 µg/ml, penicillin 100 IU /ml, streptomycin 100 µg /ml and fetal bovine serum 10%) to prepared maturation media and this media with component consider stock media. Four valgon from stock media were prepared, the one valgon from stock media determined to control group, other three valgon used supplemented with (5, 10, 15) mg/ ml Royal Jelly determined to treatment groups. All four valgon closed tightly with a parafilm tape to prevent contamination of integrated media, which was protected in the refrigerator at 4 °C until used

Oocyte collection

In the laboratory, the ovaries were washed in phosphate-buffered solution with antibiotics three times. Oocytes were collected using the aspiration and slicing technique described by other studies (Wang *et al.*, 2007; Saleh, 2017). In the slicing method, the ovaries slice into small parts with a surgical scalpel in a graded plastic petri dish containing a collection medium (TCM199 with an antibiotic). In the aspiration method, the visible follicles were aspirated using 18 gauge hypodermic needle attached with a sterile disposable syringe containing a 1ml harvesting medium. The cumulus oocyte complexes (COCs) were isolated under inverted microscope and transferred to a 35 mm petri dish containing an oocyte collection medium and wash three times to isolate oocytes from debris. Then the oocytes collected stained with trypan blue to detect the viability of oocytes before being used in the study. The petri dish containing viable oocytes was examined under a light microscope to determine the number of oocytes harvested and the collection rate. The oocytes collected are categorized into 4 grades depending on the layer cells cumulus moreover the homogenous cytoplasm designated by Wani *et al.* (2013) the grades A when oocytes are enclosed by cumulus cells completely and homogenous the cytoplasm, grade B when oocytes enclosed by cumulus cells partially with homogenous the

cytoplasm, grade C when oocytes without the cumulus cells with homogenous cytoplasm and grade D when oocytes degeneration with heterogeneous cytoplasm. Grade A with B considered the normal oocytes use in the study, while grade C with D considered abnormal oocytes so they rejected

Sperms collection and capacitation

In the laboratory the testicle is washed with normal saline with 100 IU/ ml penicillin and 1mg/ml streptomycin antibiotics, Epididymal caudal spermatozoa were collected by aspiration method, as mentioned by other studies (De Lamirande *et al.*, 1997; Ali and Saleh, 2020) when the 2-3 ml TCM media was loaded in a 10 ml plastic syringe connected to an 18 gauge needle and injected then aspired more than one time. The sample evaluates under a light microscope for individual motility and less than 60% was rejected. Spermatozoa samples were incubated in a 5% CO₂ incubator at 39°C for 4 hours for sperm maturation, the absence of distal cytoplasmic droplet was regarded as sperm maturation criteria. The sperm capacitation process achieved by adding 50 IU/ml of heparin was added to the matured spermatozoa and then incubating for 45-60 minutes in a CO₂ incubator at 39°C as mentioned by Lone *et al.*, (2011). The evaluation of sperm viability to differentiates live or dead immotile spermatozoa. The staining technique by eosin-nigrosin is the most common method analyzing of sperm viability based on the integrity of the sperm cell membrane (Agarwal *et al.*, 2016).

In vitro oocyte maturation and fertilization

The good oocytes (grade A and grade B) were divided into four groups randomly, one group culture in TCM media alone (stock media), other three groups culture in (5, 10, 15) mg/ml royal Jelly add to culture media in three separated treatment groups. In each group, the selected COCs were placed in maturation droplets (15 oocytes / 50 μ l) and covered by sterile paraffin oil in a 30 mm petri dish and then incubate under an atmosphere of 5% CO₂ with 95% humidity at 39 °C for 24 hrs. The oocytes examination was carried out after 24 hrs under inverted microscope in 4 Petri dishes to differentiate between mature and immature oocytes according to the existence of the first polar body in the perivitelline space in the mature oocyte (MII) or expansion of the cumulus cells around oocytes, while the absence of the polar body in the oocyte determined immature and rejected.

Matured oocytes of four groups were washed twice with TCM medium supplied with antibiotics and cumulus cells are eliminated by micropipette rejected gently before transferred to a glass petri dishes containing medium with the same supplementation. Capacitated spermatozoa sample prepared after dilution to yield 1×10^6 / sperm/ ml according Wani (2013). The mixture of gametes was incubated in the CO₂ incubator at 39 °C and at 95% relative humidity for 24 hrs. Fertilized oocytes were removed from the culture medium after diagnosis by an inverted microscope. Fertilization rate was evaluated based on pro-nucleus formation, presence of either sperm head in the perivitelline space, emission of a second polar body and present two cell cleavage.

Statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study percentage. Chi-square test was used to significant compare between percentages in this study.

Results and Discussion

The effect of oocyte collection method on oocytes recovery rate explained (Table 1) when used two methods oocytes collection aspiration and slicing in local Iraqi ewes. In the present study we brought 112 ovaries divided equally randomly to collected (181 and 275) oocyte in aspiration or slicing method respectively. After used statically methods we found the high significant effect ($P \leq 0.01$) in recovery rate to slicing than aspiration (4.91, 3.23) oocyte per ovary respectively. This result agreement with Wani *et al.* (2000) when study the effect of oocyte collection techniques in sheep to in vitro fertilization, the authors used three methods in oocytes collection puncture, slicing and aspiration, he confirmed the three methods can be applied in IVF but high significant in recovery rate between these three methods to puncture and slicing than aspiration, he explore the low number in oocytes recovery rate in aspiration because some follicle found deeply in cortex not prominent on the surface of the ovary.

Table 1: Effect of oocyte collection method on recovery rate and grad of oocytes

Type of collection method	No. of ovaries	Oocytes recovery	Recovery rate	Type of oocyte			
				Normal oocytes		Abnormal oocytes	
				Grad A	Grad B	Grad C	Grad D
Aspiration	56	181	3.23	86 (47.51%)	32 (17.67%)	36 (19.88%)	27 (14.91%)
Slicing	56	275	4.91	68 (24.72%)	67 (24.36)	66 (24%)	74 (26.90%)
P-value	---	----	0.0001 **	0.0081 **	0.079 NS	0.217 NS	0.041 *

* ($P \leq 0.05$), ** ($P \leq 0.01$).

Also the present study investigation the effect of oocyte collection method (aspiration and slicing) on type of oocytes illustrate (Table 1) when the oocytes classified four grad (A, B, C and D) according the number of cumulus cells around oocyte and type of cytoplasm in oocytes, when consideration the grad (A and B) normal oocytes and used in this present study, conversely (C and D) abnormal and rejected. In the present study we show the high significant effect ($P \leq 0.01$) between grad A oocyte rate in two oocytes collection method aspiration and slicing (47.51% , 24.72%) respectively. while we did not find any significant effect in grad B oocyte rate between two collection method aspiration and slicing (17.67% , 24.36%) respectively .Also the comparative between two oocyte collection methods in quality abnormal oocytes (C and D), we showed non-significant effect between oocytes collected rate in grad C in two type collecting aspiration and slicing (19.88% , 24%) respectively, but in oocyte collected in grad D we showed significant effect ($P \leq 0.05$) between two collection method to slicing than aspiration (26.90%, 14.91%) respectively. These results agreement with Wani *et al.* (2000) when study the effect of oocytes collection on maturation and fertilization in

ovine, this result demonstrated the good oocytes quality grad A was high in aspiration collection method than slicing and puncture method, while not significant different between slicing and puncture in quality oocytes recovery. Other agreement with Sianturi *et al.* (2002), when study the effect of oocytes collection and type of grading oocytes to in vitro maturation and fertilization in cow this result indicate the oocyte collection by aspiration method the normal oocytes (A+ B) high significant than abnormal (C+D). Also agreement with Wang *et al.* (2007) he explores the high number of grad D in slicing oocytes collection attributed to degeneration oocytes during chopping the ovary to small parts in scrapple surgery.

In the present study improve the local Iraqi ewes during in vitro maturation by supplement royal jelly in three different concentration treatment groups (5, 10 and 15) mg/ml and one group without supplement in royal jelly to control group in (Table 2) the result of present study demonstrated the addition RJ to culture media used to in vitro oocyte maturation in Iraqi ewes to improve the maturation rate by increase the number oocytes reach the metaphase II , when protrusion the first polar body in perivitelline space. The result indicated the addition low concentration (5) mg/ml increase the maturation rate to (46.55%), but it wasn't any significant effect with the control group (40.98%) also the result indicated the maturation rate in third group (10) mg/ml increase the maturation rate to (53.96%), there was significant effect ($P \leq 0.05$) than control group (40.98%) without significant difference with the two group (5) mg/ml (46.55 %) and fourth group (15) mg/ml (56.33%) in the maturation rate. While the fourth group (15) mg/ml the maturation rate (56.33%), there was a significant difference ($P \leq 0.05$) than control group (40.98%) and the second group (46.55%), while there was no significant difference than the third group (53.96%). This result of present study agreement with Valiollahpoor *et al.* (2016) when confirmed the addition of RJ in concentration 10 mg/ml to the media used in vitro oocytes maturation in sheep act benefit environment and increase the maturation rate in ewes. He explores the positive effect of RJ to anti-oxidant effect and scavenging the hydroxyl radical property. Also the present study agreement with Abd-Allah (2012) when demonstrate the supplement of culture media used in vitro maturation in Egyptian ewes, found the positive effect to improve the viability oocytes and increase the maturation rate and the fertilization rate in ewes oocytes was high significant in all concentration used with fetal calf serum .The positive effect of RJ attributed to content of nutrient and protein (albumin, globulin) addition to contain hormone such as (estradiol, gonadotrophin) act to improve the oocyte maturation and improve synthesis pronuclear growth factor . Also my result agreement with Kaabi *et al.* (2020) used of bee product to ameliorative effect to improve ovine maturation oocyte, he conclude all natural honey increase number oocytes reaching metaphase II stage, which lead increase glutathione concentration and improve the gene expression to control cell division.

Table 2: Effect of Royal Jelly supplement on in vitro maturation and fertilization in local Iraqi ewes

Group	No. of collected oocyte	Oocyte maturation	Maturation rate %	No. of fertilized oocyte	Fertilization rate %
Control group (TCM alone)	61	25	40.98 % c	5	20.00% b
TCM +5mg Royal Jelly	58	27	46.55% bc	7	25.92% b
TCM+10 mg Royal Jelly	63	34	53.96% ab	15	44.11% a
TCM+15 mg Royal Jelly	71	40	56.33% a	19	47.5 % a
Chi-Square -x ² (P-value)	---	---	5.147 * (0.0266)	---	8.592 ** (0.0052)

* (P≤0.05), ** (P≤0.01).

In the present study the result indicate the benefit effect of RJ in the fertilization stage by increasing the fertilization rate after sperm penetration the oocytes and started cleavage stage after 24 hours culture in three treatment group compared with control in(table 2). But the low concentration (5 mg/ml) had no significant effect compared to the control group (25.92%, 20 %) respectively, in the other two concentrations (10 and 15 mg/ml) had a high significant effect (P≤0.01) than control group and (5 mg/ml). also not found significant effect between (10 and 15 mm/ml) in fertilization rate (44.11%, 47.5 %), respectively .This result agreement Kaabi *et al.* (2020) when study the addition RJ to supplement sheep oocyte maturation medium and improve the fertilization rate, he explore the positive effect RJ to some component such as essential and nonessential amino acid to stimulating RNA and DNA synthesis with increase the cell division addition to increase intracellular level cAMP. Also agreement with Eshtiyaghi *et al.* (2016) confirmed the supplement of culture media with 10 mg/ml improve the oocytes maturation and fertilization rate by increase blastocyst formation and increase the concentration the glutathione with superoxide dismutase addition decrease apoptosis rate in both cumulus cell and oocytes. In goats Veshkini *et al.* (2018) report the RJ addition to maturation media in concentration (5, 10) mg/ml improve maturation rate and embryo division and increase formation of blastocyst rate. The positive effect of RJ to improve maturation and fertilization rate attribute to the RJ improve the candidate gene expression and change in vitro maturation condition and increase the competence oocytes development (Mazangi *et al.*, 2015). When supplementation of maturation medium royal jelly with 5 mg/mL improve the embryo cleavage rate and increase formation of blastocyst, this effect associated with improve the cytoplasmic maturation and nuclear maturation rate , enhancing of glutathione concentration and increase the expression key genes of oocyte to support the embryo development (Veshkini *et al.*, 2018). Conclusion the aspiration oocytes collection method was low in quantity of oocytes harvested but was high quality (normal oocytes) when compared with slicing, it high in quantity of oocytes harvested but the oocytes was low quality compared with aspiration. Also the result demonstrated the

addition of royal jelly improves the maturation rate and in fertilization rate but the concentration (5 mg/ml) was non-significant effect than control. But when increase the concentration to (10 mg/ml) was significant effect than control, but without found significant effect between the (15 mg/ml) in maturation rate and fertilization rate, so the concentration (10 mg/ml) the best concentration recommended in the future IVF study in country.

Acknowledgment

Thanks and gratitude to all the Section the Surgery and Obstetrics at the Veterinary Medicine College and all those who helped us in carrying out this research.

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