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Royal Jelly Improved in Vitro Maturated Bovine Oocytes

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Abstract

The aim of this study was to see how royal jelly (RJ) supplementation to IVM media influenced the maturation of bovine oocytes in vitro. The cumulus oocyte complexes (COCs) were extracted from slaughterhouse bovine ovaries and divided into three groups (control group, 5mg/ml RJ, and 10mg/ml RJ) .All oocytes then maturated in vitro for 24 hours and evaluated morphologically under the microscope depend on the expansion of cumulus cells and viability of maturated oocytes. Results revealed that RJ causing insignificant improvement in the percentage of the in vitro maturated cow oocytes in compare to the control group (72.04% ± 6.46, 83.15% ± 4.02 and 60.21% ± 9.08) respectively, with the superiority of the 10mg/ml RJ comparing to 5mg/ml RJ. In respect to the viability of in vitro maturated cow oocytes , the results reveals a significance ($P<0.05$) superiority of RJ groups in compare to the control (83.15% ± 4.02, 72.04% ± 6.46 and 60.21% ± 9.08), with best viability in the 10 mg group. In conclusion , RJ improves percentage of in vitro maturated cow oocytes and their viability post maturation in a level of 10 mg / ml and 5 mg / ml, with superiority to 10 mg /ml.

Key words. Royal jelly, IVM, bovine oocytes,

Introduction

The methods known as assisted reproductive technology (ART) are typically those linked manipulation of gametes and embryos (Galli et al., 2003, Mapletoft and Hasler, 2005). Tremendous advances have been made in (ART), especially during the last decades years. The most successful tool for achieving genetic improvement in cattle populations remains artificial insemination (AI) (Mapletoft and Hasler, 2005). Advanced ARTs have been useful in fostering livestock productivity by maintaining genetic diversity, repopulating endangered breeds and inducing genetic alterations for the development of novel products for human health applications in livestock species (Singh etal, 2009) . IVP (In vitro production) is a member of important ART in different animal species including bovine. All the experimental work undertaken to establish procedures for in vitro embryo production (IVP) was basically based on oocytes that were retrieved in large numbers from slaughterhouse ovaries. It was, however, obviously desirable to recover oocytes from living donors of proven genetic value for functional application (Galli, et al; 2014). The time needed for maximum in vitro nuclear maturation of oocytes, which means the arrival of chromosomes at the stage of Metaphase II, may be 18 to 24 hours (Gasparrini et al., 2008). Maturation periods longer than 24 hours can lead to inappropriate configurations of chromatin, oocyte aging and a decrease in the development of skills, thus showing greater sensitivity toward time affecting oocyte quality comparing to other species (Kumar and Anand, 2012). Royal jelly (RJ) in many countries is a member of the most appealing functional foods that was a marketting commodity for some time, especially in dietetics and cosmetics (Isidorov et al., 2009). RJ consists primarily of major health-promoting and biological compounds such as sugars, lipids, vitamins, proteins, free amino acids , and minerals (Nakajima et al., 2009). Fatty acids, acetylcholine, adenosine

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monophosphate (AMP), polyphenols, proteins, and hormones including estradiol, progesterone, testosterone, and prolactin have all been confirmed to be found in RJ (Ramadan and Al-Ghamdi, 2012).

RJ has a multitude of pharmacological activities: antioxidant, neurotrophic, hypoglycemic, hypocholesterolemiant, antibiotic, hepatoprotective, regulatory blood pressure and hypotensive , anti-inflammatory, antitumor , modulatory and anti-allergic immune, anti-aging, general tonic, etc (M.rghita , etal , 2008). RJ antioxidant activity has also been demonstrated and oxidative stress safety has been verified in laboratory animal experiments (Kanbur, etal 2009 ; Silici , etal , 2009). Royal jelly's antioxidant ability has been shown to be efficient in protecting female and male gametes and cryopreserved semen (Moradi et al., 2013; Shahzad et al., 2016). RJ will scavenge a wide range of free radicals such as that of hydroxyl, DPPH and superoxide radicals (Watanabe, et al., 2013). The first important step for the successful IVP of any mammalian animal is the IVM of oocytes (Bavister et al., 1992) . The maturation of oocytes is the process of complex protein phosphorylation changes that converts a primary oocyte into a mature secondary oocyte (ATomek et al., 2002). The oocytes must have a culture medium during IVM that enables the cytoplasmic and nuclear maturation is achieved (Virginia and Novoa, 2012).
In vitro maturation (IVM) and culture processes need to be improved to produce embryos with good quality and high developmental competence (Hansen and Block, 2004). Consequently, our study conducted to investigate the impact of supplementation of RJ on bovine oocytes maturated in vitro.

Materials and methods

1.Collecting ovaries sample

After slaughter, fresh cattle ovaries were collected and transported to the laboratory within 1 to 2 hours in isotonic saline solution supplemented with(50. 5 l/ml) streptomycin and (100 IU/ml) of penicillin ,maintained at 30-35°C in box retained (Singh, et al., 2020).

2. COCs recovery:

Bovine cumulus-oocyte complexes (COCs) were retrieved using an 18-gauge needle attached to a 10 mL disposable syringe by aspirating follicles with large size (3-8 mm) diameter, and slicing the ovaries into small sections using a sterile surgical scalpel, then analyzing the sediment under stereomicroscopes.

3. Preparation of media for oocyte recuperation:

TCM-199 and 0.6 percent (v/w) bovine serum albumin in aspiration medium consisting of (BSA). Follicular fluid was collected and retained for 15 minutes in the tube. In the 60 mm petri dish, the sediment was collected and oocytes were searched under the stereo zoom microscope (Dharmendra, etal; 2011), washed in IVM medium three times. Oocytes were classified into three classes based on the ooplasm morphology and number of cumulus cell(CC) layers as: (1) best COCs; (2) strong partial oocyte complexes (POCs); (3) fair denuded oocytes (DO) (Dadashpour, etal, 2012).

Grade A: Many layers of CC with uniform cytoplasm cover the entire surface of the oocytes.

Grade B: oocytes having homogenous cytoplasm with several layers of CC surrounding at least

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70% of the body.

Grade C: oocytes with few CC. (Di Francesco, et al; 2012)

4. Prepare the (IVM) media:

For IVM, a fraction of the immature COCs were allocated at 38.5 °C , 5 percent CO_2 in the air and high humidity for 24 hours. TCM199, 200µ/mL pregnant mare serum gonadotropin (PMSG), 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, and 50 g/mL gentamicin make up the IVM medium.

The recovered COCs were distributed into three sterilized petri dishes, and the royal jelly was added in two concentrations of (5 mg/ml) and (10 mg/ml) while the 3rd dish left free from RJ as a control group. All Oocytes in all groups of study the incubated in CO2 incubator optimized on 5 percent CO2, 38.5 °C, and 95 percent humidity for a period of (20 - 24)hours. Before usage, all media solutions were sterilized by going through the Millipore membrane filter (0.22 µm) equipped with a 10 ml syringe in diameter to extract bacterial particles (Zoheir, etal; 2007) .

All visual assessments were completed. Cumulus cell expansion was considered when at least a few morphological changes in the cell distribution of each COC were observed after the in vitro maturation process (Zhang, et al. 2010; Caixeta ,et al. 2013).

A common indirect indicator of oocyte efficiency is cumulus expansion post maturation, which occurs after 20 to 24 hours of culture in an in vitro maturation setting.

5-Matured oocyte viability (trypan blue staining):

Trypan Blue staining is a technique for identifying and evaluating the viability and competence of oocytes. It aids in the selection of oocytes with greater cumulus expansion. COC in each dish was washed and stained with 0.4 percent TB for 2 minutes at 37 °C, 5 % CO_2 in a humidified environment before being washed in media to determine viability. Despite the fact that some CC were stained, just COCs with unstained oocytes were deemed alive. (Quezada et al., 2018). The staining solution was made by dissolving the dye in a proper quantity of phosphate buffer saline and adjusting the pH to 7.

Individual COC was then immersed for 2 minutes in a 50mm droplet of prepared solution.

Statistical analysis:

SPSS's one-way ANOVA model was used to evaluate the data in this analysis (SPSS Science, Chicago, USA). Differences were compared by Tukeys multiple comparison post hoc test. All data were presented as mean ± SEM and the differences were considered as significant at $P< 0.05$

Results:

The total number of recovered COCs in all sessions of study was 281 COCS, the largest number of them were graded as A, then as grade B and the last as grade C (118 (41.99%), 109 (38.79%), and 54 (19.22%)) respectively.

Results also showed that the supplementation of a RJ in a level of 5mg / ml and 10mg / ml to the TCM199 media causing an improvement in cow oocytes maturated in vitro in compare to the control group in percentage of maturation (72.04±6.46, 83.15±4.02 and 60.21±9.08) respectively, with the superiority of the group of 10 mg/ ml comparing to the group of 5 mg /ml, but the differences were not significant at $P<0.05$ (Table 1).

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Groups	Recovered COCs NO.	Matured oocytes No. (%±SEM)	Non-matured oocytes No. (%±SEM)	Degenerated oocytes No. (%±SEM)
Control	93	56 (60.21±9.08)[a]	3 (3.22±1.85)[a]	34 (36.55±7.98)[a]
5mg RJ	93	67 (72.04±6.46)[a]	0[a]	26 (27.95±6.46)[a]
10mg RJ	95	79 (83.15±4.02)[a]	0[a]	16 (16.84±4.02)[a]

Table 1 The percentage of in vitro maturated cow oocytes in different groups of experiment.
*The high letters denote important differences between groups at P<0.05.

The percentage of viable in vitro maturated cow oocytes stained with trypan blue showed a substantial (P<0.05) superiority of RJ supplemented groups (10 mg / ml and 5 mg /ml) over the control group (83.15±4.02, 72.04±6.46, and 60.21±9.08), with the 10 mg group having the better viability (Table 2).

Groups	Matured oocytes No. (%±SEM)	Viable oocytes No. (%±SEM)	Non-viable oocytes No. (%±SEM)
Control	56 (60.21±9.08)[a]	12 (21.42±6.77)[a]	44 (78.57±6.77)[a]
5mg RJ	67 (72.04±6.46)[a]	36 (53.73±7.38)[b]	31 (46.26±7.38)[b]
10mg RJ	79 (83.15±4.02)[a]	49 (62.02±4.59)[b]	30 (37.97±4.59)[b]

Table 2 The percentage of viability of in vitro maturated cow oocytes in different groups of experiment.
* The high letters denote important differences between groups at P<0.05.

Discussion

The results showed that the supplementation of a RJ in a level of 5mg / ml and 10mg / ml to the TCM199 media causing an improvement in cow oocytes maturated in vitro in compare to the control group in percentage of maturation (72.04±6.46, 83.15±4.02 and 60.21±9.08) respectively, with the superiority of the group of 10 mg/ ml comparing to the group of 5 mg /ml, but the differences were not significant (P<0.05). This result is consistent with the findings of (Eshtiyaghi et al., 2016 and Amiri et al., 2016) who recorded a same improvement in ovine oocytes maturated in vitro in media supplemented with 10 mg / ml RJ. They suggest that Improved oocyte

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maturation and subsequent growth were associated with improved redox status in the oocytes and activation of glucose metabolic pathways in the surrounding cumulus cells in the RJ group, because the oocytes analyzing glucose mainly depending on cumulus cells (Eshtiyaghi et al., 2016). The same result was recorded by (Veshkini et al., 2018) in goat, when they recorded that the RJ supplementation to IVM media leading to improved maturation ratio, but they found a superior effect in the level of 5mg / ml of RJ in compare to 10 mg /ml suggesting that this effect of RJ on IVM of caprine oocytes is due to reducing gene expression especially genes induced apoptosis and subsequently improving the microenvironment of in vitro maturation.

The trypan blue stain is a widely used supravital tool for assessing cell viability (Santos et al. 2016). Furthermore, trypan blue staining has been described as a method of evaluating the developmental competence and viability of oocytes, with no detrimental impacts on the health or viability of oocytes (Alcoba, et al. 2016). When investigate the percentage of the viability of in vitro maturated cow oocytes stained by trypan blue, our results reveals a significance ($P<0.05$) superiority of RJ supplemented groups (10 mg / ml and 5 mg /ml) in compare to the control group with best viability in the 10 mg group. Several previous studies recorded a positive impact to the RJ in protect both female and male gametes (Moradi et al., 2013; Shahzad et al., 2016). The superiority of viable in vitro maturated bovine oocytes in the RJ groups may results from highly antioxidant and free radical scavenging capability of RJ due to its content of specific antioxidant proteins which causing elevation of antioxidant enzymes (Khazaei et al., 2017) and due to health-promoting and biological compounds of RJ such as sugars, vitamins, lipids , proteins, free amino acids and minerals (Nakajima et al., 2009).

Conclusions: In conclusion , there is evidence of a good improvement activity for the RJ on the in vitro maturated cow oocytes and their viability post maturation in a level of 10 mg / ml and 5 mg / ml, with superiority to 10 mg /ml.

References

1. **Galli C, Duchi R, Crotti G, Turini P, Ponderato N, Colleoni S, Lagutina I, Lazzari G.** (2003) Bovine embryo tecnologies. Theriogenology;59:599-616.
2. **Mapletoft RJ, Hasler JF.** (2005) Assisted reproductive technologies in cattle: a review. Rev Sci Tech;24:393-403.
3. **Singh, B., Chauhan, M. S., Singla, S. K., Gautam, S. K., Verma, V., Manik, R. S., and Mukesh, M.** (2009). Reproductive biotechniques in buffaloes (Bubalus bubalis): status, prospects and challenges. *Reproduction, Fertility and Development*, *21*(4), 499-510.
4. **Galli, C., Duchi, R., Colleoni, S., Lagutina, I., & Lazzari, G.** (2014). Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology*, *81*(1), 138-15**.**
5. **Gasparrini B, De Rosa A, Attanasio L, Boccia L, Di Palo R, Campanile G, Zicarelli L.**(2008) . Influence of the duration of in vitro maturation and gamete co- incubation on the efficiency of in vitro embryo development in Italian Mediterranean buffalo (Bubalus bubalis). Anim Reprod Sci, 105(3-4):354-64.
6. **Kumar D, Anand T.** (2012) . In vitro Embryo Production in Buffalo: Basic Concepts. Journal of Buffalo Science, 1: 50-54.
7. **Isidorova, V. A., Czyzewskaa, U., Isidorovab, A. G., & Bakier, S.**(2009). Gas chromatographic and mass spectrometric characterization of the organic acids extracted from some preparations containing lyophilized royal jelly. Journal of Chromatography. B,

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Received 05 March 2021; Accepted 01 April 2021.

877, 3776–3780.

8. **Nakajima Y, Tsuruma K, Shimazawa M, Mishima S, Hara H.** 2009. Comparison of bee products based on assays of antioxidant capacities. BMC Complement Altern Med. 26:9(4). doi: 10.1186/1472- 6882-9-4.
9. **M. F. Ramadan and A. Al-Ghamdi,** (2012) "Bioactive compounds and health-promoting properties of royal jelly: a review," Journal of Functional Foods, vol. 4, no. 1, pp. 39–52 . 34.
10. **M.rghita., L. A., Produsele apicole .i principalelelor însu.iri terapeutice.** In: Albinele .i produsele lor. L. A. M.rghita., second ed., Ceres, Bucharest, 2008, pp. 280-378 .
11. **Kanbur, M., Erasian, G., Beyaz, L., Silici, S,. Liman, B.C., Altinordulu, S., Atasever, A.,** (2009) The effects of royal jelly on liver damage induced by paracetamol in mice, Exp. Toxicol. Pathol., , 61(2),123-132.
12. **Silici, S., Ekmekcioglu, O., Eraslan, G., Demirtas, A.,** (2009) Antioxidative effect of royal jelly in cisplatin induced testes damage, Urology, , 74(3), 545-551.
13. **Moradi A, Malekinejad H, Farrokhi-Ardabili F, Bernousi I**. 2013. Royal Jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small Rumin Res, 113:346-352.
14. **Shahzad Q, Mehmood MU, Khan H, ul Husna A, Qadeer S, Azam A, Naseer Z, Ahmad E, Safdar M, Ahmad M.** 2016. Royal jelly supplementation in semen extender enhances post-thaw quality and fertility of Nili-Ravi buffalo bull sperm. Anim Reprod Sci, 167:83- 88.
15. **Watanabe S, Suemaru K, Takechi K, Kaji H, Imai K, Araki H.** 2013. Oral mucosal adhesive films con taining royal jelly accelerate recovery from 5-fluorouracil–induced oralmucositis. J Pharmacol Sci. 121:110–118.
16. **Bavister, B. D., Rose-Hellekant, T. A., and Pinyopummintr, T.** (1992). Development of in vitro matured/in vitro fertilized bovine embryos into morulae and blastocysts in defined culture media. *Theriogenology*, *37*(1), 127–146.
17. **ATomek, W., Sterza, F. A. M., Kubelka, M., Wollenhaupt, K., Torner, H., Anger, M., & Kanitz, W.** (2002). Regulation of translation during in vitro maturation of bovine oocytes: the role of MAP kinase, eIF4E (cap binding protein) phosphorylation, and eIF4E-BP1. *Biology of Reproduction*, *66*(5), 1274–1282.
18. **Virginia, M., and Novoa, S.** (2012). University of The Tuscia Doctorate Schools in Genetic and Cell Biology XXIII Cycle analysis of critical factors affecting the ovum pick-up and in vitro embryo production efficiency in buffalo species ubalus bubalis.
19. **Hansen PJ, Block J.** (2004). Towards an embryocentric world: The current and potential uses of embryo technologies in dairy production. Reprod Fertil Dev 16:1–14.
20. **Singh, A. K., Prakash, C., Rohit, K., Santra, A., and Das, S. K.**(2020) Cattle embryos development through in vitro techniques using thyroxine hormone as a culture media supplement.
21. **Dharmendra, K., Anand, T., Singh, K. P., Singh, M. K., Shah., R. A., Chauhan, M. S., Palta, P., Singla, S. K., & Manik, R. S.** (2011). Derivation of buffalo embryonic stem-like cells from in vitro-produced blastocyst on homologous and heterologous feeder cells. Journal of Assisted Reproductive Genetic, 28:679–688.
22. **Dadashpour, D.N., H. Kohram and S. Zainoaldini . (**2012) Cumulus cell layers as a critical factor in meiotic competence and cumulus expansion of ovine oocytes. Small

Rumin. Res., 102: 37-42.

23. **Di Francesco, Serena, Novoa, M. V. S., Vecchio, D., Neglia, G., Boccia, L., Campanile, G., Zicarelli, L., and Gasparrini, B.** (2012). Ovum pick-up and in vitro embryo production (OPU-IVEP) in Mediterranean Italian buffalo performed in different seasons. *Theriogenology*, *77*(1), 148–154.
24. **Zhang K, Hansen PJ, Ealy AD.** (2010) . Fibroblast growth factor 10 enhances
25. bovine oocyte maturation and developmental competence in vitro. Reproduction. 140:815–826.
26. **Caixeta ES, Sutton-McDowall ML, Gilchrist RB, Thompson JG, Price CA,**
27. **Machado MF, Lima PF, Buratini J.** (2013) . Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. Reproduction. 146:27–35.
28. **Eshtiyaghi M, Deldar H, Pirsaraei ZA and Shohreh B.** (2016). Royal jelly may improve the metabolism of glucose and redox state of ovine oocytes maturated in vitro and embryonic development following in vitro fertilization. Theriogenology, 85: 2210-2221.
29. **Amiri MV, Deldar H and Pirsaraei Z.** (2016). Impact of supplementary royal jelly on in vitro maturation of sheep oocytes: genes involved in apoptosis and embryonicdevelopment. Syst Biol Reprod Med, 62(1): 31-38.
30. **Veshkini A, Mohammadi-Sangcheshmeh A, Ghanem N, Abazari-Kia AH, Mottaghi E, Kamaledini R, Deldar H, Ozturk I and Gastal EL**. (2018). Oocyte maturation with royal jelly increases embryo development and reduces apoptosis in goats. Anim. Reprod., 15(2): 124-134.
31. **Santos ECS, Pradieé J, Madeira EM, Pereira MM, Mion B, Mondadori RG, Vieira AD, Pegoraro LMC, Lucia JT.(** 2016). Selection of porcine oocytes in vitro
32. through brilliant cresyl blue staining in distinct incubation media. Zygote. 25:49–55.
33. **Alcoba D, Conzatti M, Ferreira G, Pimentel A, Kussler A, Capp E, Corleta H, Brum I.** (2016). Safety of brilliant cresyl blue staining protocols on human granulosa and cumulus cells. Zygote. 24(1):83–88.
34. **Khazaei M, Ansarian A and Ghanbari E.** (2017). New findings on biological actions and clinical applications of royal jelly: a review. Journal of Dietary Supplements. (Online) journal homepage: http://www.tandfonline.com/loi/ijds20.
35. **Nakajima Y, Tsuruma K, Shimazawa M, Mishima S, Hara H.** 2009. Comparison of bee products based on assays of antioxidant capacities. BMC Complement Altern Med. 26:9(4). doi: 10.1186/1472- 6882-9-4.