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Full Length Article

Gene Expression and *In Vitro* Maturation of Sheep Oocytes using Bee Pollen and Bee Honey as Medium Supplements

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Abstract

This study was conducted to investigate the effects of raw honey obtained from black seed or Sider and honeybee pollen as an additive in sheep oocyte maturation medium on the oocyte maturation rate, changes in oocyte glutathione (GSH) levels and expression of developmental candidate genes (GDF-9, MPF, C-MOS, IGF-1, BAX). Healthy immature oocytes of Najdi sheep were cultured in a medium supplemented with 5.0% Sider or *Nigella sativa* (black seed) honey + 1.0 μg/mL honeybee pollen, and after 24 h of incubation, the effects on the improvement of *in vitro* oocyte maturation were evaluated. Results demonstrated that the mean oocyte maturation rate was the best in group treated with 5% *N. sativa* (Group 3) compared with group treated with Sider or *N. sativa honey* (Group 1A and B, respectively). Mean GSH level was higher in Group 3 oocytes (11.09 ± 0.29 nmol) than in Group 2 oocytes (honey alone; 10.93 ± 0.57; $P \leq 0.05$). Mean GSH levels were significantly decreased in Group 1. Expression analysis of candidate genes showed significant upregulation of GDF-9, cyclin B, C-MOS and IGF 1 genes in Group 3 and downregulation of BAX compared with control Group 1. In conclusion, addition of 1.0 μg/mL honeybee pollen along with one of two types of bee honey (Sider and *N. sativa*) at 5% concentration to the *in vitro* maturation medium of Najdi sheep oocytes has a beneficial effect in improving the maturation rate and gene expression and increasing the glutathione concentration in matured oocytes.

Keywords: Sheep oocytes; Honeybee; Bee pollen; Gene expression; *In vitro* maturation; Glutathione

Introduction

In vitro embryo production (IVP) is one of the most critical biotechnologies in animal breeding, in which several factors can influence the efficiency and contribute toward varying production quality in embryos, irrespective of whether they are produced *in vivo* or *in vitro*. Oxidative stress is an important factor (El-Aziz *et al*. 2016; Premkumar and Chaube 2016) that results from the generation of free radicals such as reactive oxygen species (ROS) produced due to cellular metabolism.

Honey is a sweet liquid composed of a complicated sugar mixture and a natural product made by *Apis mellifera* (honeybees) using nectar collected from plants and some bee secretions (Sowa *et al*. 2019). Honey also consists of small amounts of bioactive constituents, including minerals, enzymes, phenolic acids, vitamins, flavonoids, and organic acids (Sime *et al*. 2015), along with low concentrations of proteins that contribute to its pharmacological activities, including anti-inflammatory (Tonks *et al*. 2003; Yusof *et al*. 2007) and antimicrobial properties (Taormina *et al*. 2001; Gomes *et al*. 2010). Furthermore, honey contains vital biologically active molecules such as glutamine, taurine, cysteine, glutamic acid, and threonine (Paramás *et al*. 2006). Although there are variations in the climatic conditions in which honey is produced, the major ingredients in most of the types are similar but might differ in concentrations of the various components. Studies have verified, for example, that honey may act as a natural antioxidant (Gannabathula *et al*. 2017; Liu *et al*. 2019), which tends to vary with differences in flower arrangements and might significantly affect the antioxidant potential due to the differences in antioxidant enzymatic activities, such as those of peroxidase, catalase, and glucose oxidase, along with variations in the content of secondary plant metabolites such as flavonoids and phenols, also with high antioxidant properties (Escriche *et al.* 2014; Sadowska *et al*. 2019).

Bee pollen is a fine powder collected by bees from various plant species. It is transformed into a complex product by mixing with nectar and bee salivary secretions (Pawar *et al*. 2014). Bee pollen consists of major components, including proteins and amino acids, sugars, and lipids, as well as minor components, including vitamins, minerals, and flavonoid glycosides (Bogdanov 2004). Although biologically active ingredients of bee pollen are present in small quantities, they contribute to its beneficial

properties (Guiné 2015). Bee pollen contains approximately 10.4% of essential amino acids, including threonine, phenylalanine, lysine, isoleucine, methionine, leucine, tryptophan, histidine and valine (Roulston and Cane 2000).

Oxidative stress arises due to the generation of ROS in *in vitro* culture (IVC) conditions, which leads to a reduction in embryonic development because of increased turnover of oocytes, thereby resulting in spontaneous damage to mitochondria and a subsequent reduction in adenosine triphosphate (ATP) synthesis, which in turn causes a decrease in the developmental competence of oocytes (Jagannathan *et al*. 2016; Khazaei and Aghaz 2017; Sasaki *et al*. 2019). The *in vivo* environment contains oxygen scavengers in the follicular and oviduct fluids to protect oocytes and embryos from oxidative stress (Duzguner *et al*. 2014). This is because the process of oocyte protection plays a critical role against the effects of ROS at the preimplantation embryonic developmental stage. The scavengers are antioxidants for ROS that help in maintaining a balance between oxidant/antioxidant in the oocytes.

Several researchers have tested the effects of antioxidant supplements such as L-carnitine (Dunning and Robker 2017), melatonin (Do *et al*. 2015), fenugreek seed extract (Barakat and Al-Himaidi 2013) and green tea extract (Barakat *et al*. 2014) through supplementation in the *in vitro* maturation medium for oocyte development of various mammalian species (Aghaz *et al*. 2015; Rodrigues-Cunha *et al*. 2016) and have indicated the importance of antioxidants and their concentrations in contributing to the improvement of the quality of embryos/oocytes in the *in vitro* culture system (Öztürkler *et al*. 2010). Previous research has demonstrated that addition of green tea leaf extract to the IVM medium at a concentration 0.3 mg/mL resulted in improvement of the maturation rate of sheep oocytes and embryo development (Barakat *et al*. 2014). In addition, Do *et al*. (2015) demonstrated that using melatonin at a concentration of 25 ng/mL as a supplement in the IVM culture medium enhanced the developmental competence of porcine embryos. Furthermore, other studies have confirmed that the addition of antioxidants such as taurine and hypotaurine toadied in maintaining the redox status in oocytes (Suzuki *et al*. 2007; Manjunatha *et al*. 2009; Premkumar and Chaube 2014).

Moreover, supplementing the IVM culture medium with superoxide dismutase, catalase, and peroxiredoxins has been reported to have beneficial effects on the development of preimplantation embryos in mice (Legge and Sellens 1991; Natsuyama *et al*. 1993), porcine (Ozawa *et al*. 2006), and bovine (Ali *et al*. 2003). Glutathione reductase is considered as one of the most important antioxidants that functions in regulating the balance of oxidation in cells and protects them from ROS toxicity (You *et al*. 2010). A previous study showed that addition of taurine to the *in vitro* maturation medium of buffalo oocytes improved the embryo production efficiency (Manjunatha *et al*. 2009). Another earlier study conducted to explore the effect of essential and nonessential amino acids on the *in vitro* development of bovine embryos demonstrated that the use of essential amino acids alone had a detrimental effect, whereas a combination of nonessential and essential amino acids promoted blastocyst hatching and formation (Liu and Foote 1995).

In recent years, there have been several uses of bee products in both traditional and modern medicine, which include honey, royal jelly, bee pollen, beebread, bee venom, and propolis (Molan 1999; Veshkini *et al*. 2018). Recent research has reported that the addition of *Nigella sativa* honey and honeybee pollen to the *in vitro* maturation medium of sheep oocytes increased both the maturation rate and gene expression and enhanced the GSH content (Barakat *et al*. 2020; Kaabi *et al*. 2020).

All bee products possess pharmacological properties because they are rich in active biological components and enzymes and can thus promote good health and prevent the development of some diseases due to their beneficial biological and functional properties (Biesalski *et al*. 2009; Pasupuleti *et al*. 2017).

In this study, we investigated the effect of honey obtained from two plant sources, Sider and *N. sativa* (black seed) and honeybee pollen on the maturation of oocytes. Moreover, we examined the changes in GSH content and effects on the expression of candidate genes associated with oocyte maturation and development using *in vitro* matured sheep oocytes as a model.

Materials and Methods

Experimental details and treatments

Chemicals and materials: All chemicals, media, dishes used for oocyte culture, and Millipore membrane filter syringes were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.) and Nunclon (Denmark) or Thermo Fisher, respectively, unless otherwise indicated.

Experimental design: We evaluated the effects of bee products during *in vitro* maturation on the maturation rate, GSH concentration, and expression of candidate genes. A preliminary study was initially conducted to evaluate the effects of *N. sativa* honey and Sider honey at 5% concentration with or without 1.0 μg/mL honeybee pollen. Excellent and good sheep oocytes were selected and divided into three groups according to the treatments to be applied as follows: Group 1: oocytes cultured in a defined maturation medium + 5% concentration of Sider (Group 1A) or *N. sativa* (Group 1 B) honey as controls; Group 2: oocytes cultured in a defined maturation medium + 5% Sider honey + 1.0 μg/mL honeybee pollen; and Group 3: oocytes cultured in a defined maturation medium + 5% *N. sativa* honey + 1.0 μg/mL honeybee pollen.

The defined maturation medium (without supplements) comprised tissue culture medium-199 with Earl's (TCM-199) + 4.0 mg/mL bovine serum albumin

(BSA) + 0.02 IU FSH/mL + 0.23 IU LH/mL + 1.0 μg/mL estradiol 17-β + 50 μg/mL streptomycin (Barakat *et al*. 2018). Oocytes in all experimental treatments were cultured for 22–24 h after treatments in a CO_2 incubator at 38.5°C with 5% CO_2 and high humidity (>90%). Each treatment was repeated three times on different days.

Experimental procedures

Oocyte collection: Najdi mature female sheep ovaries were collected from Riyadh slaughterhouses, Saudi Arabia, and transferred to the laboratory within 1–2 h in warmed physiological saline (35°C–37°C) supplemented with antibiotics. Oocytes were aspirated from visible follicles on the ovary surface (2–8 mm diameter) using 20 G needles attached to a 10 mL disposable syringe. Then, all oocytes having several layers of cumulus cells and homogeneous cytoplasm (healthy oocytes) were selected as described by Kharche and Birade (2013) for *in vitro* maturation (IVM) experiments.

***In vitro* maturation of oocytes:** Selected oocytes were washed two to three times with the collection medium (TCM-199 + 50 μg/mL kanamycin + 0.5 m*M* sodium pyruvate + 50 μg/mL heparin + 4 mg/mL fatty-acid-free BSA) and washed three times with maturation medium. Then, the oocytes were cultured in their respective groups (Groups 1–3) in 35-mm Petri dishes; each group contained 15–20 cumulus oocyte complexes in droplets of IVM medium; each drop was ~100 μL of IVM medium overlaid with mineral oil and placed into 5% CO_2 incubator at least 2 h before culture.

Examination of oocyte nuclear maturation: After treatments and allowing for the elapse of the maturation period, hyaluronidase (100 I.U/mL) and a mechanical force-by-mouth procedure were used to gently pipette and clean the oocytes from cumulus cells and then fix them in acetic acid/ethanol (1:3) for 24–48 h. Next, denuded oocytes were stained with 1% aceto-orcein in 45% acetic acid (Prentice-Biensch *et al*. 2012). Oocyte nuclear division stages were divided into germinal vesicle breakdown (GVBD), germinal vesicle (GV), metaphase I (MI), anaphase (anaph.) and metaphase II (MII). Oocytes in the MII stage were recorded as mature oocytes.

GSH assay: The GSH concentration in matured oocytes was estimated according to the instruction provided in the GSH estimation kit (Sigma, Cat. CS0260) and the linear equation of the standard solution that accompanied the kit was applied.

Measurement of gene expression: Total RNA was prepared from each oocyte treatment group, and cDNA was synthesized in two steps according to the instructions of the manufacturer kits. RNA was isolated using the PureLink RNA Mini Kit (Cat.No.12183018A) and cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Cat.No.4368813) for real-time PCR. The candidate genes of interest were GDF-9, BAX, Cyclin B, C-MOS and IGF1. The sequences of specific primers used in the reverse transcription for the candidate genes are shown in Table 1 and the cycle conditions of RT-PCR are shown in Table 2.

The expression of each gene was measured using the comparative Ct ($2^{-\Delta CCt}$) method (Livak and Schmittgen 2001), according to the following equations:

$$\Delta \text{ Ct (treated)} = \text{Ct (target)} - \text{Ct (reference)}$$

$$\Delta \text{ Ct (non-treated)} = \text{Ct (target)} - \text{Ct (reference)}$$

$$\Delta\Delta \text{ Ct} = \Delta \text{ Ct (treated)} - \Delta \text{ Ct (non-treated)}$$

$$\text{Gene Expression} = 2^{-\Delta\Delta Ct}$$

Statistical analysis

All data were statistically analyzed using the SPSS program (v. 20.0, S.P.S.S. Inc., Chicago, I.L., U.S.A.). The preliminary experimental data were analyzed using a two-way analysis of variance (ANOVA) and the second experimental data were analyzed using one-way ANOVA. After the statistical analysis, the differences between mean values were performed by Duncan's test, considering $P \leq 0.05$ to be statistically significant. All results were expressed as mean ± SEM (standard error of the mean).

Results

Effect of supplementing maturation medium with bee pollen and black seed honey on *in vitro* maturation rate

As shown in Table 3, the maturation rate of sheep oocytes after the addition of bee pollen along with bee honey to the maturation medium was significantly better than that achieved using black seed honey alone, whereas when black seed honey was used, the mean maturation value of oocytes in the MII stage was 0.49 ± 0.04 compared to that observed with using black seed honey alone (0.40 ± 0.04). The same results were also found with the trait GV in both treatments and for the trait MI when using Sider honey alone or bee pollen with Sider honey (0.05 ± 0.02 or 0.14 ± 0.02). The opposite result was observed for the other traits, where the mean values in the treatment with black seed honey alone were significantly higher than those observed using the combination of black seed honey and bee pollen as medium supplements. Hence, it was inferred that the addition of bee pollen to black seed honey improved the maturation rate of sheep oocytes *in vitro*, as it significantly increased the mean value of the oocytes in the MII stage.

Effect of adding bee pollen along with honey to maturation medium on GSH content in *in vitro* matured Najdi sheep oocytes

As shown in Fig. 1, adding bee pollen along with black seed honey to the maturation medium significantly increased the concentration of GSH in matured oocytes compared to that observed with using black seed honey alone. In contrast, the addition of bee pollen to Sider honey did not improve the

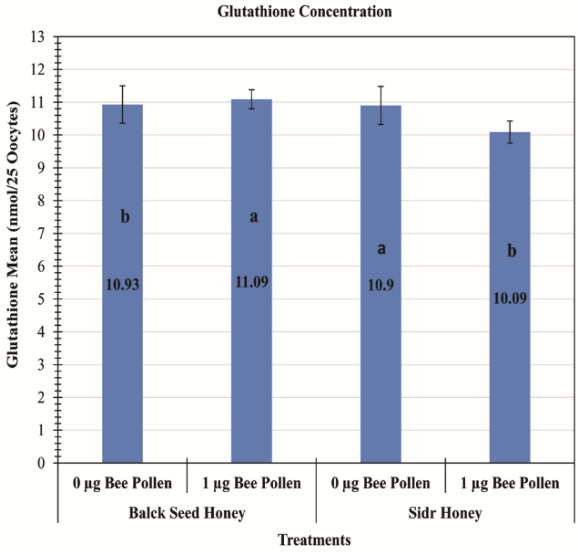

Fig. 1: Effect of adding 1 μg/mL bee pollen + 5% black seed honey to the maturation medium on the mean concentration of glutathione (GSH) content in matured sheep oocytes

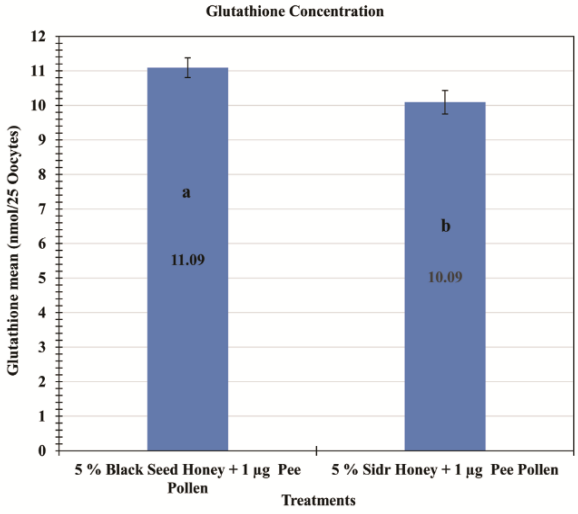

Fig. 2: Effect of supplementing maturation medium with either black seed honey combined with bee pollen or Sider honey combined with bee pollen on glutathione (GSH) content of matured Najdi sheep oocytes

maturation rate. Therefore, adding bee pollen was beneficial when added in combination with black seed honey.

Effect of adding bee pollen along with black seed honey to maturation medium on the expression of candidate genes

The expression levels of the candidate genes are shown in Table 4, which indicate that the addition of bee pollen at 1 μg/mL concentration in the presence of black seed or Sider honey at 5% concentration to the maturation medium resulted in significantly increased mean expression levels of all the examined genes, except BAX gene (apoptotic gene), whose expression was significantly decreased with the combination of black seed honey and bee pollen IVM medium supplement and thus, improving that the expression of the genes responsible for the development and regulation of oocyte maturation, and by the same token repressed physiological programmed cell death (apoptosis).

Comparison of the effect of adding black seed honey combined with bee pollen and Sider honey along with bee pollen on *in vitro* maturation rate

The results of the comparison between the use of black seed honey combined with bee pollen and Sider honey along with bee pollen revealed no significant differences between the two treatments in all traits, despite the increase in the mean values in the case of the former treatment in relation to the MII trait and the decrease was observed in the mean values of the other traits compared to the mean values of the second experimental treatment (Table 5).

Comparison of the effect of adding black seed honey combined with bee pollen and Sider honey along with bee pollen on GSH content

As shown in Fig. 2, using black seed honey at a concentration of 5% combined with bee pollen at a concentration of 1 μg/mL as supplements in the maturation medium for culturing Najdi sheep oocytes *in vitro* was more favourable toward oocyte maturation than using Sider honey combined with bee pollen at the same concentrations (11.09 ± 0.29 *vs*. 10.09 ± 0.34), respectively.

Comparison of the effect of adding black seed honey combined with bee pollen and Sider honey along with bee pollen on the expression of candidate genes

As shown in Table 6, adding black seed honey combined with bee pollen to the *in vitro* maturation medium of Najdi sheep oocytes was better than using Sider honey combined with bee pollen because it resulted in significantly higher mean expression levels of all the examined genes in matured oocytes, except for the mean expression level of the apoptotic gene (BAX), for which the opposite result was obtained. BAX expression level was significantly higher in the treatment with Sider honey combined with bee pollen IVM medium supplement than in the former treatment (black seed honey combined with bee pollen) (7.61 ± 0.244 *vs*. 5.18 ± 0.707; $P \leq 0.05$, respectively). Therefore, using the maturation medium supplemented with the combination of black seed honey and bee pollen leads to the best outcome concerning the maturation of Najdi sheep oocytes.

Discussion

To our knowledge, this study is the first investigation to

Table 1: Primer sequences and functions of the studied candidate genes

Gene	Forward Primer	Accession Numbers	Function
β-Actin	Forward: AGGCCAACCGTGAGAAGATG Reverse: AATCGCACGAGGCCAATCTC	NM_001009784.1	Housekeeping gene; cell motility, structure, and integrity
GDF-9	Forward: AGCTGAAGTGGGACAACTGG Reverse: ACACAGGATGGTCTTGGCAC	NM_001142888.2	Granulosa cell development
BAX	Forward: TGCATCCACCAAGAAGCTGAG Reverse: AGGAAGTCCAATGTCCAGCC	XM_004015363.1	Apoptotic gene
Cyclin B	Forward: GAGGGGATCCAAACCTTTGTAGTGA Reverse: CTTCTTTACATGGGAGGTCTTTAAC	L48205	Cell cycle regulation
C-MOS	Forward: CTTGGACCTGAAGCCAGCGAACATT Reverse: GTTAGAGGCAGGCAGGGAGAGCCGC	X78318	Cell cycle regulation
IGF1	Forward: TGTGGAGACAGGGGCTTTTA Reverse: CAGCACTCATCCACGATTCC	NC 022297.1	Cell development and differentiation

Table 2: RT-PCR cycle conditions

Hold Stage	PCR Stage	Melt Curve Stage
50°C 2 min	95°C 15 s	95°C 15 s
95°C 10 min	60°C 1 min	60°C 1 min
	70°C 30 min	95°C 15 s

Table 3: Effect of adding bee pollen (1 μg/mL) to the maturation medium supplemented with 5% honey on the maturation rate of Najdi sheep oocytes

Trait Treatment #	GV	GVBD	MI	Anaphase	MII	Degenerated
Black Seed Honey	0.02±0.01^{b}	0.32±0.03^{a}	0.12±0.02^{a}	0.02±0.01^{a}	0.40±0.04^{b}	0.12±0.02^{a}
Black Seed Honey + Bee pollen	0.08±0.02^{a}	0.27±0.03^{b}	0.10±0.02^{b}	0.00±0.00^{b}	0.49±0.04^{a}	0.06±0.02^{b}
Sider Honey	0.05±0.02^{b}	0.22±0.03^{a}	0.05±0.02^{b}	0.02±0.01^{a}	0.44±0.04^{b}	0.23±0.03^{a}
Sider Honey + Bee pollen	0.14±0.02^{a}	0.15±0.03^{b}	0.14±0.02^{a}	0.01±0.01^{b}	0.48±0.04^{a}	0.10±0.02^{b}

* Different letters (a, b) within each column are significantly different at $P \leq 0.05$
* Values represent mean ± SE (standard error of mean)
# Comparisons between each type of bee honey and itself + bee pollen
GV: Germinal vesicle, GVBD: Germinal vesicle break down, MI: Metaphase I, MII: Metaphase II

Table 4: Effect of adding bee pollen (1 μg/mL) to the maturation medium supplemented with 5% honey on the expression of candidate genes

Trait Treatment #	GDF-9	MPF	C-MOS	IGF-1	BAX
Black Seed Honey	7.85±0.30^{b}	9.28±0.43^{b}	9.19±0.08^{b}	8.32±0.07^{b}	5.97±0.27^{a}
Black Seed Honey + Bee Pollen	8.70±0.06^{a}	9.66±0.43^{a}	9.63±0.55^{a}	9.09±0.77^{a}	5.18±0.71^{b}
Sider Honey	4.62±0.57^{b}	5.80±0.90^{b}	6.73±0.66^{b}	6.16±0.75^{b}	2.85±0.02^{b}
Sider Honey + Bee Pollen	6.04±0.01^{a}	6.49±0.14^{a}	7.12±0.43^{a}	6.57±0.34^{a}	7.61±0.24^{a}

* Different letters (a, b) within each column are significantly different at $P \leq 0.05$
* Values represent mean ± SE (standard error of mean)
# Comparisons between each type of bee honey and itself + bee pollen

Table 5: Mean values ± SEM of nuclear stages when using black seed honey with bee pollen versus Sider honey with bee pollen

Trait Treatment	GV	GVBD	MI	Anaphase	MII	Degenerated
5% Black Seed Honey+ 1 μg Bee Pollen	0.08±0.019^{a}	0.27±0.031^{b}	0.10±0.021^{ab}	0.00±0.000^{a}	0.49±0.035^{a}	0.06±0.017^{a}
5% Sider Honey+ 1 μg Bee pollen	0.14±0.024^{a}	0.15±0.025^{a}	0.14±0.024^{b}	0.01±0.007^{a}	0.48±0.035^{a}	0.10±0.021^{a}

* Different letters (a, b) within each column are significantly different at $P \leq 0.05$
* Values represent mean ± SE (standard error of mean)
GV: Germinal vesicle, GVBD: Germinal vesicle break down, MI: Metaphase I, MII: Metaphase II

Table 6: Mean values ± SEM of candidate gene expression when using black seed honey with bee pollen and Sider honey with bee pollen

Trait Treatment	GDF-9	MPF	C-MOS	IGF-1	BAX
5% Black Seed Honey+ 1 μg Bee Pollen	8.70±0.058^{a}	9.66±0.428^{a}	9.63±0.552^{a}	9.09±0.772^{a}	5.18±0.707^{b}
5% Sider Honey+ 1 μg Bee pollen	6.04±0.012^{b}	6.49±0.142^{b}	7.12±0.429^{b}	6.57±0.340^{b}	7.61±0.244^{a}

* Different letters (a, b) within each column are significantly different at $P \leq 0.05$
* Values represent mean ± SE (standard error of mean)

demonstrate how the application of the combination of black seed honey and bee pollen as oocyte maturation supplements could markedly enhance Najdi sheep oocyte maturation and thus promote embryo production. Bee pollen and black seed honey consist of vitamins, proteins, antibiotics, antioxidants, enzymes, amino acids, sugars, fats, minerals, glycosides and flavonoids (Bogdanov 2004; Pawar *et al*. 2014; Veshkini *et al*. 2018).

An agricultural medium is the key determinant factor for IVP success (Greve *et al*. 1987; Rizos *et al*. 2002; Sutton *et al.* 2003). There are different methods to improve the process through the use of medium additives such as antioxidants, hormones, and vitamins, which enhance the nuclear and cytoplasmic oocyte maturation to increase the formation rate of the blastocyst (Shabankareh *et al*. 2012; Mishra *et al*. 2016; Dunning and Robker 2017).

According to the present study results, the addition of bee pollen combined with black seed honey to the maturation medium of sheep oocytes resulted in an increase in the number of oocytes that reached the MII stage in a shorter time compared with the addition of bee pollen combined with Sider supplement and honey alone supplement controls, thus indicating an improvement in the maturation rate. Furthermore, there was an increase in the GSH content of the matured oocytes. Hence, the elevation of the intracellular GSH levels and oocyte maturation rate correlated positively, and therefore, they could act as indicators for evaluating the efficiency of oocyte development (Eppig 1996; Luberda 2005; Veshkini *et al*. 2018). Moreover, the combination of black seed honey and bee pollen treatments led to the upregulation of the expression of oocyte developmental candidate genes, which was not observed with the black seed honey alone treatment.

Bee pollen and black seed honey are enriched with active ingredients and antioxidants, which might have yielded positive effects in improving the maturation rate and gene expression (Boselli *et al.* 2003; Kodai *et al.* 2007; Tamura *et al.* 2009; Valiollahpoor *et al.* 2016; Prazina and Mahmutovic 2017; Spulber *et al.* 2017). Consistent with the present study results, several previous studies have used various components and reported beneficial effects. For instance, addition of vitamins to the maturation medium of goat oocytes (Bormann *et al*. 2003) and sheep oocytes (Shabankareh *et al.* 2012) was found to be effective in improving oocyte maturation, as well as embryonic growth. In another study in which sugars were added through the use of fructose and glucose, each at 5.5 mmol concentration, to support the IVM of swine oocytes, it was observed that fructose supplement is better than glucose in the *in vitro* production of swine embryos (Wongsrikeao *et al.* 2006). Furthermore, the addition of quercetin (QT), a component of bee pollen with antioxidant properties, at a low concentration (1.0 μg/mL) to the maturation medium of swine oocytes resulted in an increase in the number of oocytes that reached the MII stage at a higher rate and a decrease in ROS levels (Kang *et al.* 2016).

The results of the present study are also consistent with those reported by Veshkini *et al*. (2018), who used royal jelly, one of black seed honey products, at a concentration of 5 mg/mL as an IVM medium supplement and observed an improvement in the maturation rate of goat oocytes, leading to an enhancement in the GSH content and a reduction in the expression of apoptosis-inducing genes. In addition, several studies have demonstrated similar results using royal jelly in culture media (Ali *et al.* 2003; Dey *et al.* 2012; Choi *et al.* 2013; Do *et al*. 2015; Fakruzzaman *et al*. 2015; Mazangi *et al.* 2015; Mishra *et al.* 2016; Valiollahpoor *et al.* 2016).

ROS are produced by oocytes and embryos through metabolism, which stimulate granulosa apoptosis, leading to a reduction in oocyte maturation and embryonic development (Khazaei and Aghaz 2017). Therefore, oxidative stress certainly has a negative impact on *in vitro* oocyte maturation and subsequent embryonic development. However, while present *in vivo*, the oviductal and follicular fluids contain natural antioxidants that neutralize their effects, thereby protecting them from oxidative stress (Wang *et al.* 2002; Gupta *et al.* 2010).

Similarly, previous studies have demonstrated that supplementing IVM media with antioxidants such resveratrol (Kwak *et al*. 2012), melatonin (Do *et al.* 2015), and L-carnitine (Mishra *et al*. 2016) led to an improvement in the maturation rate and embryonic development, an increase in the GSH content, and a reduction in ROS levels. Moreover, cytoplasmic maturation was found to be improved through the alleviation of oxidative stress during IVM (Khazaei and Aghaz 2017).

The results of the present study also indicated an improvement in the oocyte GSH content due to the supplementation of black seed honey to the IVM medium, which thereby protected the oocytes from ROS due to the antioxidant effect and enhanced the expression of candidate genes; this finding was consistent with other studies that have used some of the components of honey and reported similar results (Ali *et al*. 2003; Dey *et al*. 2012; Kwak *et al*. 2012; Choi *et al.* 2013; Do *et al*. 2015; Fakruzzaman *et al*. 2015; Mazangi *et al*. 2015; Valiollahpoor *et al*. 2016; Veshkini *et al*. 2018).

Conclusion

The addition of a combination of 1.0 μg/mL bee pollen and 5% black seed honey as supplements to the maturation medium of Naidi sheep oocytes had a positive effect by enhancing their *in vitro* maturation rate, GSH content that protects against free radical damage, and expression of oocyte developmental candidate genes. These beneficial effects are attributable to the enriched components of bee pollen and black seed honey, consistent with previous investigations.

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Author Contributions

Conceptualization, methodology and investigation by IMK;

formal analysis by IAHB; Original draft written by IMK and IAHB; write up improvement and editing by IAHB, RAA and RAA; funding acquisition by IAHB; supervision by IAHB and RAA

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