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Ameliorative effect of bee products on *in vitro* maturation of sheep oocytes

Aaishah Kaabi[1], *Ibrahim Barakat*[2]*, *Reem Alajmi*[1], *Areej Al-Khalaf*[3], *Ommati Mehdi*[4]

[1]*Zoology Department, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia*
[2]*Cell Biology Department, National Research Centre, Dokki, Giza, Egypt*
[3]*Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, Riyadh, Kingdom of Saudi Arabia*
[4]*Department of Bioinformatics, College of Life Sciences, Shanxi Agricultural University, Taigu, P.R. China*

**Corresponding author: ibrahimahb@yahoo.com*

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Abstract: The present study was undertaken to investigate the possible stimulating effects of *Nigella sativa* (*N. sativa*) honey, natural Saudi Sider honey, and honeybee pollen to the *in vitro* maturation (IVM) medium of sheep oocytes on their subsequent development. Hence, immature oocytes were exposed to various concentrations of natural *Nigella sativa* (*N. sativa*), and Saudi Sider honey (5, 10, and 20%), as well as honeybee pollen (1, 10, 50 µg/ml) during an *in vitro* maturation period (24 hours). After the exposure time, the maturation rate, glutathione (GSH) concentration, and candidate gene expression (*GDF-9*, *MPF*, *CMOS*, *IGF-1*, and *BAX*) were evaluated. Our results showed that the maturation rate was higher in the groups challenged with the lowest level of the bee products (5% and 1 µg/ml) when compared with that in the control group; where the mean number of oocytes in the metaphase II stage reached 0.360 for the honeybee pollen-treated group, 0.293 for the *N. sativa*-treated group, and 0.203 for the natural Saudi Sider honey-treated group. The glutathione level was significantly increased in the group exposed to *N. sativa* honey when compared with the other groups. Concerning the gene expression results, the Saudi Sider honey treatment showed the best results for all the genes except the *CMOS* gene, which was significantly higher than the GI and GII groups and lower than the GIV group and the *BAX* gene which did not show a significant difference when compared with the other groups. In conclusion, the addition of natural honey and honeybee pollen at a low concentration to an IVM medium improved the *in vitro* maturation rate, increased the glutathione level, and gene expression of the *in vitro* matured ovine oocytes.

Keywords: honeybee pollen; gene expression; glutathione; *Nigella sativa*; nuclear maturation; Sider honey

In vitro embryo production (IVEP) is a valuable tool for increasing the offspring and refers to a number of procedures performed in the laboratory, including *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), and *in vitro* culture (IVC) (Viana et al. 2018). Oocyte *in vitro* maturation (IVM) is a promising technology and useful for human infertility treatment and animal production; it might be considered as an estimation of improving the genetic gain (Dunning and Robker 2018).

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The IVM technology requires the recovery of immature oocytes from small visible follicles that are found on the ovary surface and then their culture in suitable conditions prepared for maturity (Wang et al. 2014). *In vitro* maturation remains inferior to *in vivo* maturation with a reduced developmental potential, having lower blastocyst formation rates (Kyasari et al. 2012). This is due to the difference between the *in vitro* and *in vivo* situations, where maturity occurs inside the female reproductive tract and the ovarian follicle has an ideal protective environment (*in vivo*). While the *in vitro* conditions can be affected by some co-factors, including the oxygen concentration (increased up to 20%, where the optimum level of oxygen ranges from 3–5%), exposure to light, centrifugation processes, pH alterations, culture medium compositions and many others that have caused oxidative stress (Du Plessis et al. 2008; Li et al. 2012; Agarwal et al. 2014).

Oxidative stress (OS) is an imbalance between the concentration of oxidants [i.e., intracellular reactive oxygen species (ROS)] and antioxidant systems (Ommati et al. 2019a; Ommati et al. 2019b; Ommati et al. 2020). It has been repeatedly reported that OS can cause adverse effects on fertility, implantation, and pregnancy rates, through the increased ROS levels, which is one of the well-known factors affecting IVEP results (Ommati et al. 2018). Hence, *in vitro* conditions require protective mechanisms against OS, such as compounds with high antioxidant properties for scavenging ROS during maturation, and improving the oocyte and embryo quality, as well as the subsequent embryonic developmental competence (Du Plessis et al. 2008; Takahashi 2012). Therefore, many studies have attempted to supplement the maturation medium with several high-potential antioxidant compounds (i.e., amino acids, peptides, herbal components, vitamins, etc.) to test different culture conditions for IVM and also alleviate the adverse effects of ROS on the oocytes and embryos, as well as to increase the oocytes developmental potency. However, there is a good body of evidence reporting that ascorbic acid, quercetin, resveratrol, honeybee pollen, and tocopherol, as well as L-carnitine, have ameliorative roles in reducing ROS levels and increasing the intracellular reduced glutathione (GSH) reservoir in oocytes (Bormann et al. 2003; Wongsrikeao et al. 2006; Shabankareh et al. 2012; Kang et al. 2016; Dunning and Robker 2018; Al-Mutary et al. 2019; Zabihi et al. 2019). The crucial role of GSH in IVM has been repeatedly reported. GSH can also play an essential role in various biological processes; for instance, it can protect the cells against OS-induced cellular injuries, it contributes to the synthesis of protein and DNA, as well as regulates the intracellular redox balance. The association between GSH and early embryo development has been reported; therefore, the intra-oocytes GSH level can be considered as a marker of the oocyte quality and developmental competence for embryos after IVF (Zabihi et al. 2019).

Many investigations have reported that bee products, such as honey and honeybee pollen, are rich in nutrients and antioxidants. Therefore, supplementing the IVM medium with honeybee pollen or honey is a possible way to improve the IVM efficiency (Barakat et al. 2020; Kaabi et al. 2020). Honey is a complex biological substance produced by *Apis mellifera* bees and composed mainly from vitamins (e.g., retinol, tocopherol, anti-haemorrhagic vitamin, thiamine), amino acids (e.g., glutamic acid, arginine, cysteine, aspartic acid, and proline), fatty acids, flavonoids, sugars, phenolic compounds, mineral salts, carotenoid contents, and antimicrobial and antioxidant substances (Abel and Baird 2018). Kaabi et al. (2020) has reported that the addition of black seed honey (5%) to the maturation medium of sheep oocytes improved the maturity and gene expression (*GDF-9*, *MPF*, *CMOS*, *IGF-1* and *BAX*) and enhanced the intracellular glutathione concentration.

Barakat et al. (2020) have demonstrated that the use of honeybee pollen at a concentration of 1 µg/ml as a supplement in an IVM medium improved the *in vitro* maturation rate of sheep oocytes, increased the glutathione concentration, and enhanced the gene expression.

Royal jelly (RJ) is also a natural product secreted by worker bees to feed young larvae and contains mineral salts, sugars, proteins, lipids, amino acids, fatty acids and vitamins, such as vitamin A, B, C, D and E and antioxidants, and is antimicrobial and antiviral (Buttstedt et al. 2013). Some studies were focused on the effect of RJ on the *in vitro* capacitation of buffalo spermatozoa. The results indicated that RJ enhanced the cryo-survival rate, sperm motility, acrosomal reaction, and the *in vitro* fertilisation capacity of buffalo spermatozoa (Shahzad et al. 2016).

Hence, the objectives of this study were to investigate the effect of bee products (two kinds of honey

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and honeybee pollen) with different concentrations on the *in vitro* maturation of sheep oocytes, the glutathione concentration, and the division and any apoptotic related gene expression to determine the best treatment and concentration that lead to the improvement of the *in vitro* maturation conditions.

MATERIAL AND METHODS

Chemicals

The media and chemicals used in the present study were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The dishes and Millipore membrane syringe filters for the oocytes culture were obtained from Nunclon (NUNC, Roskilde, Denmark), and Thermo Fisher Scientific Inc. (Logan, UT, USA). The salts for preparing the buffer solutions were of an analytical grade and obtained from Merck (Darmstadt, Germany).

Experimental design

This study was designed to assess the effects of bee products, including two kinds of honey (Sider and *N. sativa*) (Turkish product; purchased from a shop called Dr. Assal with the assurance of being natural and not adulterated) and honeybee pollen (Canadian product; purchased from Chemco Group through Qassim apiaries) during the IVM of sheep oocytes on the maturation rate, GSH concentration, and the expression of the understudied genes to improve the established defined maturation medium. For this purpose, immature oocytes were divided into four groups according to the type of treatment with three replicates in each group as follows:

- Group I (GI): *In vitro* maturation (IVM) defined medium without any supplements (control group).
- Groups II (GII): Oocytes were cultured in a defined maturation medium plus *N. sativa* honey at different concentrations (5, 10, and 20%; first concentration, second concentration, and third concentration, respectively).
- Groups III (GIII): A defined maturation medium plus various concentrations of Saudi Sider honey (5, 10, and 20%; first concentration, second concentration, and third concentration, respectively).
- Groups IV (GIV): Various concentrations of honeybee pollen (1, 10, and 50 µg/ml; first concentration, second concentration, and third concentration, respectively), were added to the defined *in vitro* maturation medium of sheep oocytes (Kang et al. 2016).

The culture conditions in all the groups were 38.5 °C, 5% CO_2, and high humidity for 22–24 hours. The defined maturation medium used in the culture was composed of TCM-199 + 4.0 mg/ml of bovine serum albumin (BSA) + 0.02 IU FSH/ml + 0.23 IU LH/ml + 1.0 µg/ml of oestradiol 17-β + 50 µg/ml of streptomycin.

Experimental procedures

OOCYTE COLLECTION AND GRADING

The ovaries were collected immediately after slaughter from non-infected Najdi ewes in a Riyadh slaughterhouse, the Kingdom of Saudi Arabia, and immediately transported to the laboratory within 2 h in a tube containing a warmed saline solution (37 °C) supplemented with 50 µg/ml of streptomycin and 100 IU/ml of penicillin. Afterwards, all the ovaries were rinsed with 70% ethanol to eliminate the blood and any infections, and they were washed twice in a fresh warmed normal saline solution and kept in a water bath under the same temperature until the cumulus-oocyte complexes (COCs) aspirated from the ovarian follicles (~ 2 mm to 6 mm) using a 10 ml syringe with an l8 gauge needle containing 500 µl of an aspiration medium [TCM-199 + 50 µg/ml of kanamycin + 0.5 mM of sodium pyruvate + 50 µg/ml of heparin + 4 mg/ml of bovine serum albumin (BSA)].

IN VITRO MATURATION OF OOCYTES

The COCs were graded as 1) Grade-A: The COCs had more than three layers of cumulus cells and a homogeneous granular grey cytoplasm; 2) Grade-B: The COCs had less than three layers of cumulus cells surrounding the zona pellucida with a homogeneous cytoplasm; and 3) Grade-C: A very tiny scattered cumulus mass and displayed

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a heterogeneous cytoplasm. The Grade-C oocytes were discarded, and only grades A and B were used for the IVM.

The selected COCs were washed three times with a maturation medium and randomly cultured in four-well plates containing a maturation medium with or without various concentrations of honey and honeybee pollen according to the experimental design of the study. Each group of oocytes (20 to 35 oocytes) was cultured for 22–24 h for maturation under the mentioned conditions.

NUCLEAR STAINING OF MATURED OOCYTES

The mature COCs of each group were denuded using hyaluronidase at a 100 IU/ml concentration by a mouth-controlled pipetting system to completely remove the cumulus cells. The denuded mature oocytes were fixed in acetic acid/ethanol (1 : 3) for at least 24 h and then stained with a 1% acetoorcein stain. Subsequently, the stained oocytes were examined with a stereomicroscope and classified according to their nuclear division into five stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase (Anaph.), and metaphase II (MII). All the oocytes in the MII stage were considered as matured oocytes.

GLUTATHIONE ASSAY

At the end of the IVM, the denuded matured oocytes were stored at –80 °C in 1.5 ml Eppendorf tubes containing 10 µl of phosphate-buffered saline (PBS). A commercial assay kit (CS0260; Sigma Aldrich, St. Louis, USA) was used to determine the nanomole levels of the reduced glutathione (GSH).

TOTAL RNA EXTRACTION AND REAL-TIME PCR (RT-PCR)

The oocyte RNA was extracted using a PureLink® RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA, USA; Cat. No. 12183018A) based on the manufacturer's instructions. The RNA quality and quantity were examined using a NanoDrop ND-1000 spectrophotometer (Nano-Drop, Wilmington, USA). Afterwards, reverse transcription (RT) was performed using a High-Capacity cDNA Reverse Transcription Kit (4368813; Applied Biosystems, Carlsbad, USA). In brief, 15 µl of the DNase I-treated RNA was mixed to the RT mixture (15 µl) containing 75 IU of a MultiScribe reverse transcriptase and the RT buffer. The RT reaction mixture was incubated (25 °C, 10 min), followed by 37 °C for 120 min, and then at 85 °C for 5 minutes. Finally, the cDNA was stored at –20 °C till the day of the experiment for the quantitative real-time polymerase chain reaction (qRT-PCR). Following the manufacturer's instructions, the quantitative real-time PCR (qRT-PCR) was conducted using a QuantiTect SYBR Green PCR kit (Takara Biological Engineering Company, Dalian, P.R. China). The mRNA expressions of the candidate genes, including *GDF-9*, *Bax*, *Cyclin B*, *C-MOS*, and *IGF-1*, were recorded using quantitative real-time PCR (qRT-PCR). The primer sequences were designed by Primer v5.0 software and come from the literature (Table 1). The qRT-PCR was performed

Table 1. Information on the primer sequences of the studied genes

Gene	Primers	Reference	Accession No.	Function
β-actin	F: AGGCCAACCGTGAGAAGATG R: AATCGCACGAGGCCAATCTC	Barakat et al. (2018)	NM_001009784.1	housekeeping gene; cell motility, structure, and integrity
GDF-9	F: AGCTGAAGTGGGACAACTGG R: ACACAGGATGGTCTTGGCAC	Barakat et al. (2018)	NM_001142888.2	granulosa cell development
Bax	F: TGCATCCACCAAGAAGCTGAG R: AGGAAGTCCAATGTCCAGCC	Barakat et al. (2018)	XM_004015363.1	apoptotic genes
Cyclin B	F: GAGGGGATCCAAACCTTTGTAGTGA R: CTTCTTTACATGGGAGGTCTTTAAC	Khalil et al. (2010)	L48205	cell cycle regulation
C-MOS	F: CTTGGACCTGAAGCCAGCGAACATT R: GTTAGAGGCAGGCAGGGAGAGCCGC	Khalil et al. (2010)	X78318	cell cycle regulation
IGF1	F: TGTGGAGACAGGGGCTTTTA R: CAGCACTCATCCACGATTCC	Fan et al. (2017)	NC 022297.1	cell development and differentiation

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Table 2. Effects of several treatments on the efficiency of the *in vitro* sheep oocyte maturation

Trait / Treatment	GV	GVBD	Metaphase I (MI)	Anaphase	Metaphase II (MII)	Degenerated
Control (GI)	0.380 ± 0.034^{a}	0.430 ± 0.013^{a}	0.025 ± 0.001^{b}	0.000 ± 0.000^{a}	0.030 ± 0.001^{c}	0.135 ± 0.006^{b}
N. sativa honey (GII)	0.082 ± 0.001^{c}	0.362 ± 0.020^{ab}	0.082 ± 0.003^{a}	0.013 ± 0.001^{a}	0.293 ± 0.013^{a}	0.178 ± 0.012^{b}
Natural Sider honey (GIII)	0.230 ± 0.012^{b}	0.273 ± 0.011^{c}	0.028 ± 0.001^{b}	0.005 ± 0.001^{a}	0.203 ± 0.007^{b}	0.287 ± 0.022^{a}
Honeybee pollen (GIV)	0.062 ± 0.003^{c}	0.322 ± 0.021^{bc}	0.072 ± 0.004^{a}	0.012 ± 0.002^{a}	0.360 ± 0.022^{a}	0.190 ± 0.014^{b}

$^{a–c}$Different letters within each column are significantly different at $P \leq 0.05$. Values represented as mean ± SEM
GV = germinal vesicle; GVBD = germinal vesicle break down

under the following thermo-cycling conditions: Hold stage (50 °C for 2 min and 95 °C for 10 min) and 40 cycles for the qRT-PCR (95 °C for 15 s, 60 °C for 60 s, and 70 °C for 30 min), and a melt curve stage (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s) to confirm the amplified product specificity. All the tests were performed in triplicate, and the mRNA quantification was analysed using the $2^{-\Delta\Delta Ct}$ method by comparing the candidate gene expression relative to the *β-actin* as a housekeeping gene (Ommati et al. 2019b; Ommati et al. 2020).

Statistical analysis

The data of the current investigation were analysed using SPSS software v20.0 (SPSS Inc., Chicago, IL, USA) using a two-way analysis of variance (ANOVA). The mean ± standard error of the mean (SEM) of at least three independent trials are described in the text. The mean difference was performed using Duncan's test (Duncan and Duncan 1955). The level of significance was set at $P \leq 0.05$.

RESULTS

Effect of treatment (two types of honey and honeybee pollen) factor

The natural Sider honey (GIII) was the worst treatment for improving the maturation rate of sheep oocytes (Table 2). However, the *N. sativa* honey (GII) and honeybee pollen (GIV) are the best treatments; albeit, there were no significant differences between them in all the traits, especially in the metaphase II stage. It could indicate that the *N. sativa* honey and honeybee pollen can significantly improve the maturation rate of the oocytes (0.293 and 0.360, respectively) (Table 2).

The GSH content, as a valid index of the OS, was considerably increased in the cells exposed to the *N. sativa* honey when compared with that in the groups challenged with natural Sider honey and honeybee pollen (Figure 1).

The effect of the honey products on the expression of the candidate genes, including *GDF-9*, *MPF*, *CMOS*, *IGF-1*, and *BAX* in the mature oocyte, is shown in Table 3. Except for *BAX*, the relative expression levels of the other genes were significantly altered in the trial groups. The relative expression levels of *GDF-9*, *MPF*, and *IGF-1* mRNA increased dramatically in the cells challenged with natural Sider honey (GIII).

Comparison of the different treatment concentration factors

A significant difference between the first and third concentration was observed in the traits of the degenerated oocytes, metaphase MII, ger-

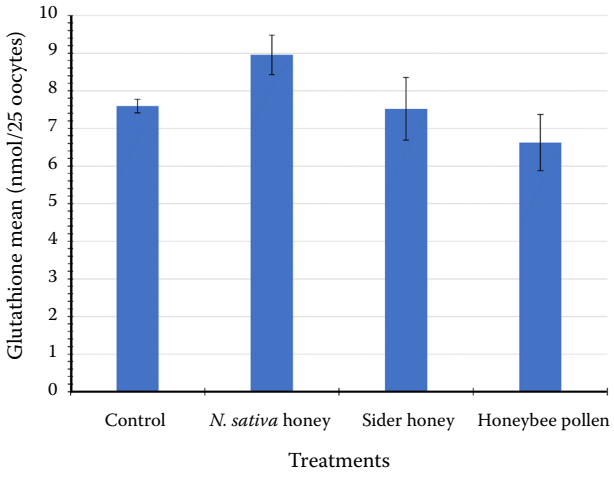

Figure 1. Effect of the honey types and honeybee pollen on the glutathione concentration in the *in vitro* matured Najdi sheep oocytes

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Table 3. Effects of several treatments on the candidate gene expression in the *in vitro* matured sheep oocytes

Trait / Treatment	*GDF-9*	*MPF*	*CMOS*	*IGF-1*	*BAX*
Control (GI)	1.007 ± 0.041^{b}	1.030 ± 0.093^{b}	1.007 ± 0.048^{c}	1.037 ± 0.089^{b}	1.153 ± 0.228^{a}
N. sativa honey (GII)	1.377 ± 0.264^{b}	2.237 ± 0.324^{b}	1.803 ± 0.368^{c}	1.629 ± 0.231^{b}	1.334 ± 0.096^{a}
Natural Sider honey (GIII)	6.051 ± 2.569^{a}	4.272 ± 1.661^{a}	4.488 ± 1.605^{b}	12.561 ± 4.588^{a}	0.971 ± 0.074^{a}
Honeybee pollen (GIV)	1.490 ± 0.218^{b}	1.471 ± 0.628^{b}	5.891 ± 1.608^{a}	4.381 ± 1.477^{b}	1.036 ± 0.212^{a}

$^{a–c}$Different letters within each column are significantly different at $P \leq 0.05$. Mean values represented as mean ± SEM

Table 4. Effect of the different concentrations of honey and honeybee pollen on the mean values of the nuclear maturation characteristics of the sheep oocytes

Trait / Treatment	GV	GVBD	Metaphase I (MI)	Anaphase	Metaphase II (MII)	Degenerated oocytes
First concentration (5% honey, 1 µg honeybee pollen)	0.114 ± 0.001^{b}	0.323 ± 0.013^{a}	0.059 ± 0.005^{a}	0.019 ± 0.002^{a}	0.330 ± 0.014^{a}	0.156 ± 0.013^{b}
Second concentration (10% honey, 10 µg honeybee pollen)	0.209 ± 0.013^{a}	0.368 ± 0.023^{a}	0.058 ± 0.003^{a}	0.004 ± 0.000^{b}	0.216 ± 0.011^{b}	0.199 ± 0.018^{ab}
Third concentration (20% honey, 50 µg honeybee pollen)	0.243 ± 0.020^{a}	0.350 ± 0.021^{a}	0.039 ± 0.002^{a}	0.000 ± 0.000^{b}	0.119 ± 0.004^{c}	0.238 ± 0.022^{a}

$^{a–c}$Different letters within each column are significantly different at $P \leq 0.05$. Values represented as mean ± SEM
GV = germinal vesicle; GVBD = germinal vesicle break down

minal vesicle (GV), and anaphase (Table 4). On the other hand, there was also a significant difference between the first and third concentration in the traits of the metaphase II (MII), anaphase, GV, and degenerated oocyte traits. However, there were no significant differences in the other stages of the nuclear division of the oocytes' germinal vesicle break down (GVBD) and metaphase I (MI) that matured *in vitro* in these concentrations. Meanwhile, MII was significantly different between the second and third concentrations (Table 4).

Altogether, it can be concluded that the best concentration is the first concentration from the bee products, where the mean oocyte number in the MII stage was significantly higher in the first concentration (0.330) when compared with that in the second and third concentrations (0.216 and 0.119, respectively) (Table 4).

The GSH content was concentration-dependent decreased. The first concentration had a decisive role on the oocyte GSH content (9.75 ± 0.45 nmol/25 oocytes), revealing another ameliorative effect of the first concentration in the oocytes matured *in vitro* (Figure 2).

The effect of various concentrations of the treatments on the candidate genes, including *GDF-9*, *MPF*, *CMOS*, *IGF-1*, and *BAX* in the oocyte matured *in vitro* is illustrated in Table 5. The relative expression levels of *GDF-9*, *MPF*, *CMOS*, and *IGF-1* in the oocytes matured *in vitro* supplemented with

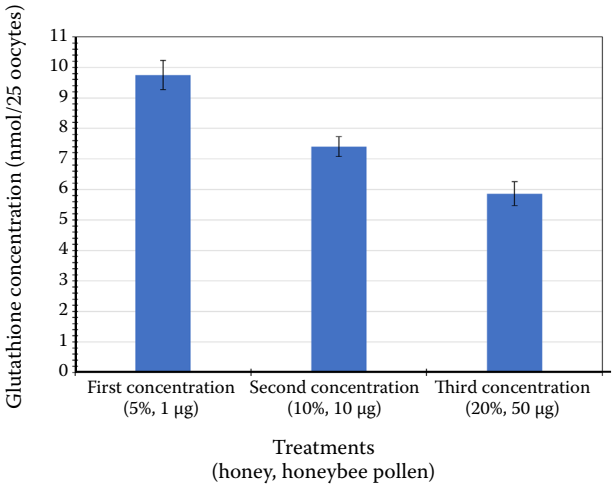

Figure 2. Comparison between the different concentrations of the treatments with the honey and honeybee pollen on glutathione concentration

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Table 5. Comparison between the different concentrations of the honey and honeybee pollen effects on the gene expression of the candidate genes

Treatment \ Trait	*GDF-9*	*MPF*	*CMOS*	*IGF-1*	*BAX*
First concentration (5% honey, 1 µg honeybee pollen)	0.916 ± 0.058^{b}	1.337 ± 0.373^{b}	0.819 ± 0.080^{b}	2.353 ± 0.309^{b}	1.042 ± 0.112^{a}
Second concentration (10% honey, 10 µg honeybee pollen)	5.330 ± 1.922^{a}	4.279 ± 1.215^{a}	5.040 ± 1.116^{a}	10.620 ± 3.707^{a}	1.354 ± 0.170^{a}
Third concentration (20% honey, 50 µg honeybee pollen)	1.197 ± 0.155^{b}	1.142 ± 0.160^{b}	4.033 ± 1.350^{a}	1.733 ± 0.401^{b}	0.975 ± 0.132^{a}

$^{a–b}$Different letters within each column are significantly different at $P \leq 0.05$. Values represented as mean ± SEM

Table 6. Effect of the interaction between the different treatments and concentrations of the honey and honeybee pollen on the nuclear maturation stage means

Treatment	Trait	GV	GVBD	Metaphase I (MI)	Anaphase	Metaphase II (MII)	Degenerated
Control (GI)		0.380 ± 0.034^{a}	0.430 ± 0.035^{a}	0.025 ± 0.011^{cd}	0.000 ± 0.000^{b}	0.030 ± 0.012^{e}	0.135 ± 0.024^{cd}
N. sativa honey (GII)	5%	0.015 ± 0.009^{d}	0.325 ± 0.033^{abc}	0.115 ± 0.023^{a}	0.025 ± 0.011^{a}	0.400 ± 0.035^{ab}	0.120 ± 0.023^{d}
	10%	0.080 ± 0.019^{cd}	0.345 ± 0.034^{ab}	0.080 ± 0.019^{abc}	0.015 ± 0.009^{ab}	0.325 ± 0.033^{bc}	0.185 ± 0.028^{bcd}
	20%	0.150 ± 0.025^{c}	0.415 ± 0.035^{a}	0.050 ± 0.015^{bcd}	0.000 ± 0.000^{b}	0.155 ± 0.026^{d}	0.230 ± 0.030^{bc}
Natural Sider honey (GIII)	5%	0.050 ± 0.015^{d}	0.220 ± 0.029^{c}	0.050 ± 0.015^{bcd}	0.015 ± 0.009^{ab}	0.435 ± 0.035^{a}	0.230 ± 0.030^{bc}
	10%	0.275 ± 0.032^{b}	0.360 ± 0.032^{ab}	0.035 ± 0.013^{bcd}	0.000 ± 0.000^{b}	0.175 ± 0.027^{d}	0.235 ± 0.030^{bc}
	20%	0.365 ± 0.034^{a}	0.240 ± 0.030^{bc}	0.000 ± 0.000^{d}	0.000 ± 0.000^{b}	0.000 ± 0.000^{e}	0.395 ± 0.035^{a}
Honeybee pollen (GIV)	1 µg	0.010 ± 0.007^{d}	0.315 ± 0.033^{abc}	0.045 ± 0.015^{bcd}	0.035 ± 0.013^{a}	0.455 ± 0.035^{a}	0.140 ± 0.025^{bcd}
	10 µg	0.100 ± 0.021^{cd}	0.335 ± 0.033^{abc}	0.090 ± 0.020^{ab}	0.000 ± 0.000^{b}	0.335 ± 0.033^{bc}	0.240 ± 0.030^{b}
	50 µg	0.075 ± 0.019^{cd}	0.315 ± 0.033^{abc}	0.080 ± 0.019^{abc}	0.000 ± 0.000^{b}	0.290 ± 0.032^{c}	0.190 ± 0.028^{bcd}

$^{a–e}$Different letters within each column are significantly different at $P \leq 0.05$. Values represent the mean ± SEM
GV = germinal vesicle; GVBD = germinal vesicle break down

the second concentrations were significantly increased (Table 5).

Also, the mRNA expression level of the *CMOS* was considerably increased in the group exposed to the third concentrations.

Effect of the interaction between the treatment and concentration factors

The results of the interaction between the treatment and the concentration factors are summarised in Table 6. The highest MII and anaphase levels in the oocytes matured with various concentrations of treatments were recorded in the lowest concentration (5% and 1 µg) of each treatment (*N. sativa*, natural Sider honey, and honeybee pollen). However, the worst condition for the *in vitro* maturation was observed in the groups treated with the third concentrations (20% and 50 µg/ml).

The mean glutathione contents were highest in the oocytes matured with the lowest concentration of the *N. sativa* honey (10.93 nmol/25 oocytes; at a 5% concentration), natural Sider honey (10.9 nmol/25 oocytes; at a 5% concentration), and honeybee pollen (9.58 nmol/25 oocytes; at a 1 µg/ml maturation medium) ($P \leq 0.05$). However, the lowest mean glutathione content in mature Najdi sheep oocytes was found in the groups challenged with the third concentration of honey and honeybee pollen treatments (20% and 50 µg/ml, respectively) (Figure 3).

Table 7 revealed that the mature oocytes *IGF-1* mRNA expression levels were markedly upregu-

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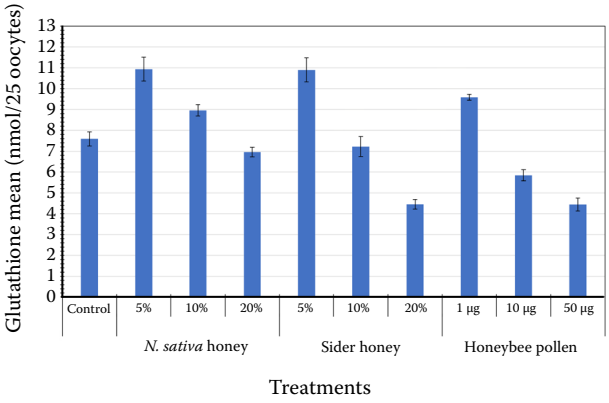

Figure 3. Mean values of the glutathione concentration in the matured sheep oocytes as a result of the interaction between two study factors

lated in the groups challenged with the second concentration (10%) of the natural Sider honey (30.167 ± 4.417) and honeybee pollen (9.940 ± 1.536).

In the mature oocytes, significant increases in the *CMOS*, *MPF*, and *GDF-9* gene expression levels were observed in the group exposed to the second concentration (10%) of natural Sider honey. However, the lowest level of the *GDF-9* and *CMOS* mRNA expression in the mature oocyte was recorded at the lowest concentration of each treatment (*N. sativa*, natural Sider honey, and honeybee pollen). Meanwhile, the highest level of the apoptosis-related gene (*BAX*) expression level was observed in the 10 µg/ml honeybee pollen (1.747 ± 0.369).

DISCUSSION

Recently, *in vitro* embryo production (IVEP) protocols have substantially progressed; however, the *in vitro* blastocyst formation rates are still low, ranging from 20% to 40%. Therefore, different approaches to the optimisation of the IVEP stages have been employed. The first strategy of the *in vitro* maturation was improved through stimulation by luteinising hormones, to stimulate their meiotic progression to metaphase II (MII) (de Oliveira Santos et al. 2018; de Oliveira et al. 2020).

As mentioned earlier, the *in vitro* oocyte maturation rates are still low. There are also discrepancies in other reports on other species. For instance, mean oocyte maturation rates of 70% have been reported in ovine (Zabihi et al. 2019), porcine (Alvarez et al. 2019), and bovine (An et al. 2019) species; as well as 45% in goats (Piras et al. 2019). Abd El-Aziz et al. (2016) reported that the reason for the discrepancies in the efficiency of the oocyte maturation might be related to the culture environment. Hence, several other components (i.e., vitamins, antioxidants, hormones, proteins, etc.) have been suggested to be added into the medium to enhance the IVM rates and reduce the oxidative stress (Shabankareh et al. 2012; Mishra et al. 2016; Dunning and Robker 2018). Bormann et al. (2003) and Shabankareh et al. (2012) added vitamins, as supplements, to the maturation medium of goat and sheep oocytes, respectively, which presented

Table 7. Effect of the interaction between the different treatments and concentrations of the honey and honeybee pollen on the gene expression mean

Treatment \ Trait		*GDF-9*	*MPF*	*CMOS*	*IGF-1*	*BAX*
Control (GI)		1.007 ± 0.082^{bc}	1.030 ± 0.185^{d}	1.007 ± 0.095^{d}	1.037 ± 0.179^{c}	1.153 ± 0.456^{ab}
N. sativa honey (GII)	5%	0.633 ± 0.084^{c}	3.277 ± 0.425^{bc}	0.793 ± 0.099^{d}	2.453 ± 0.136^{c}	1.123 ± 0.032^{ab}
	10%	1.847 ± 0.301^{bc}	1.737 ± 0.176^{cd}	3.210 ± 0.090^{c}	1.337 ± 0.288^{c}	1.597 ± 0.182^{ab}
	20%	1.650 ± 0.561^{bc}	1.697 ± 0.485^{cd}	1.407 ± 0.166^{cd}	1.097 ± 0.130^{c}	1.283 ± 0.139^{ab}
Natural Sider honey (GIII)	5%	0.957 ± 0.069^{bc}	0.983 ± 0.200^{d}	0.623 ± 0.041^{d}	3.687 ± 0.295^{c}	1.140 ± 0.090^{ab}
	10%	16.283 ± 0.829^{a}	10.800 ± 1.039^{a}	10.723 ± 1.077^{a}	30.167 ± 4.417^{a}	0.920 ± 0.188^{ab}
	20%	0.913 ± 0.009^{c}	1.033 ± 0.180^{d}	2.117 ± 0.205^{cd}	3.830 ± 0.704^{c}	0.853 ± 0.020^{ab}
Honeybee pollen (GIV)	1 µg	1.067 ± 0.020^{bc}	0.057 ± 0.029^{d}	0.853 ± 0.288^{d}	2.237 ± 0.441^{c}	0.750 ± 0.035^{ab}
	10 µg	2.183 ± 0.393^{b}	3.550 ± 1.144^{b}	5.220 ± 0.479^{b}	9.940 ± 1.536^{b}	1.747 ± 0.369^{a}
	50 µg	1.220 ± 0.220^{bc}	0.807 ± 0.193^{d}	11.600 ± 1.229^{a}	0.967 ± 0.225^{c}	0.610 ± 0.135^{b}

$^{a–d}$Different letters within each column are significantly different at $P \leq 0.05$. Resulting values are represented as a mean ± SEM

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a positive effect on improving the oocyte maturation and embryonic growth.

Meanwhile, Wongsrikeao et al. (2006) have showed that using a fructose-supplemented medium in producing swine embryos *in vitro* was better than glucose. On the other hand, it has been well reported that natural honey and honeybee pollen is made up of valuable compounds, such as sugars, amino acids, vitamins, enzymes, proteins, antioxidant compounds, antibiotics, glycosides, organic acids, minerals, phenolic compounds, and flavonoids (Solayman et al. 2016). As mentioned, ascorbic acid (AA) is a vital antioxidant component of natural honey. Khanday et al. (2019) has been reported that AA, as an additive to the maturation medium of goat oocytes, can cause a high maturation rate. On the other hand, Gustina et al. (2020) reported that the addition of 150 μM of α-tocopherol, as another antioxidant component, to the IVM of sheep oocytes could increase the number of activated oocytes. Based on the above literature, it can be concluded that supplementing the oocyte maturation medium with antioxidants can improve the rate of maturation, increase the glutathione concentration and reduce the reactive oxygen species (ROS) levels (Khazaei and Aghaz 2017). For this purpose, in the current study, a conventional culture medium supplemented with various concentrations of natural honey and honeybee pollen was investigated for the first time in the *in vitro* maturation of sheep oocytes.

Overall, this study showed the possibility of enhancing the *in vitro* maturation of oocytes supplemented with natural honey or honeybee pollen through the upregulation of some candidate genes and increase the GSH content, which might be beneficial to improve the maturation medium of ewe oocytes and finally the oocyte efficiency. This observation is consistent with *in vitro* previous studies (Veshkini et al. 2018).

Piras et al. (2019) have been reported that the *in vitro* maturation medium of caprine oocytes supplemented with 1 μM of resveratrol caused the high average development of the blastocysts and increased the glutathione levels. Also, Zabihi et al. (2019) observed that the addition of resveratrol at a 0.25 and 0.5 μM concentration, as a supplement of the IVM of sheep oocytes, led to an improvement in the meiotic competence and early embryonic development. Quercetin (QT) has also been used as a supplement to IVM of swine oocyte which led to an increasing oocyte maturation rate and reduced the ROS levels (Kang et al. 2016).

The data of the current study were in line with our previous study (Barakat et al. 2020) and that of Kaabi et al. (2020), who used honeybee pollen and black seed natural honey (*N. sativa*) in the IVM medium of sheep oocytes.

Briefly, bee products have a decisive role in the improvement of the *in vitro* maturation rate, increasing the glutathione concentration, and upregulating the gene expression.

In summary, our results demonstrate that the use of natural honey or honeybee pollen increased the mean number of oocytes reaching the MII stage (maturation rate), which led to an improvement in the glutathione content and enhancement of the gene expression controlling the cell cycling. Evaluating the antioxidant status of the matured oocytes, however, would better clarify the antioxidative effects of these compounds. Although it is speculated that the higher regulation of these candidate genes in matured oocytes challenged with these supplements might have partly contributed to improving the vital related intracellular routes, though the underlying mechanism(s) responsible for this improvement is (are) yet to be determined.

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Conflict of interest

The authors declare no conflict of interest.

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