

## Original Article

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Effect of royal jelly on *in vitro* fertilization and early embryo development following nicotine treatment in adult female ratsZhila Khodabandeh<sup>1</sup>, Vahid Nejati<sup>1</sup>, Ali Shalizar–Jalali<sup>2✉</sup>, Gholamreza Najafi<sup>2</sup>, Fatemeh Rahmani<sup>1</sup><sup>1</sup>Department of Biology, Faculty of Science, Urmia University, Urmia, Iran<sup>2</sup>Department of Veterinary Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

## ABSTRACT

**Objective:** To scrutinize the protective role of royal jelly as an anti-oxidant on nicotine-induced changes in malondialdehyde (MDA) level, p53 expression, *in vitro* fertilization (IVF) rate, and early embryo development in adult female rats.

**Methods:** A total of 56 adult female Wistar rats were divided into 8 groups ( $n=7$  in each group). Group 1 served as an untreated control group, group 2, 3 and 4 received nicotine at a dose of 0.50, 1.00 and 2.00 mg/kg respectively, group 5 received royal jelly at a dose of 100.00 mg/kg, and group 6, 7 and 8 received 0.50, 1.00 and 2.00 mg/kg nicotine, respectively, with 100.00 mg/kg body weight royal jelly. Nicotine and royal jelly were administered daily for 49 days in the experimental groups intra-peritoneally and orally, respectively. At the end of the experimental period, p53 expression, IVF rate and early embryo development as well as MDA concentration were measured.

**Results:** The IVF rate, number of cumulus oocytes, two-cell embryos and blastocysts decreased in the nicotine-treated groups in a dose-dependent manner. In addition, p53 mRNA expression and MDA levels increased in the nicotine-treated groups. Royal jelly co-administration led to partial improvement in the aforementioned parameters.

**Conclusions:** Royal jelly may have a repro-protective effect in nicotine-administered female rats in terms of its anti-oxidant and anti-apoptotic properties.

**KEYWORDS:** Fertility; Nicotine; Ovary; Rat; Royal jelly; *In vitro* fertilization; Embryo development

## 1. Introduction

Nicotine, an organic nitrogenous compound found in plants like tobacco[1], is a highly toxic substance being rapidly absorbed from the respiratory tract, oral mucosa and skin[2]. Studies on the physiological and toxic effects of nicotine in humans have been carried out relatively broadly, and today, nicotine is definitely in the rank of toxins with serious harmful effects on human health. It is widely recorded that nicotine consumption is associated with male

and female fertility disorders. Cotinine is the main metabolite of nicotine in human follicular fluid and luteal granulosa cells. Thus, evidence suggests that nicotine can affect the sex cells functions[3,4].

Nicotine can change the adrenal axis of the pituitary-hypothalamus and gonads secretion through protein absorption reduction. The follicular growth and ovulation are dependent on estrogen secretion from granulosa cells[5], stimulated with follicle-stimulating hormone (FSH) secreted from the pituitary gland[6]. Nicotine inhibits the gonadotropins release *via* ovulation prevention and follicular growth delay[1] and nicotine exposure in the perinatal period can lead to an increase in ovarian apoptosis along with steroidogenesis and fertility alterations in female offspring[7].

Increasing evidence supports the role of oxidative stress in nicotine toxicity[8]. Oxidative stress has been broadly shown to regulate apoptosis and to exert agonistic and antagonistic effects on apoptotic signaling[9]. It has been reported previously that nicotine is involved in apoptosis induction[10]. Several studies have examined the effects of nicotine on apoptosis both *in vitro* and *in vivo*, suggesting a correlation between nicotine exposure and apoptosis[11].

The p53 is known to regulate several cellular activities including cell cycle arrest and apoptosis and it is the most commonly mutated gene in human cancers[12]. Accordingly, p53 has been introduced as a female reproduction regulator being mostly involved in implantation[13]. It has been also indicated formerly that nicotine triggers mitochondria-dependent apoptosis resulting in p53 over-expression[14].

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It was also found that nicotine causes oxidant/anti-oxidant imbalance in ovarian tissue resulting in oxidative stress and anti-oxidant defense machinery debilitation eventually leading to ovarian cells DNA damage and apoptosis[15,16].

Royal jelly, a principal food of the honeybee queen, is produced by the hypo-pharyngeal glands of worker honeybee and consists of 66% water, 15% sugars, 5% lipids, and 13% proteins along with essential amino acids and vitamins[17]. It has been shown that royal jelly has pharmacological properties such as anti-tumor, anti-inflammatory, immunomodulatory, anti-oxidant and repro-protective activities[18]. It has been also suggested that royal jelly can promote folliculogenesis through its estrogenic potential[19].

Based on this concept, this study was implemented to determine the possible protective effects of royal jelly on *in vitro* fertilization (IVF) rate and early embryo development following nicotine treatment in adult female rats.

## 2. Materials and methods

### 2.1. Animals

Fifty-six adult female Wistar rats weighing ( $175\pm 3$ ) g and aged 12 weeks were obtained from Animal House of Faculty of Science, Urmia University, Urmia, Iran. The rats were maintained under controlled room temperature of ( $22\pm 3$ ) °C with 12 h light/dark cycles and the humidity level of 50%-60%. All animals had access to laboratory chow and tap water.

### 2.2. Preparation of nicotine and royal jelly

The nicotine solution was provided from Sigma-Aldrich Company (Sigma-Aldrich, USA). Fresh royal jelly was obtained from Agricultural Research and Education Center (Urmia, West Azerbaijan, Iran) and stored at  $-20$  °C until use. The substances were dissolved in normal saline.

### 2.3. Treatments

After two-week acclimation, the rats were divided into 8 groups, with 7 rats in each group. Group 1 served as an untreated control group, groups 2, 3 and 4 received nicotine respectively at a dose of 0.50, 1.00 and 2.00 mg/kg, group 5 received royal jelly at a dose of 100.00 mg/kg, and groups 6, 7 and 8 received 0.50, 1.00 and 2.00 mg/kg nicotine respectively with 100.00 mg/kg royal jelly. Nicotine and royal jelly were administered daily for 49 days in experimental groups intra-peritoneally (*i.p.*) and orally, respectively[14].

### 2.4. *In vitro* maturation (IVM)

Following anesthesia with ketamine (75 mg/kg; *i.p.*; Alfasan International, Woerden, Holland) 24 h after the experiment period[20], both ovaries were isolated and placed in tissue culture medium 199 (TCM199) (Thermo Fisher Scientific, USA; supplemented with 5% fetal calf serum 7.50 IU/mL human chorionic gonadotropin and 100 IU/mL rFSH) containing dishes. The oocytes were transferred to the oocyte dish under a stereomicroscope (Olympus, Japan), the follicles were perforated using the G30 spiked needles, and the germinal vesicle (GV)-stage oocytes were transferred to the medium. In the next step, GV-stage oocytes containing 3 to 4 rows of cell mass were selected and transferred by oral pipette containing the TCM199. The dish of the drops was incubated for 24 h. Twenty-four hours after GV presence in IVM culture media, oocytes were examined. At this time, most oocytes were entered the metaphase II stage (MII) and their first polar bodies were released.

### 2.5. *In vitro* fertilization

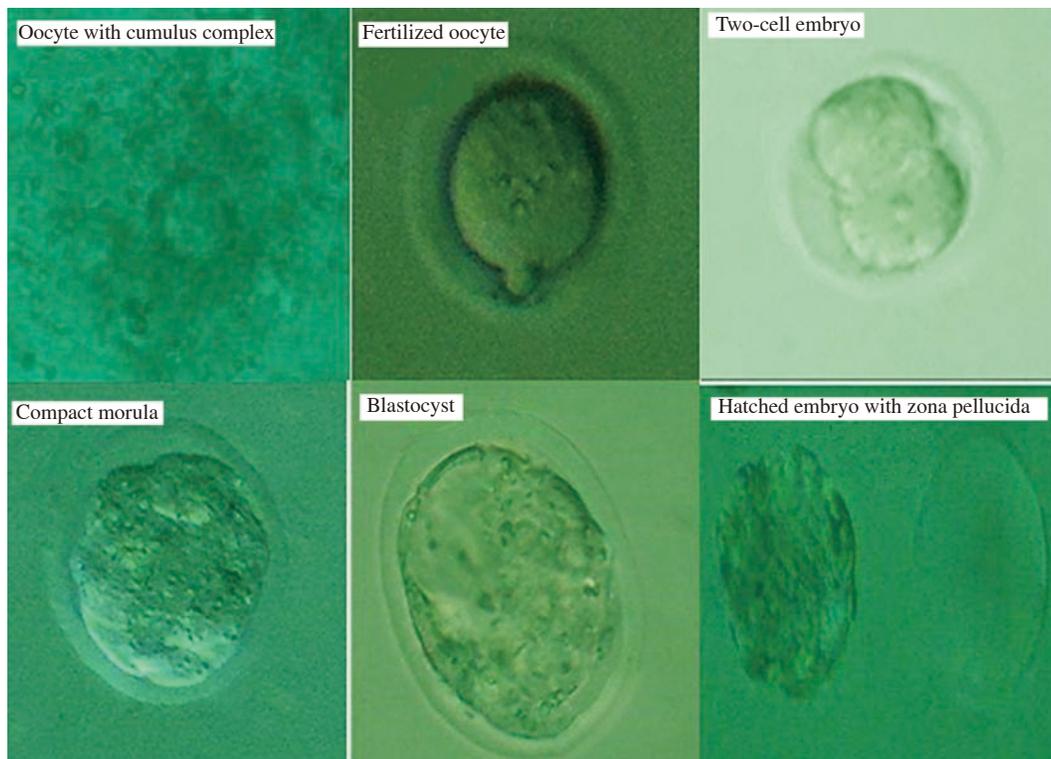
Oocytes were transferred to the modified rat 1-cell embryo culture medium (mR1ECM) after washing with mR1ECM medium under mineral oil. Then, the sperms that passed the capacity stage were added to culture medium ( $10^6$ /mL)[20]. Fertilization occurred 9 h after sperm addition with the observation of two pronuclei (Figure 1). The zygotes were transferred to a fresh culture medium that had already reached the equilibrium and cultured for 5 days[21].

### 2.6. Malondialdehyde (MDA) measurement

Lipid peroxidation levels were measured as described formerly[22]. The homogenized ovarian tissue samples were centrifuged at  $17\ 709 \times g$  for 5 min, 150  $\mu$ L of the supernatant was transferred to the test tube, then 300  $\mu$ L of 10% trichloroacetic acid was added and it was centrifuged. Then, 300  $\mu$ L of the supernatant solution was transferred to the test tube and incubated with 300  $\mu$ L of 0.67% thiobarbituric acid at 100 °C for 25 min. After cooling the solution for 5 min, the pink color of MDA with a thiobarbituric acid was detected by spectrophotometer at 535 nm. The MDA level was calculated by MDA absorption coefficient and expressed as nmol/g tissue.

### 2.7. RNA extraction

Two hundred mg of powdered ovarian tissue was added to 1 mL of TRIzol and mixed with vortex for 15 s. Samples were placed at room temperature for 5 min. For each mL of TRIzol, 0.20 mL of chloroform was added and the tubes were placed at room



**Figure 1.** Photomicrographs of *in vitro* embryo development under an inverted microscope (Olympus, Japan) at 200× magnification.

temperature for 3 min. Samples were centrifuged at  $24\,104 \times g$  for 15 min and the upper blue phase was transferred to the new tubes. Five hundred  $\mu\text{L}$  of isopropyl alcohol was added and they mixed well and kept at room temperature for 10 min. The RNA pellet was washed with 1 mL of 70% ethanol, centrifuged for 5 min at a temperature of  $4^\circ\text{C}$  and the supernatant was discarded. The RNA pellet was dried at room temperature and 30 mL of sterilized deionized water was added and mixed well to completely dissolve the RNA[23].

### 2.8. cDNA synthesis

One  $\mu\text{g}$  of extracted mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (PrimeScript 1st strand cDNA Synthesis Kit, Takara Bio Inc., Japan) in 20  $\mu\text{L}$  reaction mixture according to the manufacturer's instructions. The resulting cDNA was kept at  $-20^\circ\text{C}$  until use[24].

### 2.9. Reverse transcription polymerase chain reaction (RT-PCR)

In 25  $\mu\text{L}$  solution containing 12.50  $\mu\text{M}$  Master Mix (Cinagen, Iran), 0.50  $\mu\text{M}$  of each primer (Forward: 5'-ATGGAGGATTCACAGTCGGATA-3'; Reverse: 5'-GACTTCTTGATAGATGGCCATGG-3'), 1  $\mu\text{L}$  of cDNA and 10.50  $\mu\text{L}$  of water, the RT-PCR reaction was performed for 27 cycles of GAPDH and p53 cDNAs and for each of them, the reactions were as follows: GAPDH and p53 denaturization:  $95^\circ\text{C}$  in 3 min, connection:  $53^\circ\text{C}$  in 40 s and elongation:  $72^\circ\text{C}$  in 45 s.

### 2.10. Statistical analysis

The data were presented as mean $\pm$ SEM. The variables were analyzed by one-way analysis of variance followed by Tukey's test for *post hoc* comparisons using Statistical Package for the Social Sciences, (version 23.0, SPSS Inc., Chicago, USA). The  $P < 0.05$  was considered as a significant difference criterion. Image J Software (version 2017, National Institutes of Health, USA) was used to process and evaluate products bands.

### 2.11. Ethics statement

This study was carried out following approval from the Ethical Committee of Urmia University, Urmia, Iran, (No. 2.291, 2017.09.27) regarding the use and care of experimental animals.

## 3. Results

### 3.1. The IVM, IVF rate and early embryo development

Number of immature oocytes reaching MII and percentage of fertilization, two-cell embryos, blastocysts and hatched embryos in the nicotine groups decreased significantly in comparison with the control and royal jelly groups ( $P < 0.05$ ). In addition, the percentage of arrested embryos in the nicotine groups showed a significant increase compared to the control group. The royal jelly supplementation caused partial improvement in aforementioned parameters (Table 1 and 2).

### 3.2. Lipid peroxidation

Nicotine caused marked lipid peroxidation in the ovarian tissue as demonstrated by a significant elevation of MDA in the nicotine groups compared to the control and royal jelly groups ( $P<0.05$ ); while, the royal jelly co-treatment could lead to reductions in nicotine-induced lipid peroxidation (Figure 2).

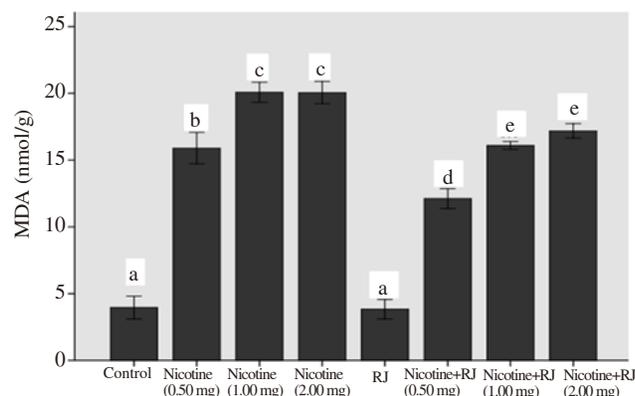
### 3.3. The RT-PCR analysis

The results showed that p53 expression in the nicotine-treated groups was not significantly different compared to the control group. In the treatment groups, only 1.00 mg/kg nicotine plus royal jelly group exhibited a significant decrease in p53 expression compared to the nicotine groups ( $P<0.05$ ; Figure 3).

**Table 1.** *In vitro* maturation in different experimental groups.

Groups	Number of GV	MII (%)
Control	46.00±5.03	87.89±1.38 <sup>a</sup>
Nicotine (0.50 mg/kg)	42.00±3.05	80.02±1.88 <sup>b</sup>
Nicotine (1.00 mg/kg)	43.00±2.08	76.19±3.34 <sup>b</sup>
Nicotine (2.00 mg/kg)	42.00±3.21	72.30±2.43 <sup>b</sup>
Royal jelly (100.00 mg/kg)	45.33±5.23	90.62±0.81 <sup>a</sup>
Nicotine (0.50 mg/kg) + Royal jelly	45.00±2.08	85.13±0.92 <sup>a</sup>
Nicotine (1.00 mg/kg) + Royal jelly	46.00±2.51	74.84±3.76 <sup>b</sup>
Nicotine (2.00 mg/kg) + Royal jelly	43.33±2.40	73.17±1.58 <sup>b</sup>

Different superscripts (a, b) in the same column show significant differences between groups ( $P<0.05$ ). GV: Germinal vesicle-stage oocyte; MII: Metaphase II stage oocyte. Royal jelly: 100.00 mg/kg royal jelly.

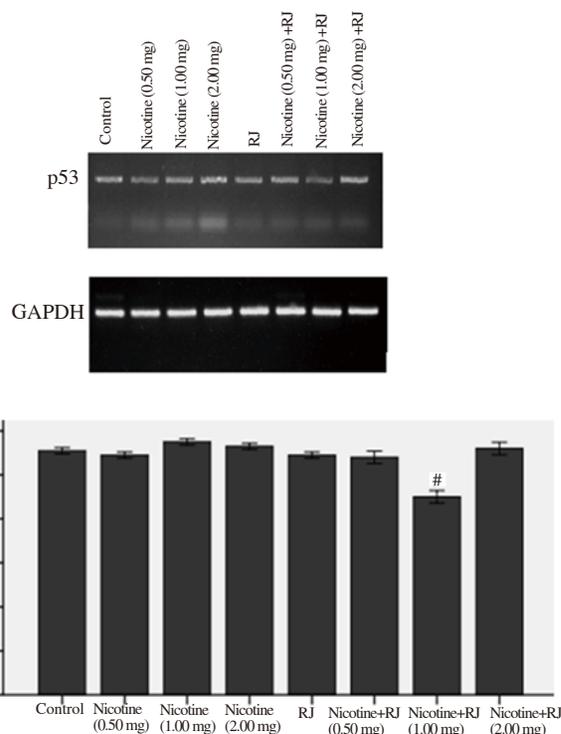


**Figure 2.** Malondialdehyde (MDA) concentration in the ovary of rats treated with nicotine (0.50, 1.00 and 2.00 mg/kg), with or without royal jelly (RJ) supplementation (100.00 mg/kg). Different superscripts (a, b, c, d, e) in the same row show significant differences between groups ( $P<0.05$ ).

**Table 2.** *In vitro* fertilization and early embryo development in different experimental groups (%).

Groups	Zygotes	Tow-cell embryos	Blastocyst	Hatched embryos	Arrested embryos
Control	87.83±0.51 <sup>a</sup>	91.14±1.43 <sup>a</sup>	78.98±2.44 <sup>a</sup>	66.24±0.90 <sup>a</sup>	14.65±0.21 <sup>a</sup>
Nicotine (0.50 mg/kg)	78.76±1.03 <sup>b</sup>	65.54±1.12 <sup>b</sup>	55.29±1.00 <sup>b</sup>	29.67±1.65 <sup>b</sup>	21.09±0.69 <sup>b</sup>
Nicotine (1.00 mg/kg)	78.06±1.02 <sup>b</sup>	65.05±3.00 <sup>b</sup>	52.65±0.50 <sup>b</sup>	30.84±0.72 <sup>b</sup>	21.66±0.91 <sup>b</sup>
Nicotine (2.00 mg/kg)	75.80±1.79 <sup>b</sup>	63.91±1.83 <sup>b</sup>	52.60±1.93 <sup>b</sup>	30.78±1.92 <sup>b</sup>	21.96±0.79 <sup>b</sup>
Royal jelly (100.00 mg/kg)	88.42±0.57 <sup>a</sup>	91.69±1.25 <sup>a</sup>	79.51±2.34 <sup>a</sup>	66.86±0.84 <sup>a</sup>	14.14±0.44 <sup>a</sup>
Nicotine (0.50 mg/kg) + Royal jelly	82.74±1.21 <sup>a</sup>	79.24±0.41 <sup>c</sup>	62.20±1.93 <sup>c</sup>	41.33±2.07 <sup>c</sup>	17.91±0.18 <sup>c</sup>
Nicotine (1.00 mg/kg) + Royal jelly	81.82±1.67 <sup>a</sup>	78.80±1.79 <sup>c</sup>	60.66±1.98 <sup>c</sup>	41.05±2.51 <sup>c</sup>	20.58±0.77 <sup>b</sup>
Nicotine (2.00 mg/kg) + Royal jelly	80.42±1.47 <sup>a</sup>	68.93±5.19 <sup>d</sup>	57.69±2.11 <sup>d</sup>	33.70±2.20 <sup>d</sup>	20.55±0.44 <sup>b</sup>

Different superscripts (a, b, c, d) in the same column show significant differences between groups ( $P<0.05$ ). Royal jelly: 100.00 mg/kg royal jelly.



**Figure 3.** Reverse transcription polymerase chain reaction analysis of p53 mRNA expression in different experimental groups. #: compared to other experimental groups,  $P<0.05$ . RJ: Royal jelly at dose of 100.00 mg/kg. p53 mRNA levels were normalized against GAPDH.

## 4. Discussion

Reportedly, more than 25% of women use cigarettes and 60% of non-smokers and children are exposed to tobacco smoke daily. Accordingly, smoking and exposure to nicotine are associated with fertility disorders[8]. Cigarette smoking affects women fertility due to its adverse effects on reproductive processes such as ovulation, fertilization and implantation[25]. The IVM of mammalian oocytes leads to an improvement in the treatment of human and animal infertility. The present study revealed that nicotine in a dose-dependent manner caused a significant reduction in the number of MII oocytes compared to the control group. Nicotine binds to the nuclear and cytoplasmic proteins, affecting follicular growth potential and leading to oocyte meiosis disturbance[26]. In the current

study, royal jelly supplementation was able to improve the number of oocytes. Correspondingly, it has been indicated that royal jelly addition to culture media can significantly improve the viability and IVM of Egyptian sheep oocytes[27]. Moreover, royal jelly addition to maturation medium was found to improve blastocyst formation and reduce apoptosis in sheep cumulus cells and the oocyte during the *in vitro* development[28]. It is well documented that appropriate *in vivo* and *in vitro* maturation of the oocyte are associated with oxidative stress prevention due to the balance in oxidant/anti-oxidant status[29]. In a parallel manner, it has been shown that royal jelly improves ovine oocyte IVM and its subsequent development through redox status amelioration in the oocytes[30].

The present study showed that nicotine administration resulted in a significant decrease in IVF rate results along with significant lipid peroxidation in the ovarian tissue compared to the control group. The growing body of evidence supports the role of nicotine in oxidative stress induction in ovarian tissue leading to oocyte damage[31]. Further, it has been revealed that nicotine can alter the normal process of bovine oocyte meiosis affecting subsequent embryonic development[32], confirming our findings. Our findings also exhibited that royal jelly as a promising anti-oxidant can exert repro-protective activity. Consistently, recent reports suggest royal jelly supplementation as an effective approach to treat female infertility and promote female fertility parameters due to its estrogenic and anti-oxidant activities[33].

Our results also showed that the 1.00 mg/kg nicotine plus royal jelly group exhibited a significant decrease in p53 expression compared to the nicotine groups, suggesting royal jelly as a promising agent against p53-evoked reproductive failures[34]. In agreement with our findings, former studies have demonstrated that royal jelly has anti-apoptotic effects against nicotine- and heat stress-induced testiculopathies respectively in mice and rats[14,35] and modulates apoptosis in liver and kidney of cisplatin-treated rats[36]. Additionally, it has been suggested that royal jelly protects human peripheral blood leukocytes against radiation-induced apoptosis[37].

In conclusion, the results of this study indicated that nicotine impairs IVM and IVF rate in rats in a dose-dependent manner through oxidative stress induction and royal jelly can have repro-protective effects in nicotine-administered female rats possibly due to its estrogenic, anti-oxidant and anti-apoptotic properties.

### Conflict of interest statement

The authors declare that there is no conflict of interest.

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### Authors' contributions

Zhila Khodabandeh contributed in investigation, writing and original draft preparation, Ali Shalizar-Jalali conducted data curation, writing, reviewing and editing, Vahid Nejati carried out conceptualization, methodology and data curation, Gholamreza Najafi performed methodology and data curation, and Fatemeh Rahmani was responsible for methodology and data curation.

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