Received: 15 December 2021 | Revised: 8 February 2022 | Accepted: 9 February 2022
DOI: 10.1002/ame2.12217

ORIGINAL ARTICLE

Royal-jelly-based apitherapy can attenuate damages to male reproductive parameter following nicotine administration

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Funding information
This research was funded by Kermanshah University of Medical Sciences (grant no. 96067).

Abstract

Background: Nicotine administration can generate severe oxidative stress and lipid peroxidation. Royal jelly, with its antioxidant properties, acts as a scavenger of reactive oxygen species. This study describes the apitherapy effects of royal jelly on testicular damage following nicotine administration.

Methods: Forty-eight male BALB/c mice were divided into 8 groups ($n = 6$): saline, 3 different doses of royal jelly (100, 150, and 200 mg/kg body weight (BW) per day), nicotine (1.5 mg/kg), and 3 different groups of Nic + Roy (1.5 mg/kg of Nic + 100, 150, and 200 mg/kg BW per day of royal jelly). Nicotine was administrated intraperitoneally, and royal jelly was prescribed orally for 10 consecutive days. Serum levels of hormones (testosterone, luteinizing hormone, and follicle-stimulating hormone), total antioxidant capacity, nitric oxide (NO) status, malondialdehyde levels, sperm DNA fragmentation, sperm parameters, histopathological changes (H&E staining), immunohistochemistry against apoptotic proteins, and gene expression of *Bcl-2*, *p53*, *Caspase-3*, and *Nrf2* (real-time PCR) were assessed to evaluate the molecular and histological changes.

Results: Hormone levels, sperm parameters, and status of antioxidants were decreased significantly ($p < .05$) following nicotine administration. Moreover, royal jelly treatment normalized hormonal and antioxidant characteristics, decreased apoptotic gene expression, increased *Nfr2* gene expression, and restored histopathological alteration to the physiological status significantly ($p < .05$).

Conclusion: Royal jelly upregulates the antioxidant status, inhibits the mitochondrial-dependent apoptosis pathway, and increases the rate of proliferation. This therapeutic agent effectively protected the testis against nicotine-associated damages by antioxidant and anti-apoptotic effects.

KEYWORDS
antioxidant, apitherapy, apoptosis, damage, male, nicotine, reproduction, royal jelly, testis

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1 | INTRODUCTION

Nicotine (Nic) as a pharmacologically active alkaloid stimulant with highly toxic effects naturally occurs in the Solanaceae family of plants. Nic constitutes 0.6%-3.0% of dry weight of tobacco, which can potentially decrease the sperm quality, increase sperm DNA damage, adversely affect testicular spermatogenesis, cause several morphological abnormalities of spermatozoa, and reduce sexual hormone levels. Nic-related infertility is reported in various studies.[1]

Royal jelly (Roy) is a honey-bee secretion that is used for nutrition of larvae and adult queens. It is secreted from the glands in the hypopharynx of nursing bees, and fed to all larvae in the colony. Roy consists of water (67%), protein (12.5%), simple monosaccharide sugars (11%), fatty acids (6%), and 10-hydroxy-2-decanoic acid (3.5%). It also contains trace minerals, antibacterial and antibiotic components, pantothenic acid (vitamin B5), pyridoxine (vitamin B6), and vitamin C. According to former reports, Roy has many pharmacological activities, including antioxidant, antiinflammatory, antitumor, antimicrobial, and immune-modulatory functions.[2] Roy is considered an important part of traditional medicine in Iran. The most crucial component of male reproduction is testis, which is related to sperm production and sexual hormone secretion. This organ structurally consists of seminiferous tubules surrounded by loose connective tissues. The specific structure of testis makes this organ potentially sensitive to damage such as dysregulation of the cellular antioxidant system. Sperm has a small amount of cytoplasm to prevent cell injury.

Thus, since Nic consumption is common among individuals in society and this substance has adverse effects on the male sexual system, in this experimental study we aimed to assess the apitherapy effects of Roy on histopathologic testicular alteration following Nic administration.

2 | MATERIALS AND METHODS

2.1 | Animal groups

Forty-eight male BALB/c adult mice (30 ± 5 g, 8 weeks) were obtained from the animal house of the university. They were kept under standard conditions (22 ± 2°C, 12/12-hours light/dark cycle, 60% relative humidity, and free access to water and food) for 2 weeks for adaptation to the new living environment.

2.2 | Experimental treatments

Nic solution was purchased (Merck-Germany) and fresh Roy was obtained from the Center of Medicinal Plants (Tehran, Iran). All substances were dissolved in normal saline before usage. The animals were randomly divided into 8 experimental groups (*n* = 6 in each group). The control group received intraperitoneal injection of normal saline (0.2 mL), in addition to the oral administration of Roy at different doses of 100, 150, and 200 mg/kg. The animals in the Nic group were administered 1.5 mg/kg Nic intraperitoneally to induce Nic toxicity. Roy was administrated at 10 AM followed by intraperitoneal administration of Nic at 10:30 AM for 10 consecutive days.[3]

2.3 | Tissue sampling

A day after the last administration (11th day of treatment), all animals were anesthetized using intraperitoneal injection (50 IU) of ketamine 10 IU/xylazine 90 IU and then euthanized by cervical dislocation. Following thoracotomy procedure, the blood sample was aspirated. Then, both testes were dissected (left one for histological staining and the right for genetic assessments). Epididymis was also dissected for sperm collection.[4]

2.4 | Serological analysis of testosterone, LH, and FSH

Sensitive enzyme immunoassay (EIA) was used for determination of plasma levels of testosterone (Tes), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) utilizing the biotin-streptavidin-peroxidase amplification system in a competitive-binding assay.[5]

2.5 | Sperm parameter assessment

Left epididymis was dissected and cut under a loop microscope (in a Petri dish containing DMEM/F12). The released sperm was incubated (7 minutes, 37°C) and then assessed on the basis of four indices, that is, motility, viability, morphology, and count, according to the protocol of Jalili et al. Motility and viability were assessed using Trypan blue staining, and morphology of sperm was assessed through Papa-Nikola staining.[6]

2.6 | Diameter of seminiferous tubules

After tissue preparation, the diameter index of seminiferous tubules was measured using a Motic camera and software (Moticam 2000; Spain) using the routine protocol of Roshankhah and coworkers.[7]

2.7 | FRAP assay

This biochemical technique was employed to assess the total antioxidant capacity of the serum after Roy administration. This technique is based on the ability of plasma to reinstate ferric ions. Following the application of the procedure, total antioxidant capacity values were obtained using standard curves.[8]

2.8 | NO and malondialdehyde (MDA) measurement

NO levels as an index of oxidative stress were measured by Griess assay. Following biochemical procedure according to the routine protocol, the optical density (OD) of samples was measured by an ELISA reader (Hyperion, USA) at a wavelength of 540 nm.[6] Also, MDA levels of testicular tissue were evaluated as an index of lipid peroxidation. In this regard, homogenized samples of testes were treated by homogenization buffer (1.15% KCl solution) and centrifuged at 252 *g* (10 minutes). Then, the homogenized samples were added to a reaction mixture containing SDS, acetic acid (pH 3.5), thiobarbituric acid, and distilled water. After boiling the mixture at 95°C for 1 hour and centrifuging at 1008 *g* (10 minutes), the absorbency of the supernatant was measured by spectrophotometry at 550 nm wavelength.[9]

2.9 | Immunohistochemical (IHC) assay and H&E staining

Protein expression of Bcl-2, p53, and Caspase-3 was measured using IHC staining. Following antigen retrieval, the slides were incubated with mouse monoclonal antibodies against these proteins. After a day, the sections were incubated with biotinylated goat anti-mouse IgG. Then, they were exposed to 3,3-diaminobenzidine substrate as a chromogen. Harris hematoxylin staining was used for counterstaining. On the basis of the published protocol of H&E staining, the histological sections were treated as follows: removal of paraffin wax using xylene, application of absolute alcohol for removal of xylene, application of alcohol with descending concentration for hydration, staining with colored solutions of hematoxylin and eosin, application of alcohol with ascending concentration for dehydration, and clarification of tissues with xylene.[10]

2.10 | Sperm DNA fragmentation (SDF) test

This test was conducted according to the Bakhtiari protocol to assess the level of sperm DNA damage using sperm chromatin dispersion (SCD) assay. After slide preparation and bright-field microscopy assessment, the spermatozoa with fragmented DNA exhibited a small halo and the spermatozoa with no DNA fragmentation had medium or big haloes.[11]

2.11 | Gene expression assessment

mRNA was isolated according to the standard TRIZOL method. The quality and purity of the extracted RNA were measured by NanoDrop-2000 spectrophotometer (Thermo Scientific, Washington, District of Columbia) at 260 nm with A260/280 nm (1.8-2.0) and A260/230 (1.8-2.0), respectively. cDNA was synthesized according to the manufacturer's protocol (Fermentas, GmbH, Germany). After the RT-PCR reaction was carried out, the PCR products were analyzed on 1.5% agarose gel electrophoresis and gel densitometry assessments of the bands were done (Syngen, InGenius 3, UK). The genes and related primers were: *p53* (F: GTACCTTATGAGCCACCCGA, R: AGAAGGTTCCCACTGGAGTC), *Bcl-2* (F: CTCGTCGCTACCGTCGTGACTTCG, R: ACCCCATCCCTGA AGAGTTCC), *Caspase-3* (F: TCTGACTGGAAAGCCGAAACTC, R: TCT GACTGGAAAGCCGAAACTC), and *Nrf2* (F: CAGCGACGGAAAGAG TATGA, R: TGGGCAACCTGGGAGTAG). Also, *GAPDH* (F: AGAACAT CATCCCTGCATCCAC, R: TCCCACTGTCTGTCTCAATGCCAC) was designed as an internal control. All obtained gene expression data were represented using the $2^{-\Delta\Delta Ct}$ method.

2.12 | Ethical considerations

All animal manipulations were conducted in compliance with the Guidelines for the Humane Care and Use of Laboratory Animals using protocols approved by the university (ethics no. KUMS.1398.738).

2.13 | Statistical analysis

Data are expressed as mean ± standard deviation (SD). The variables were analyzed by one-way analysis of variance followed by Tukey post-hoc comparisons using SPSS (version 18.0, SPSS Inc., Chicago, Illinois). The statistical significance level was considered $p < .05$.

3 | RESULTS

3.1 | Sperm parameter alteration

Following administration of Nic, all indices of viability, motility, count, and morphology were decreased significantly ($p < .05$) in the Nic group compared with control animals. Sperm viability, motility, and morphology were increased significantly ($p < .05$) in the Nic + Roy 100 mg/kg and Nic + Roy 150 mg/kg groups compared with animals of the Nic group. Also, the count of sperm was increased significantly ($p < .05$) in animals of the Nic + Roy 200 mg/kg group compared with the Nic group (Table 1).

3.2 | Diameter of seminiferous tubules

This index was decreased significantly ($p < .05$) in the Nic group compared with control animals and was increased significantly ($p < .05$) in the Nic + Roy 200 mg/kg group compared with the Nic group (Table 1).

TABLE 1 Effect of Nic and Roy on sperm parameters and seminiferous diamete

Experimental groups	Viability (%)	Motility (%)	Count (10^6)	Normal morphology (%)	Diameter of seminiferous tubules (μm)
Saline	69.51 ± 1.68	15.01 ± 1.8	8.68 ± 1.4	47.78.1 ± 0.7	189.9 ± 3.2
Nic	30.18 ± 0.68 #	2.68 ± 0.2#	0.26 ± 0.7#	13.1 ± 0.5#	108.3 ± 2.8#
Roy 100 mg/kg	67.98 ± 0.68	13.88 ± 0.8	8.68 ± 1.8	48.08 ± 0.8	186.8 ± 5.01
AS 150 mg/kg	72.28 ± 0.88	15.68 ± 1.7	8.68 ± 1.9	47.58 ± 0.6	191.3 ± 2.9
AS 200 mg/kg	68.98 ± 0.98	14.91 ± 1.1	8.45 ± 0.3	47.6 ± 0.4	192.04 ± 1.9
Nic + Roy 100 mg/kg	29.66 ± 0.6	2.01 ± 0.4	2.28 ± 0.4	14.38 ± 0.14	108.4 ± 4.1
Nic + Roy 150 mg/kg	49.51 ± 0.58*	12.68 ± 0.7*	2.38 ± 0.01	28.28 ± 0.4*	104.7 ± 4.12
Nic + Roy 200 mg/kg	50.02 ± 0.38*	13.18 ± 0.5*	7.18 ± 1.08*	43.32 ± 0.3*	188.09 ± 3.2*

Note: Data were presented as mean ± SD.

Abbreviations: Nic; nicotine, Roy; royal jelly.

*$p < 0.05$ compared to Nic group.

#$p < 0.05$ compared to the control group.

TABLE 2 Effect of Nic and Roy on levels of serum indices and sperm DNA fragmentation in male mice

Experimental groups	Tes (ng/mL)	FSH (ng/mL)	LH (ng/mL)	FRAP (nmol/L)	NO (OD)	MDA (μmol/L)	SDF (%)
Control	1.2 ± 0.08	5.3 ± 0.4	0.69 ± 0.02	2.2 ± 0.1	28.5 ± 1.2	0.5 ± 0.02	37.17 ± 0.2
Nic	0.3 ± 0.02#	1.5 ± 0.77#	0.02 ± 0.01#	0.3 ± 0.02#	48.02 ± 3.2#	18.4 ± 2.1#	63.5 ± 0.6#
Roy 100 mg/kg	1.1 ± 0.04	5.3 ± 0.18	0.73 ± 0.03	2.8 ± 0.2	31 ± 0.6	0.08 ± 0.00	36.12 ± 0.1
Roy 150 mg/kg	1.1 ± 0.01	5.2 ± 0.08	0.78 ± 0.02	2.7 ± 0.9	28.01 ± 1.1	0.01 ± 0.00	37.6 ± 0.3
Roy 200 mg/kg	1.2 ± 0.03	4.87 ± 0.13	0.83 ± 0.12	1.9 ± 0.8	27.02 ± 2.2	0.01 ± 0.00	36.10 ± 0.2
Nic + Roy 100 mg/kg	0.21 ± 0.28	4.93 ± 0.12	0.05 ± 0.13	1.82 ± 0.1#	30 ± 2.01#	3.1 ± 0.90#	48.3 ± 0.4#
Nic + Roy 150 mg/kg	0.27 ± 0.18	4.2 ± 0.2#	0.03 ± 0.23	1.92 ± 0.8#	29.6 ± 1.6#	3.00 ± 0.7#	49.01 ± 0.3#
Nic + Roy 200 mg/kg	0.9 ± 0.98#	48 ± 0.59#	0.53 ± 0.45#	2.3 ± 0.5#	32.3 ± 1.8#	2.01 ± 0.91#	47.6 ± 0.1#

Note: Data were presented as mean ± SD.

Abbreviations: FSH, follicular stimulating hormone; LH, leutenizig hormone; MDA, Malondialdehyde; Nic; nicotine; NO, nictric oxide; OD, optical density; Roy, royal jelly; SDF, sperm DNA fragmentation; Tes, testesteone.

*$p < 0.05$ compared to Nicotine group.

#$p < 0.05$ compared to the control group.

3.3 | Testosterone, LH, and FSH measurement

Whole serum levels of Tes, LH, and FSH were decreased significantly ($p < .05$) after Nic administration in the Nic group compared with control animals. These values were increased significantly ($p < .05$) in the Nic + Roy 200 mg/kg group compared with the Nic group. FSH levels revealed an increase trend in the Nic + Roy 150 mg/kg group compared with Nic animals (Table 2).

3.4 | Serum levels of FRAP

Serum levels of FRAP were significantly decreased in the Nic group ($p < .05$) compared with the animals in the control group. Also, in Nic + Roy animals receiving full doses of Roy, the FRAP levels showed a significant increase ($p < .05$) compared with the Nic group (Table 2).

3.5 | Measurement of NO and MDA

NO and MDA levels were significantly higher in animals of the Nic group than in the control ($p < .05$). Also, following administration of Roy in Nic + Roy 100, 150, and 200 mg/kg groups, the NO and MDA levels were decreased significantly ($p < .05$) compared with the Nic group (Table 2).

3.6 | SDF index

Fragmentation of sperm DNA was significantly higher in the Nic group ($p < .05$) than in control animals. This value decreased significantly ($p < .05$) following Roy administration in Nic + Roy 100, 150, and 200 mg/kg groups compared with Nic animals (Table 2).

3.7 | Histopathological observations

Following Nic administration, the density of testicular interstitial tissue and the number of cells were decreased. Also, the aggregation of spermatogenic lineage cells was decreased, exhibiting various intercellular gaps. Compared with the Roy group, in the Nic group, damaged testicular tissue was largely repaired and restored to normal shape, and Leydig cells filled the space among the seminiferous tubules in interstitial tissue (Figures 1 and 2).

3.8 | Gene expression of p53, Bcl-2, Caspase-3, and Nrf2

P53 gene expression showed a significant increase ($p < .05$) in the Nic group compared with control animals, and a significant decrease ($p < .05$) in Roy 150 and 200 mg/kg groups and in Nic + Roy 150 and 200 mg/kg groups, respectively, compared with control and Nic groups. The gene expression of *Bcl-2* was decreased significantly ($p < .05$) in the Nic group compared with control animals. Also, this value was increased significantly ($p < .05$) in groups receiving 100, 150, or 200 mg/kg Roy compared with control. *Bcl-2* gene expression showed a significant increase in Nic + Roy 150 mg/kg and Nic + Roy 150 mg/kg compared with the Nic group ($p < .05$). Following administration of Nic, the gene expression of *Caspase-3* was increased significantly ($p < .05$) in the Nic group compared with animals in the control group. This value was decreased significantly ($p < .05$) in Nic + Roy groups (with doses of 100, 150, and 200 mg/kg) compared with Nic animals. The expression of *Nrf2* gene was upregulated significantly ($p < .05$) in groups receiving 100, 150, or 200 mg/kg Roy, as well as in Nic + Roy 100, 150, and 200 mg/kg groups, compared with control and Nic groups, respectively (Figure 3).

4 | DISCUSSION

Reactive oxygen species (ROS) affect the quantity and quality of seminal fluid. One of the possible causes of testicular atrophy is the factors that disrupt spermatogenesis, resulting in a decreased number of sperms. In this study, we found that the oxidative stress caused by Nic can damage the male reproductive system. An antioxidant agent can scavenge the ROS accumulated in testicular tissue, leading to the proper functioning of the organ. We found that Roy, with its antioxidant properties, is useful in the restoration of male reproductive function following Nic administration. Thus, we propose that Roy administration be strictly recommended for Nic-consuming individuals.

Various studies showed that the Nic consumption increases oxidative stress levels and consequently ROS status, leading to cell cycle arrest and cell apoptosis,[12] as we confirmed in the present study. We also found that Roy, as a stimulant of LH, FSH, and Tes production, induces the proliferation of germinal epithelium of seminiferous tubules and also interstitial tissue surrounding these tubules. These processes can accelerate Leydig cell activity, leading to an increased rate of spermatogenesis and elevated levels of Tes. ROS increases the amount of malondialdehyde enzyme activity and

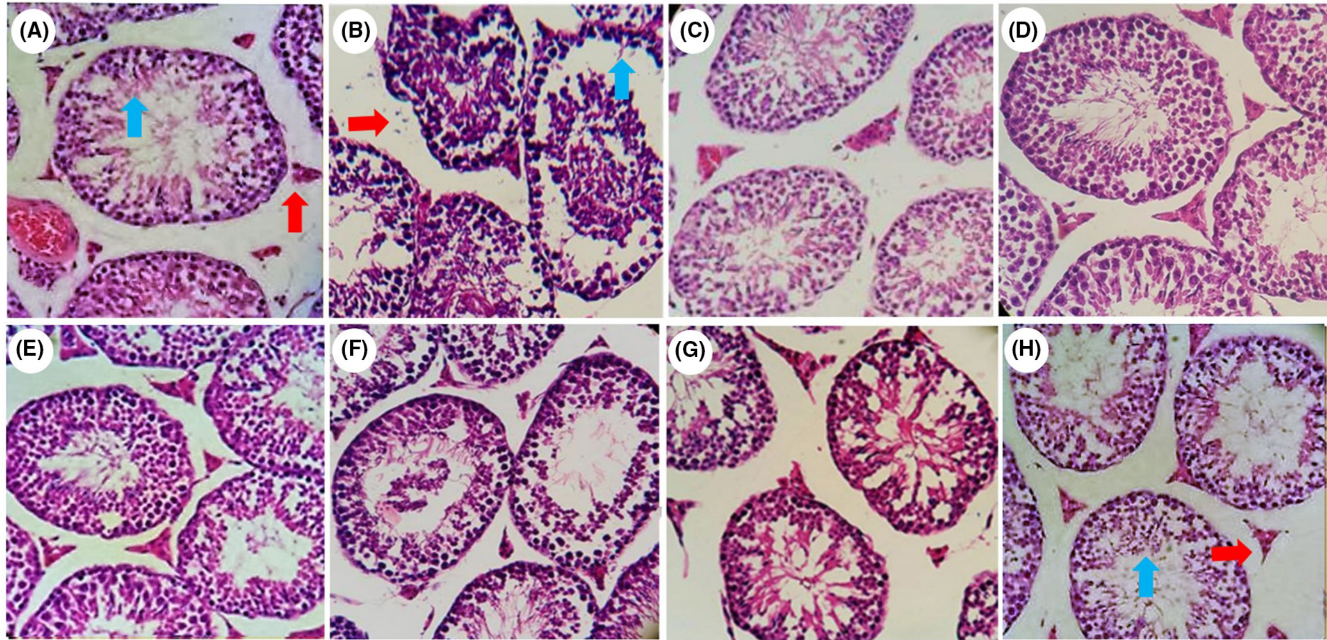

FIGURE 1 Histopathological images of testes in control and treatment groups including A: control, B: Nic, C: Roy 100 mg/kg, D: Roy 150 mg/kg, E: Roy 200 mg/kg, F: Nic + Roy 100 mg/kg, G: Nic + Roy 150 mg/kg, H: Nic + Roy 200 mg/kg. N = 6 mice in each group. Red arrow indicated the interstitial tissue in normal group (A), damaged group (B), and restored group (H). Blue arrow represented the normal germinal epithelium in control group (A), damaged dispersed germinal epithelium in Nic group (B), and repaired damaged epithelium after Roy administration (H). Nic, nicotine; Roy, royal jelly. Scale bar: 100 μm (H&E staining, 400x)

FIGURE 2 Immunohistochemistry images of testes in control and treatment groups against apoptotic proteins of p53, Bcl-2, Caspase-3. Nic, nicotine; Roy, royal jelly. Scale bar: 100 μm (IHC staining, 400x)

disrupts the integrity of the cell membrane.[13] Excessive amounts of ROS cause lipid peroxidation in membranes of cells and mitochondria. Lipid peroxidation can disrupt cell membrane integrity, resulting in disruption of whole-cell activity.[14] High concentrations of ROS play an important role in the pathophysiological damage of human spermatozoa. Thus, oxidative stress is a major cause of male infertility. Oxidative stress, lipid peroxidation, and Nic-induced membrane changes cause germ cell apoptosis at different stages of development.[15] Najafi and coworkers conducted an experimental study on antioxidative features of Roy on accumulated ROS induced by oxymetholone in testicular tissue. Oxymetholone is an androgen and anabolic steroid medication that is used primarily in the treatment of anemia. The results showed that the administration of this medication agent can induce degeneration of seminiferous epithelium,it can also affect the interstitial tissue of testes, leading to decreased levels of Tes. However, in the group receiving Roy, a protective effect on seminiferous tubules against oxidative stress toxicity caused by oxymetholone was detected. It also increased serum levels of Tes following a restorative effect on interstitial tissue.[16] Al-Lahham and coworkers assessed the oxidative effects of Nic and the role of *Nicotiana tabacum* L. with antioxidative features. In line with our findings, Al-Lahham et al. found Nic to cause ROS accumulation in cells. They also suggested that biochemicals with natural sources such as *Nicotiana tabacum* L. have antioxidant characteristics.[17] This finding was also confirmed in the present study, where we found Roy as a natural agent to have powerful antioxidant features.

Antioxidant agents like Roy protect sperm from ROS attacks. Roy also has anti-apoptotic features that protect the testicular germ cells from the toxic effects of Nic. Because sperm lose a large amount of their cytoplasm in spermatogenesis, they lack a large number of cellular antioxidant systems. Thus, they are more sensitive to increased levels of ROS than somatic cells. The first consequence of ROS invasion to membranous structures of cells is the incidence of cellular peroxidation. The application of antioxidants such as Roy to remove ROS inhibits the lipid peroxidation, resulting in maintenance of the normal biochemical structure of cells.[18] Mammalian sperm membranes contain large amounts of unsaturated fatty acids that are sensitive to lipid peroxidation due to oxidative stress, which causes rapid loss of intracellular ATP and, thus, reduced sperm motility and viability.[19] Therefore, it can be concluded that changes in biochemical intracellular contents of sperm can reduce the viability in Nic-affected mice. This phenomenon is related to the lipid peroxidation of sperm membranes. As a powerful antioxidant, Roy partially prevents the damaging effects of Nic on sperm motility and viability. In

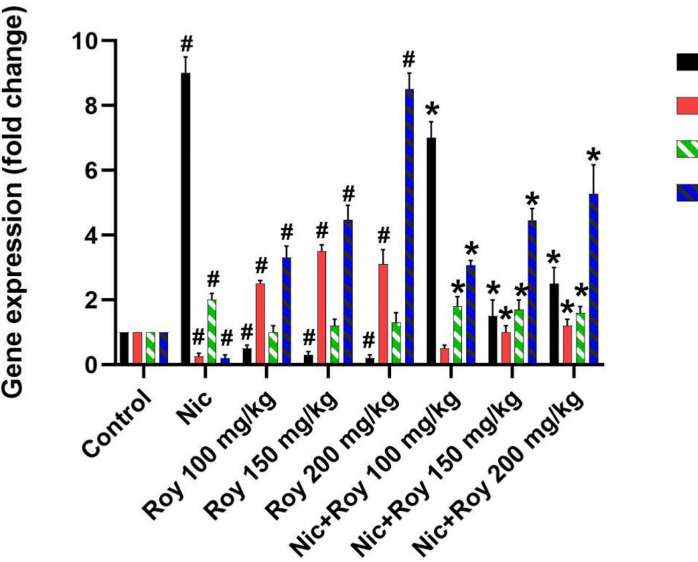

FIGURE 3 Genes expression of *p53*, *Bcl-2*, *Caspase-3*, *Nrf2* in control and treatment groups. N = 6 mice in each group. Nic, nicotine; Roy, royal jelly. $p < 0.05$ as Significancy. # and * represented significant changes than control and Nic groups, respectively

addition, Roy alone increased sperm motility in the groups receiving Roy compared with the control group. The results of this study show that there is a significant decrease in the mean size of the inner diameter of the seminiferous tubules in the Nic group compared with the control group. This feature is due to the fact that the wall cells of the seminiferous tubules in the groups receiving Nic and Nic + Roy are rapidly differentiated and released from the tubular wall, which slightly increases the inner diameter of the tubes. Nitric oxide increases the release of gonadotropin-releasing hormone by increasing the release of gonadotropins via activating the neuronal nitric oxide synthesis enzyme in the pituitary gland.[20] Nitric oxide activates the enzyme guanylate cyclase, which releases cyclic guanosine monophosphate and increases the secretion of gonadotropins and LH and FSH, thus increasing sperm motility. Because testosterone is an androgenic hormone produced by testicular Leydig cells in response to stimulation with LH secreted by the pituitary gland, it is possible that the testosterone levels are increased after Roy administration owing to a direct effect of this antioxidant on cells. Testosterone controls the secretion of LH from the anterior pituitary gland through a negative feedback mechanism, and Roy may indirectly increase the secretion of gonadotropin-stimulating hormones from the hypothalamus, followed by an increase in LH secretion in the pituitary gland.[21] Nitric oxide also regulates reproductive and sexual function in mammalian species. Numerous studies have shown that nitric oxide is related to the acrosome and tail in mouse and human sperm, which seem to be involved in sperm motility and acrosomal function, important factors in the reproductive process. Elevated NO levels interfere with the lipid peroxidation of unsaturated fatty acids located in the plasma membrane of sperm. These unsaturated acids are very sensitive to ROS attack because of their chemical structure, which contains hydrogen.[22] As a result, the increase in nitric oxide leads to a cascading reaction, followed by the production of free radicals. BCL-2 protein is located in the mitochondrial membrane and endoplasmic reticulum. BCL-2 protein, as a proto-oncogene in germ cells, is involved in the regulation of cell apoptosis.[23] Thus, this protein is generally involved in inhibiting cellular apoptosis. If *BCL-2* gene expression is reduced, the process of programmed cell death or apoptosis begins with activation of other oncogenes such as *P53*. Considering the role of caspases in different cells, studies have shown that BCL-2 can also inhibit the process of apoptosis by inhibiting the synthesis and production of caspases. Caspase-3 protein generally occurs under different conditions of cellular apoptosis with different mechanisms.[24] Thus, under conditions of DNA damage and growth factor disorders, the internal pathway, or, in other words, BCL2-protein-dependent pathway, is activated. This pathway is dependent on mitochondria, and the involvement of cysteine proteins or caspases (caspases 3, 8, 9, and 6) is essential. *Nrf2* is a critical gene involved in expression of antioxidant proteins in cells.[25] It is obvious that, following ROS attack to the cells, the levels of Nrf2 gene expression and protein generation could be elevated. As we found in this study, Nic had a suppressing effect on gene expression of *Nrf2*. Also, due to the high protective effect of this gene in Roy as a powerful antioxidant, the gene expression of *Nrf2* represented accelerated levels following Roy administration in Nic-treated groups. Lin and coworkers evaluated the antioxidant effect of curcumin in RAW264.7 cells by increasing the activation of the Nrf2-Keap1 pathway. Lin and coworkers assessed the role of Nrf2 in activation of intracellular system following administration of curcumin. Thus, they concluded that the gene expression of Nrf2 leads to activation of antioxidant system in cells.[26] We also found that this gene expression was increased following Roy administration, which showed a critical role in protecting against oxidative features of Nic.

On the basis of the results of the present study, it can be stated that the administration of Roy, especially in high doses, as a strong antioxidative agent can affect spermatogenesis and fertility in Nic consumers. Moreover, we showed the antioxidant properties of Roy to have a protective effect on sperm parameters against free radicals and apoptosis associated with Nic.

ACKNOWLEDGMENTS

We are grateful to the Research Council of Kermanshah University of Medical Sciences for their financial support (grant number 96067).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.N.Z. and A.N.F. conceived and designed the study, supervised the data collection, interpreted the results, and revised the manuscript. A.G.H. conducted the data analysis, prepared the tables, and wrote the statistical analysis methods and the results. C.J. and M.B. approved and submitted the final manuscript.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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How to cite this article: Nazar-Zadeh M, Jalili C, Nikgoftar Fathi A, Ghanbari A, Bakhtiari M. Royal-jelly-based apitherapy can attenuate damages to male reproductive parameter following nicotine administration. *Anim Models Exp Med*. 2022;5:133–140. doi:10.1002/ame2.12217