Accepted: 3 January 2017

DOI: 10.1111/and.12804

ORIGINAL ARTICLE

WILEY ANDROLOGIA

Honey improves spermatogenesis and hormone secretion in testicular ischaemia–reperfusion-induced injury in rats

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Summary

This study was conducted to survey the protective effect of pre-treatment with Persian honey during post-ischaemia reperfusion on ischaemia–reperfusion (IR)-induced testis injury. Animals were divided into four groups of IR, honey + ischaemia-reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR). The testes were examined for spermatogenesis index. Detection of single- and double-stranded DNA breaks at the early stages of apoptosis was performed. Total serum concentration of FSH, LH and testosterone was measured using ELISA. All data were expressed as mean ± *SD* in each group, and significance was set at $p \leq .05$. Spermatogenesis index was significant in the HIR group ($p < .001$). Serum levels of FSH and LH were significantly higher in the CIR and HIR groups. Serum levels of testosterone were significantly higher in VIR and HIR groups. Apoptotic cells in IR and CIR groups increased significantly statistically ($p < .001$), while in HIR and VIR groups, the number of apoptotic cells decreased and the positive cells of TUNEL staining were detected in spermatocytes and spermatid. The present study indicates that honey decreases the cellular damage and apoptosis during testicular I/R injury, with significant protective effects on reproductive hormone production.

KEYWORDS
honey, ischaemia/reperfusion, spermatogenesis, testis

1 | INTRODUCTION

Testicular torsion is a common severe acute urological emergency that occurs when the spermatic cord (from which the testicle is suspended) twists. When this occurs, blood supply is cut off to the testicles and leads to testicular ischaemia (Fehér & Bajory, 2016). With early detection, testicular torsion can be cured in almost 90% of cases. In late identification, however, infarction will occur, and if testicular torsion is not treated within 4–6 hr, may lead to the loss of the testicles (Drlík & Kočvara, 2013). Thus, time is the most critical factor in the surgical detorsion that is currently the only treatment and allows blood reperfusion. Delay in surgical detorsion can be fatal and may lead to impaired sperm production and decrease male fertility and cause loss of the testicles (DaJusta, Granberg, Villanueva, & Baker, 2013).

During testicular torsion following detorsion, the main pathophysiology is ischaemia–reperfusion injury of the testis. The testes may suffer from reactive oxygen species (ROS), hypoxia, or a great volume of blood flow, which cause oedema in the testicle. Remarkable increase of reactive oxygen species such as superoxide anions, hydrogen peroxide, hydroxyl radicals and peroxynitrite anion may damage the cell membrane lipids, proteins and even the DNA (Elshaari, Elfagih, Sheriff, & Barassi, 2011).

Antioxidants have been beneficial in testicular ischaemia–reperfusion (IR) injury. The flavonoids are the main group of antioxidants present in honey. There is a mixture of different compounds, including antioxidants, amino acids, vitamins (B2, B4, B5, B6, B11 and vitamin C), carbohydrates, proteins, minerals and 18 free amino acids in honey. Also, honey contains several enzymes, such as acid phosphorylase,

catalase, invertase and glucose oxidase (Ediriweera & Premarathna, 2012). Several studies indicate that honey, like other antioxidant agents (Golalipour, Khori, Ghafari, & Gharravi, 2006; Golalipour, Gharravi, Ghafari, & Afshar, 2007), has many medicinal effects, including antibacterial, hepatoprotective, hypoglycaemic, reproductive and antihypertensive. It may act via amelioration of oxidative stress in tissues (Kadirvelu & Gurtu, 2013; Mijanur Rahman, Gan, & Khalil, 2014). Natural honey prevents ischaemia–reperfusion-induced injury of gastric (Ali et al., 1997) cardiac arrhythmias (Najafi, Shaseb, Ghaffary, Fakhrju, & Eteraf Oskouei, 2011), streptozotocin-induced diabetes (Erejuwa et al., 2011).

The Persian honey, *Apis mellifera meda*, was first described by Skorikov in 1985 (Ruttner, 1988).

Based on the literature review, till now, there is no report regarding the impacts of *Apis mellifera meda skorikov* on IR-induced testis injury. Hence, the present survey is conducted to survey the protective effect of pre-treatment with *mellifera meda skorikov* during post-ischaemia reperfusion on IR-induced testis injury.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All experiments were performed in accordance with the principles of laboratory animal care of Lorestan University of Medical Sciences. As the welfare of animals used in this research was very important, every effort was made to reduce the suffering and the number of animals.

2.2 | Animals and study design

This experimental study was conducted at Razi Herbal Medicine Research Center of Lorestan University of Medical Sciences. Forty adult male Wistar rats weighing 250–300 g were acclimatised for 1 week to the condition. Animals had free access to standard laboratory feed chow and water. The animals were randomly divided into four groups (experimental and control) of 10 rats.

Sham: no IR, received vehicle alone, exposed to sham operation and served as sham control.
IR: Received the vehicle alone during ischaemia followed by 50 days' reperfusion.
HIR: Received oral honey (5%) during ischaemia followed by 50 days' reperfusion.
VIR: Received vitamin C, 20 mg/kg oral during ischaemia followed by 50 days' reperfusion.
CIR: Received carbohydrates-equal calories with honey (5%) during ischaemia followed by 50 days' reperfusion.

Honey used in this study was *Apis mellifera meda skorikov*. To prepare the required concentrations, honey was completely dissolved in the drinking water of rats, and then, the rats were fed with the solution.

2.2.1 | Ischaemia induction

The rats were anaesthetised intraperitoneally with ketamine HCl (50 mg/kg) and xylazine (5 mg/kg) in accordance with the protocol approved by the Animal Care and Use Committee (Gholami et al., 2008). The groin region was shaved, and the femoral artery/vein was carefully separated from the femoral nerve. The artery and vein were knit using a silk suture 6/0 and slipknot technique for 3 hr (Iida, Schmelzer, Schmeichel, Wang, & Low, 2003; Saray, Apan, & Kisa, 2003). Honey or the vehicle was given just before reperfusion in I/R groups as well as in the sham-operated control group in which clamping was not performed.

2.2.2 | Tissue perfusion

As shown above, four groups (10 rats per group) received 50 days of reperfusion after honey or the vehicle, carbohydrates. The sham group without IR was selected as the control group.

2.3 | Histopathological studies

After fixation in 10% formalin, testes were embedded in paraffin, cut, dehydrated and stained with haematoxylin–eosin. About 30 rounds or nearly round cross sections of seminiferous tubules were randomly chosen in each rat. The seminiferous tubules were rated for their modified spermatogenesis index (SI) by Johnson's score on a scale of 0–10 in accordance with the range from no cells to complete spermatogenesis.

2.4 | Sperm testing

Sperm count and motility were evaluated by light microscopy at a magnification of 400× and nonprogressive motility and immotility of spermatozoa were reported as percentages. To conduct sperm motility analysis, the cauda epididymis was cut, and spermatozoa were released in 5 ml of medium Ham's F-10 medium (Sigma, USA) containing 0.5% bovine serum albumin and incubation at 37°C (with 5% CO_2) for 20 min. Then, the cauda epididymis sperm reserves were determined and the total sperm count was determined using a haemocytometric method. Sperm motility was analysed and reported as the mean of motile spermatozoon in accordance with the World Health Organization's (WHO's) method. Sperm viability was evaluated by eosin–nigrosin staining test. Aniline blue staining was applied for morphology assessment. The slides were assessed for morphological abnormality in tail, neck or head.

2.5 | TUNEL

TUNEL (TdT-mediated dUTP-X nick end labelling) assay was performed in accordance with the manufacturer's instructions (Roche Company). Detection of single- and double-stranded DNA breaks at the early stages of apoptosis was performed using the In-Situ Cell Death Detection Kit, POD (11684817910). The apoptosis percentage was calculated as the ratio of apoptosis-positive seminiferous tubules to the total number of seminiferous tubules.

2.6 | Serum FSH, LH and testosterone hormone measurement

Blood samples were taken from the heart and were centrifuged at the rate of 5,000 per min to separate blood serum. Total serum concentrations of FSH, LH and Testosterone were measured using ELISA.

2.7 | Statistics

Statistical analysis was conducted with SPSS V.22 and the variables were tested using the Kruskal–Wallis test and Mann–Whitney *U*-test. All data were expressed as mean ± *SD* in each group and significance was set at $p \leq .05$.

3 | RESULTS

3.1 | Histopathological studies

In the Spermatogenesis index (SI) by Johnson's score, an increase in the spermatogenic index was significant in the HIR group ($p < .001$). Therefore, the SI shifted to a high score during the treatment with honey during ischaemia (Figure 1).

3.2 | Sperm count

The values of the mean sperm count—as shown in Figure 2—were significantly higher in HIR group ($p < .001$) when compared to other groups.

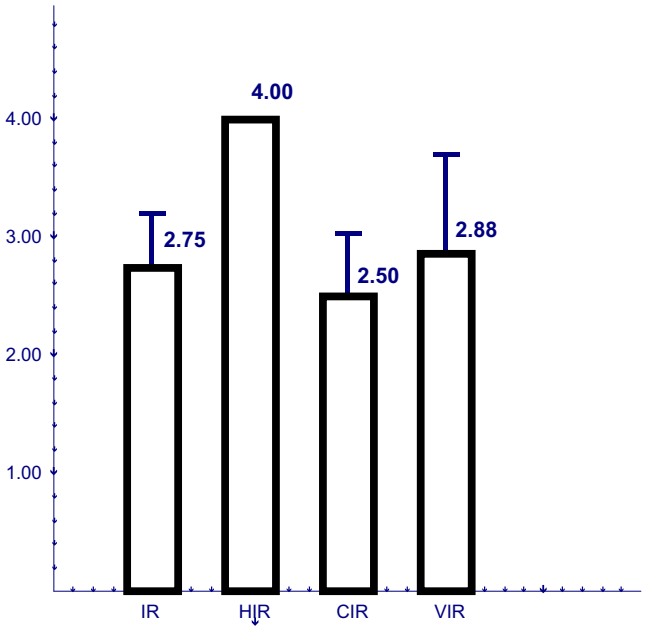

FIGURE 1 Spermatogenesis index (Mean Johnsen's score) compared among the study groups. Ischaemia- reperfusion (IR), honeyreperfusionischaemia- reperfusion (HIR), vitamin C + ischemia- reperfusion (VIR) and carbohydratesreperfusionischaemia- reperfusion (CIR)

3.3 | Serum FSH, LH and testosterone hormone

Figures 3, 4 and 5 show serum levels of testosterone, FSH and LH in groups. There was a significant difference in serum levels of testosterone, FSH and LH among groups ($p < .001$).

Serum levels of FSH and LH were significantly higher in the CIR and HIR groups. Serum levels of testosterone were significantly higher in VIR and HIR groups.

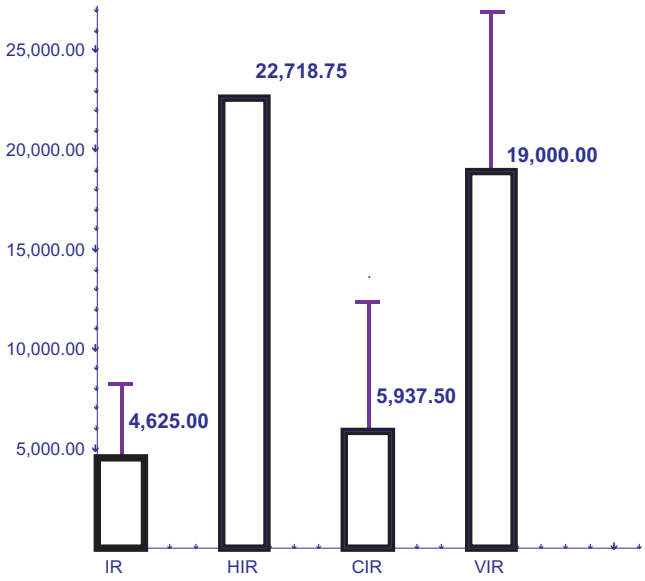

FIGURE 2 Effects of ischaemia/reperfusion and treatments with honey, carbohydrate and vitamin c on sperm count (10^6/ml). Ischaemia- reperfusion (IR), honeyreperfusionischaemia-reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR)

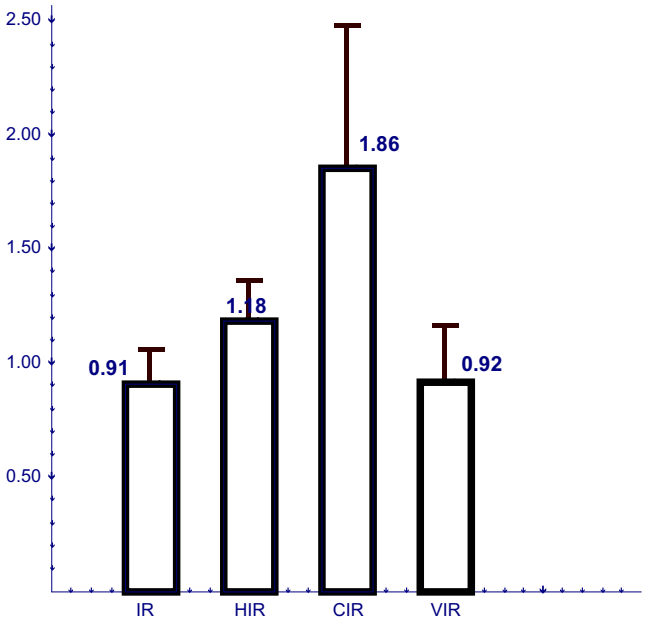

FIGURE 3 Serum levels of FSH (ng/ml) compared among the study groups. Ischaemia- reperfusion (IR), honey + ischaemia-reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR)

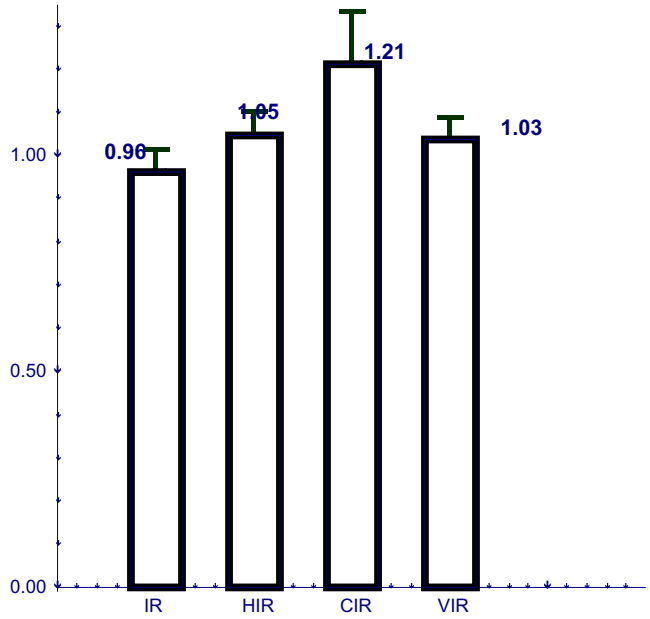

FIGURE 4 The serum level of LH (ng/ml) compared among the study groups. Ischaemia- reperfusion (IR), honey + ischaemia-reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR)

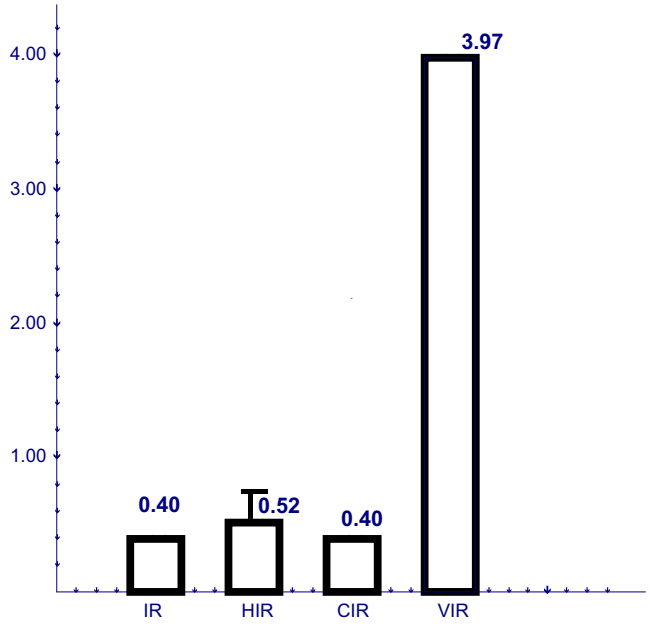

FIGURE 5 Serum levels of testosterone (ng/ml) compared among the study groups. Ischaemia- reperfusion (IR), honey + ischaemia-reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR)

3.4 | Apoptosis analysis

Evaluation of germ cell apoptosis showed that apoptotic cells, as shown in Figure 6 in IR and CIR groups, increased significantly statistically ($p < .001$), while in HIR and VIR groups, the number of apoptotic cells decreased significantly in comparison with IR and CIR groups ($p < .001$) (Figures 6 and 7).

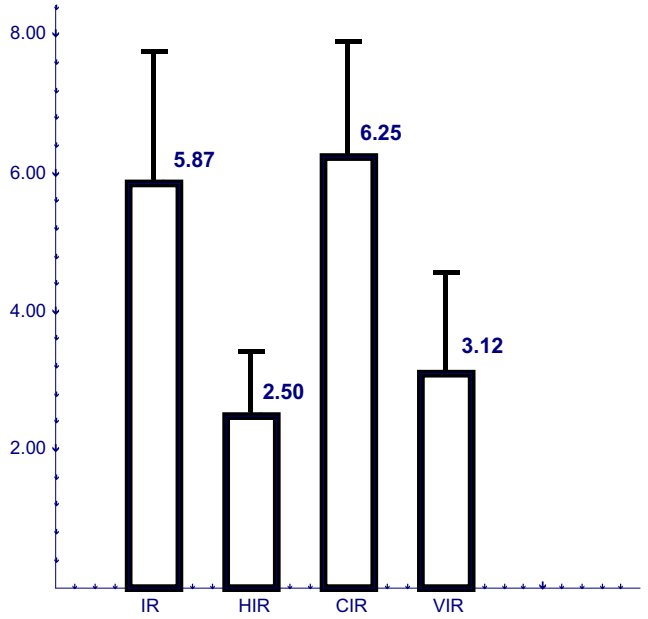

FIGURE 6 Quantitative analysis of apoptosis in testis. The number of seminiferous tubules containing three or more apoptotic cells was calculated by TUNEL stain. Ischaemia- reperfusion (IR), honey + ischaemia- reperfusion (HIR), vitamin C + ischaemia-reperfusion (VIR) and carbohydrates + ischaemia- reperfusion (CIR)

4 | DISCUSSION

In the present study, honey improved spermatogenesis and mean sperm count in IR-induced testis injury. The results are consistent with earlier research of testes of animals treated with honey. It revealed normal testicular architecture with complete normal spermatogenesis and treatment with honey induces spermatogenesis (Abdul-Ghani, Dabdoub, Muhammad, Abdul-Ghani, & Qazzaz, 2008). Several studies indicate that damage to spermatozoa by oxidative stress biomarkers during IR-induced testis injury plays a key role (Taylor, 2001). Owing to the high polyunsaturated fatty acid content of sperm membranes, a testicular tissue is susceptible to oxidation. Higher levels of seminal ROS and malondialdehyde (MDA) as oxidative stress biomarkers lead to oligoasthenozoospermia and poor sperm fertilisation potential (Sanocka & Kurpisz, 2004). Previous studies concluded that the use of oral antioxidant could improve sperm concentration. Overproduction of ROS may induce sperm cell injury through several pathways, motility and morphology in infertile males (Elmussareh, Mahrous, & Kayes, 2015).

It is well established that FSH and LH are essential for the maintenance of spermatogenesis. The process of spermatogenesis is regulated by gonadotropin-releasing hormone (GnRH) to produce FSH and LH that then act on the testis to regulate spermatogenic potential. The binding of LH to its receptors on the surface of Leydig cells stimulates the production of testosterone that diffuses into the seminiferous tubules. Within the germinal epithelium of seminiferous tubules, testosterone and FSH receptors are found only in Sertoli cells; hence, these cells regulate spermatogenesis (Walker & Cheng, 2005).

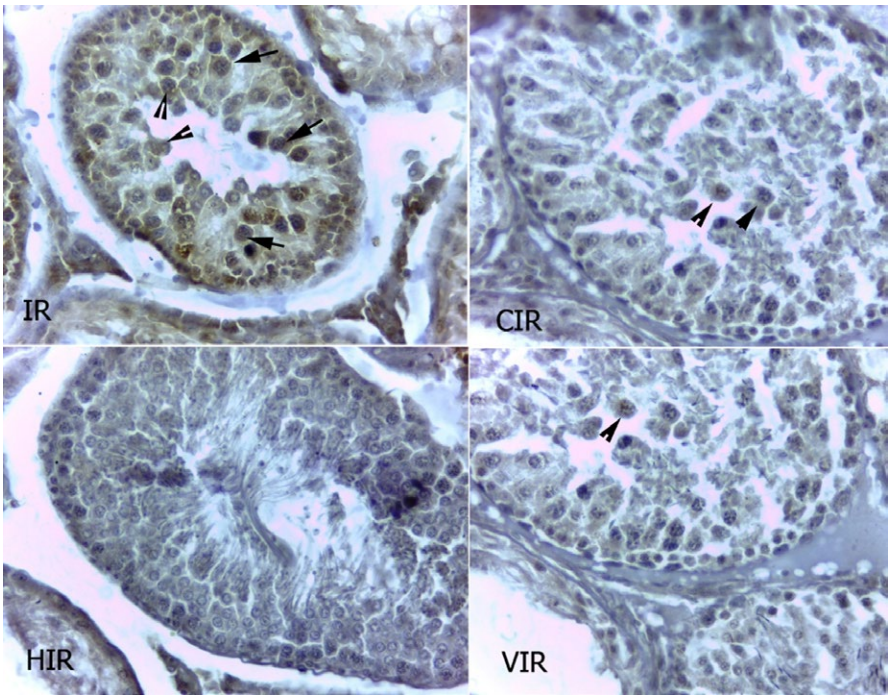

FIGURE 7 Apoptotic cells in the rats testis identified by in-situ end labelling of fragmented DNA. Arrows indicate positively labelled cells which appear as very darkly stained cells in this micrograph. Ischaemia- reperfusion (IR), honey + ischaemia- reperfusion (HIR), vitamin C + ischaemia- reperfusion (VIR) and carbohydrates + ischaemia-reperfusion (CIR). Original magnification 400×

In the present study, treatment of animals with honey during ischaemia followed reperfusion led to an increase of FSH and LH and testosterone. Previous studies using honey also reported effects of honey on reproductive hormones, including testosterone, LH and FSH in rats, and suggested that honey enhanced sperm count in rat by increasing testosterone production (Salman et al., 2013). Other researchers reported that honey that originated from Malaysia or Palestine has no significant effects on reproductive hormones including testosterone, LH and FSHin rats (Gill-Sharma et al., 2003; Mohamed, Sulaiman, Jaafar, & Sirajudeen, 2012). Oxidative stress reduces enzymatic and nonenzymatic levels in Leydig cells and leads to testosterone reduction. In IR group, oxidative stress in testicular tissue inhibited androgenesis by Leydig cells. But in HIR and VIR groups, honey and Vitamin C could up-regulate the testosterone level and subsequently protected the spermatogenesis process.

LH and FSH activity depends on both the quantity of these hormones and the number of specific receptors in the testis. The reduction in serum testosterone found in IR group seems to be the result of a depression in gonadal steroidogenesis due to reduced FSH and LH secretions. Therefore, it is possible to suggest that the treatment of animals with honey during ischaemia followed reperfusion is able to improve the FSH, LH and testosterone levels in HIR group.

As previously reported, increase in TNF-α after testis reperfusion led to the activation of NFκB in Sertoli cells as well as seminiferous pertitubular cells. The findings suggest a role for the pro-inflammatory cytokines as early mediators of IR injury in the testis (Lysiak, 2004). Pre-treatment with honey reduces the expression of phosphorylated JNK, IKkβ and IRS-1, thereby significantly reducing the expression of TNF-α (Popa Morariu, Schiriac, Ungureanu, & Cuciureanu, 2012).

Testis is sensitive to enhanced formation of reactive oxygen species during ischaemia–reperfusion injury, which results in testicular cell damage and apoptosis (Ozbal et al., 2012). The process of programmed cell death, or apoptosis, occurs in both physiologic and pathologic stages (Elmore, 2007). It has previously been demonstrated by other workers that apoptotic events appeared to occur predominantly in spermatocytes, early and late spermatids, and Sertoli cells. In contrast, spermatogonia seldom underwent apoptosis (Hadziselimovic, Geneto, & Emmons, 1998). In the present study, all groups had apoptosis in primary and secondary spermatocytes and spermatids, but significantly fewer apoptotic bodies were detected in spermatids in the honey-treated group. It was seen that the honey pre-treatment had a positive effect and decreased ROS and improved antioxidant defences, consequently leading to inhibition of apoptosis.

These findings were probably due to the antioxidant protective effects of honey, as supported by previous studies which reported that honey has a large amount of antioxidant properties derived from its constituents such as flavonoles (quercetin, kaempferol), flavanones (naringin, naringenin), flavones (apigenin, acacetin), phenolic acids (caffeic acid, chlorogenic acid) and coumarins (Abubakar, Abdullah, Sulaiman, & Suen, 2012; Ediriweera & Premarathna, 2012).

Our review of the literature did not reveal any studies that investigate the anti-apoptotic effects of honey on testis tissue. To our knowledge, this is the first report of the anti-apoptotic effects of honey against testicular ischaemia–reperfusion injury in rats.

5 | CONCLUSION

It was determined through our study that honey increased FSH and LH and testosterone and decreased the cellular damage and apoptosis during testicular IR injury in rats. Additionally, administration of honey showed significant protective effects against antioxidants. These results suggest that pre-treatment with honey has beneficial effects in the

prevention of IR injury of the testis. However, further studies are needed for a better understanding of the exact mechanism of honey as well as the beneficial effects on spermatogenesis in mammalian spermatozoon.

ACKNOWLEDGEMENTS

This study was approved in the Lorestan University of Medical Sciences. Special thanks to Lorestan University of Medical Sciences for the financial support, Khorramabad, Iran. The authors thank the head and staff of Razi Herbal Medicines Research Center of Lorestan Medical University.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

The authors contributed equally in the study concept and design, acquisition of data, statistical analysis, analysis and interpretation of data, and drafting of the manuscript.

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How to cite this article: Gholami M, Abbaszadeh A, Khanipour Khayat Z, Anbari K, Baharvand P, Gharravi AM. Honey improves spermatogenesis and hormone secretion in testicular ischaemia–reperfusion-induced injury in rats. *Andrologia*. 2017;00:e12804. https://doi.org/10.1111/and.12804