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Effects of bee honey on promotion of peripheral nerve regeneration in an experimental model of nerve crush injury

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Abstract: Peripheral nerve injuries are commonly encountered within clinical settings because of accidental trauma. This study aimed to examine the therapeutic effect of bee honey on peripheral nerve crush injury through a histological and physiological perspective. In this study, forty Wistar rats were divided into four groups. Rats were subjected to surgical operations to expose the sciatic nerve. Animals of the first group were operated without inducing any lesion to the nerve. The other three groups were subjected to induction of nerve crush injury. Two groups of them were treated with honey solution locally and intraperitoneally respectively. The other group served as injured nontreated group. Two physiological tests were performed to examine the living animals' nerve functions. At the end of the experimental period, the rats were sacrificed, and samples from the sciatic nerve and gastrocnemius muscle were obtained for histological, immunohistochemical and ultrastructural examination. Physiological indicators and structural investigations demonstrated considerable amelioration of the function and structure of nerves and muscles in the two treated groups compared with the injured nontreated group. The findings indicate that the bee honey has a curative effect on the peripheral nerve crush injury in the rat model.

Keywords: Bee honey, nerve crush injuries, nerve regeneration, non-surgical treatment.

INTRODUCTION

The rate of peripheral nerve injuries is prevalent within clinical practice because of accidental trauma. Transaction nerve injuries usually require surgical repair, including grafting and tissue engineering (Lee and Wolfe 2000, Campbell 2008). In contrast, non-surgical therapies are considered the treatment of choice for nerve crush injury, especially that of moderate severity (Raso *et al.,* 2005, De Albornoz *et al.,* 2011). Non-surgical therapies for nerve crush injury that have been tested on animal models included physical therapies, application of growth factors, transplantation of neural stem cells, and some protein intervention at the site of the lesion (De Albornoz *et al.,* 2011). Few human studies have been concentrated on non-surgical conservative therapies. However, the human studies dealt only with physical therapy modalities, and their efficiency was not proved (Gigo-Benato *et al.,* 2005, Rochkind 2009, De Albornoz *et al.,* 2011).

Honey has been known as a sweetened natural substance produced by honeybees from blossoms nectar or from the secretions or excretions of living parts of plants. It is a natural antioxidant containing ascorbic acid, catalase, tocopherols, flavonoids and phenolic compounds (Ball 2007). The honey has been used for a long time as a conventional treatment for infected wounds. Recently, medical professionals have reused this treatment, especially where ordinary modern medications have failed (Stokes *et al.,* 1993, Subrahmanyam 1991). Several studies have described honey's efficiency in promoting the healing process and clearing infection from contaminated wounds (Molan *et al.,* 1988, Jeddar *et al.,* 1985, Subrahmanyam 1991). Moreover, the neuroprotective effect of honey and honeybee products was recorded (Ali and Kunugi, 2020, Balaha *et al.,* 2021). Ferulic acid has been known as a potent polyphenol found in honey responsible for providing neuroprotection against injury and ischemia-associated apoptosis (Cheng *et al.,* 2008). It has been clearly stated that the brain's cholinergic system is improved as a result of the antioxidant activities of honey through excitatory amino acids (Azman *et al.,* 2016). Honey is, also, beneficial to prevent memory impairments and cognitive dysfunction of vascular dementia (Azman *et al.,* 2018; Qaid *et al.,* 2020).

Despite modern therapeutic agents, different natural remedies have been rediscovered to overcome the challenges associated with the treatment. In a similar context, the present study has aimed to examine the possible therapeutic effects of bee honey on peripheral nerve crush injury through histological and physiological perspectives.

MATERIALS AND METHODS

Animals

Forty adult, male, pathogen-free, and 8-weeks old Wistar rats, weighing 220-250 g have been used. Animals were kept in an air-conditioned room (26±3°C) with a standard light/dark cycle. The animals were allowed free access to

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food pellets and water. Ethical approval of this study was issued by Institutional Animal Care and Used Committee (HUIACUC), College of Medicine, Taif University, Taif, Saudi Arabia.

Experimental design

Animals were divided randomly into four groups; ten rats were placed in each group.

- In the first group (sham group), the surgery was performed by exposing the sciatic nerve without inducing any lesion or ligation.
- In the second group (injured nontreated group), the surgery was performed, and the sciatic nerve was crushed as described below and the animals were left without any local or systemic treatment.
- In the third group, the animals were operated as the second group, and the perineurium of the crushed area of the nerve was inoculated by a single dose of 5µl fresh honey without dilution using a glass cannula.
- In the fourth group, the animals were operated as the second group and administrated intraperitoneally daily with 10 mg/kg body weight of the honey solution (reference). These treatments lasted for eight weeks.

The nociceptive function and motor nerve recovery were evaluated by measuring the withdrawal reflex latency (WRL) and sciatic function index (SFI), respectively. The tests were done for all animals before operations (week 0) and repeated weekly through the 8-week. At the end of agent treatments, the animals were sacrificed, and samples from the sciatic nerve and gastrocnemius muscle were taken and prepared for histological, immunohistochemical and ultrastructural investigations.

Chemicals and reagents

All the chemicals and drugs used were obtained from MilliporeSigma (St. Louis, MO, USA) unless otherwise specified.

Composition and preparation of honey

Yemeni natural honey, Salam-Tehama (*Acacia ehrenbergiana*) type was used. The bees were fed on *Acacia ehrenbergina* flowers (purchased from the local market, Taif, Saudi Arabia). The composition of honey is distributed as follows: glucose (30.0%), fructose (38.0%), sucrose (5.5%), maltose (6.5%) and water (18.0%). Fructose/glucose ratio 1.27. The total phenolic content is 246.21mg /100g of honey as catechin equivalent (CE) and the antioxidant activity is 65.4% inhibition (Al-Mamary *et al.,* 2002). Fresh, pure honey was used in this study without any change in its natural composition. It was diluted with distilled water (1/10 volume) and prepared freshly for intraperitoneal administration. For local treatment, it was used fresh without dilution. Choosing this type of honey is based on its higher antioxidant activity and polyphenolic content than the other Yemini types (Al-Mamary *et al*., 2002).

Surgical procedure

Sodium pentobarbital 3% was used for anesthesia (doses ranged from 38mg/kg to 48mg/kg, ip). Before surgery, rats were injected intramuscularly with Gentamycin (100 mg/kg b.w.) and treated with Duratears lubricant eye ointment to prevent eye dryness. During surgery, an aseptic technique was followed, and the rats were put on a water-heated platform (37±0.5°C) to maintain body temperature. The right sciatic nerve of all animals was operated, and the contralateral unoperated side was left as normal control. About 1.0 cm distal to the sciatic foramen of the hip bone, the nerve was exposed and double crushed with small forceps for 30 seconds in two points with a distance of 0.5 mm in between (Jiang *et al.,* 2009, Dallo *et al.,* 2007). The distance between the two crushed points represented a short-lasting reservoir of perineurium, through which inoculations by honey were carried out for the locally treated group. On the covering muscle, the level of the crush lesions was marked using a 9/0 non-absorbable silk suture. The muscle was closed using Vicryl 6/0, and the skin was closed using Mersilk 5/0 (Dowdall *et al.,* 2005, Luís *et al.,* 2007).

Nociceptive function

For evaluation of the sciatic nerve nociceptive function (perception or sensation of pain), withdrawal reflex latency (WRL) was measured. The rat was covered with a surgical towel around the upper part of the body and then placed to stand on a hotplate at 56◦C with the operated hind paw. As the extremity warms, heat sensitive nociceptive afferents are stimulated. Using a stopwatch, WRL was measured as the elapsed time from the instant of hotplate contact to the paw's withdrawal. The test was repeated three times, with two minutes intervening time. The average of three latencies was calculated and recorded (Luís *et al.,* 2007; Masters *et al.,* 1993).

Motor nerve recovery

For the assessment of motor nerve recovery, the sciatic function index (SFI) was determined. SFI demonstrates the grade of nerve dysfunction on a scale varying from 0 (for normal function) to 100 (for complete dysfunction). The procedure and analysis were performed, as described previously (Bain *et al.,* 1989, Jiang *et al.,* 2009). The rats were allowed to follow a specified walking track. The track was 8.2 X 100 cm with a white paper sheet placed at its bottom. The hind paws of the rat were dipped in red ink to leave prints on the paper sheet. After the walking trial, the footprints were subjected to several measurements; The print length (PL) is the interval between the heel and third toe. The toe spread (TS) is the interval between the first toe and fifth toe. The intermediary toe spread (IT) is the interval between the second toe and fourth toe. The prefix (E) was used to

indicate the operated side, and the prefix (N) indicates the non-operated side. SFI was calculated by the formula (Bain *et al.,* 1989).

$$SFI = 38.3 \frac{(EPL-NPL)}{NPL} + 109.5 \frac{(ETS-NTS)}{NTS} + 13.3 \frac{(EIT-NIT)}{NIT} - 8.8$$

Light and transmission electron microscopy

The animals were killed using euthanasia methods designed to cause minimal distress and pain to the animal. In this procedure, a seizure medication (sodium pentobarbital 3%) was given to the animal in large doses (100 mg/kg ip) that makes it unconscious. As the seizure medication reacts, the animal's heart and brain functions were shut down within 2-3 minutes. After this, the gastrocnemius muscles from both the operated and contralateral sides were excised, weighed and several slices were cut at the mid-belly of the muscle and fixed in 4% paraformaldehyde. Thereafter, the specimens were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. 5µm thick sections were cut and stained with hematoxylin & Eosin (H&E) for histological observation and assessment. The sciatic nerve was dissected out just distal to the operated part, fixed in phosphate-buffered 2.5% glutaraldehyde, postfixed in phosphate-buffered 1% osmium tetroxide and dehydrated in ascending grades of ethanol. The samples were oriented longitudinally to obtain cross-sections and embedded in epoxy resin. 2 µm thick sections were cut and stained with hematoxylin & Eosin for light microscopic examination. Ultrathin sections (80-90nm) were cut and stained with lead citrate and uranyl acetate for transmission electron microscopic examination.

Immunohistochemistry

Immunohistochemistry was used in all groups for the examination of regenerated sciatic nerve fibers. Neurofilament protein in the nerve cells was expressed using streptavidin-peroxidase immunohistochemistry (Song *et al.,* 2013). The sciatic nerve was postfixed and sectioned into 5 µm thick sections. The slides were incubated overnight at 4°C with mouse anti-neurofilament protein polyclonal antibody (1:50 dilution), followed by incubation for 30 minutes at 37°C with undiluted biotin-labeled goat anti-rabbit/mouse IgG (Beijing Zhongshan Golden Bridge Biotechnology Co Ltd, Beijing, China). Immunolabeling of sections was gained through incubation with diaminobenzidine substrate (Sigma-D5905). Finally, the glial nuclei were counterstained with hematoxylin. The negative control sections were incubated with phosphate-buffered saline instead of the primary antibody. The binding of antibodies with neurofilament protein was represented as brown-yellow particles in the sciatic nerve neurites. Three randomly selected fields of view from each section were subjected to quantitative analysis using an image analyzer system (Leica Qwin 500; Germany). The strength of staining with antibodies was documented by the main value of positive products ratio to the field of view.

STATISTICAL ANALYSIS

The data were expressed as means ± SD. SPSS 20.0 was used in the study for data analysis. One-way analysis of variance (ANOVA) was applied to analyze the statistical differences between groups. with subsequent Turkey's tests. A *P* value below 0.05 is considered statistically significant.

RESULTS

Withdrawal reflex latency (WRL)

The mean values of WRL in seconds were determined preoperatively and every week after surgery until week eight (table 1). The injury produced a severe deficit in pain sensation. There are significant increases in WRL of the second group (injured nontreated) at weeks 1, 2, 3, and 4 as compared to the preoperative values ($p<0.05$). The locally treated group showed rapid relief reaching less than four seconds latency time at week three postoperative. The intraperitoneally treated group showed relative amelioration as compared with the injured nontreated group.

Sciatic function index (SFI)

The main value of SFI for the control group and at the preoperative week ranged between -6.7±2.01 and -14.1± 2.74. The second group (injured nontreated) showed a significant decrease at weeks 1, 2, 3, 4 and 5 as compared with the preoperative values ($p<0.05$). The third group (locally treated group) showed a rapid increase within the first three weeks after nerve lesion reaching the preoperative values. The fourth group (intraperitoneally treated group) showed a relatively gradual increase compared with the injured nontreated group (table 2).

Histopathological observations

The gastrocnemius muscle sections of the control group showed normal structure of muscular bundles, formed of myofibrils rimed peripherally with their nuclei. The results related to the injured nontreated group showed mild histological changes. The injured nontreated group's muscle displayed neurogenic atrophy characterized by dark blue nuclear clumps with angulated atrophic myofibers. The muscles of the third and fourth groups (treated groups) were identical and showed restored muscular architecture and efficiently regenerated (fig. 1). A normal nerve structure made up of a large fascicle with its surrounding perineurium and axons was found in the sciatic nerve's cross-section of the control group.

A damaged and disintegrated myelin sheath was found in the sections of the crushed nontreated sciatic nerve. Nerve regeneration and Schwann cell proliferation were found in the nerve sections of the two treated groups (fig. 2).

Table 1: Mean values of withdrawal reflex latency (WRL), time in seconds ± standard error

Week	Groups			
	Control	Injured non-treated	Injured locally treated	Injured i.p. treated
0	1.8 ± 0.01	1.7 ± 0.31	1.7 ± 0.34	1.8 ± 0.01
1	1.7 ± 0.31	8.6 ± 1.01*	7.2± 1.01*	8.5 ± 1.12*
2	1.7 ± 0.21	6.7 ± 1.01*	4.1 ± 1.01*	6.3 ± 1.02*
3	1.8 ± 0.02	5.1 ± 1.01*	3.4 ± 0.34	4.7 ± 1.11*
4	1.8 ± 0.22	4.6 ± 1.01*	2.8 ± 0.23	4.1 ± 1.03*
5	1.7 ± 0.34	3.0 ± 0.23	2.3 ± 0.41	2.7 ± 0.21
6	1.9 ± 0.01	2.3 ± 0.12	2.2 ± 0.13	2.2 ± 0.20
7	1.7 ± 0.41	2.2 ± 0.23	2.1 ± 0.15	1.9 ± 0.16
8	1.7 ± 0.01	2.2 ± 0.22	2.1 ± 0.03	1.8 ± 0.10

WRL was measured as the elapsed time (in seconds) from the instant of hotplate contact by the rat hind paw to the paw's withdrawal.
*Significant as compared with the control ($p<0.05$). Differences were considered statistically significant at $p<0.05$

Table 2: Main indices ± standard error of sciatic nerve function (SFI) for each experimental group

Week	Groups			
	Control	Injured non-treated	Injured locally treated	Injured i.p. treated
0	-13.1 ± 3.01	-6.7 ± 2.01	-11.7 ± 3.21	-13.7 ± 2.21
1	-11.8 ± 3.11	-72.7 ± 5.82*	-65.6 ± 4.12*	-69.3 ± 3.11*
2	-11.7 ±2.71	-71.9 ± 4.56*	-31.3 ± 5.02*	-65.2 ± 3.31*
3	-14.0 ± 3.16	-42.0 ± 6.11*	-14.7 ± 3.34	-33.4 ± 2.43*
4	-10.6 ± 3.15	-29.8 ± 4.23*	-14.3 ± 6.42	-24.4 ± 2.45*
5	-12.2 ± 3.14	-28.7 ± 6.01*	-11.2 ± 6.51	-25.5 ± 1.96*
6	-10.9 ± 2.83	-21.1 ± 5.14*	-13.7 ± 4.36	-17.2 ± 3.11
7	-14.1 ± 2.74	-14.7 ± 3.34	-10.7 ± 2.34	-9.1 ± 3.23
8	-9.9 ± 3.12	-14.5 ± 2.45	-11.7 ± 2.01	-6.7 ± 4.41

*Significant as compared with the control ($p<0.05$). Differences were considered statistically significant at $p<0.05$

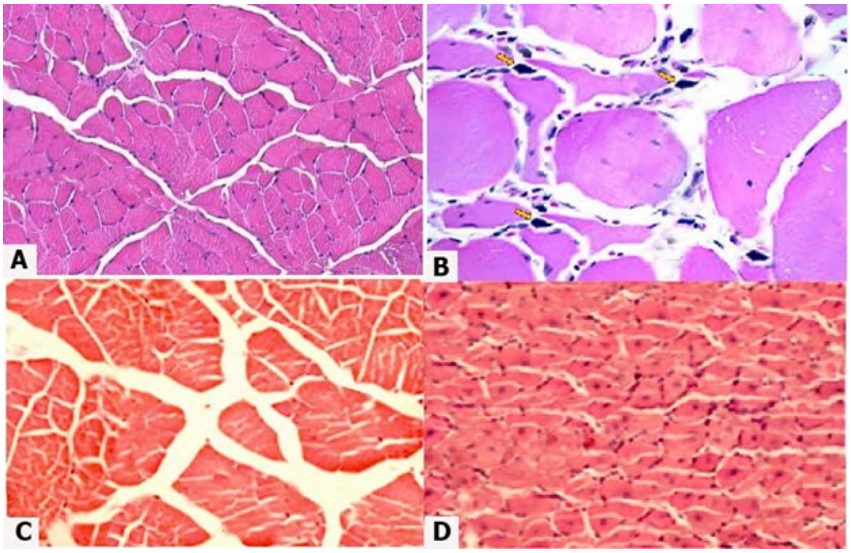

Fig. 1: Photomicrographs of gastrocnemius muscle specimens showed normal muscular bundles, formed of myofibrils rimed peripherally with their nuclei, in control group rat (A). A photomicrograph of gastrocnemius muscle of rat undergoing crushed sciatic nerve (B) displayed neurogenic atrophy of skeletal muscle characterized by dark blue nuclear clumps (yellow arrows) with angulated atrophic myofibers. The damaged muscular architecture in locally treated and intraperitoneally treated rats (C& D respectively) is virtually restored and efficiently regenerated (indicated by the presence of centrally located nuclei in myofibrils) [H & E x40 in A, C & D, and x100 in B].

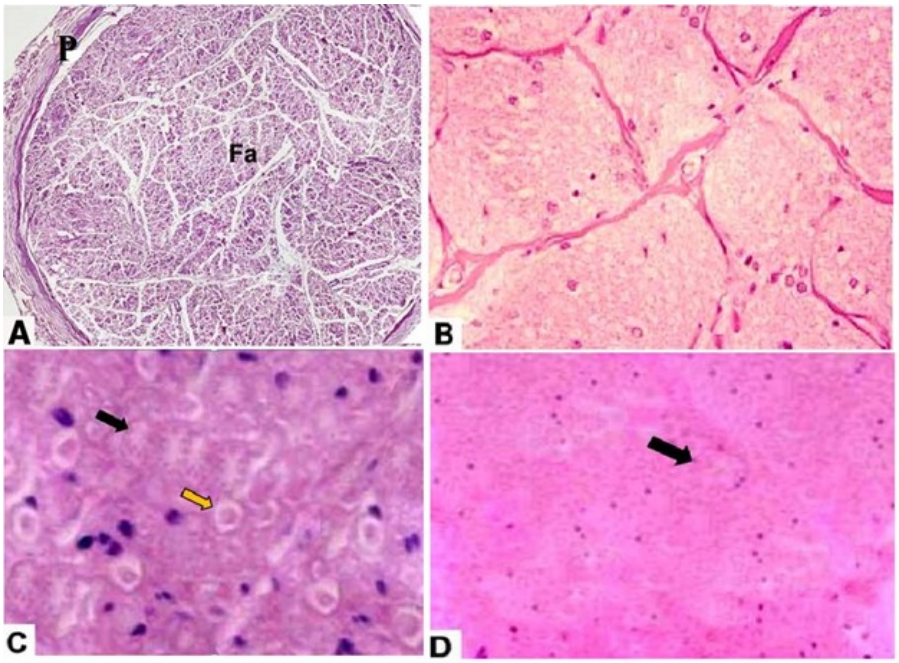

Fig. 2: Photomicrographs of sciatic nerve specimens. Cross-section of normally sciatic nerve structure in the sham group rat (A) formed of a large fascicle (Fa) with its axons and surrounding perineurium (P). Photomicrograph of a crushed sciatic nerve (B) revealed damaged and disintegrated myelin sheath. In locally and intraperitoneally treated animals (C & D respectively), there are nerve regenerations (black arrows) as well as Schwann cell proliferation (C; yellow arrow) [H & E x40 in A, B & D and x 100 in C].

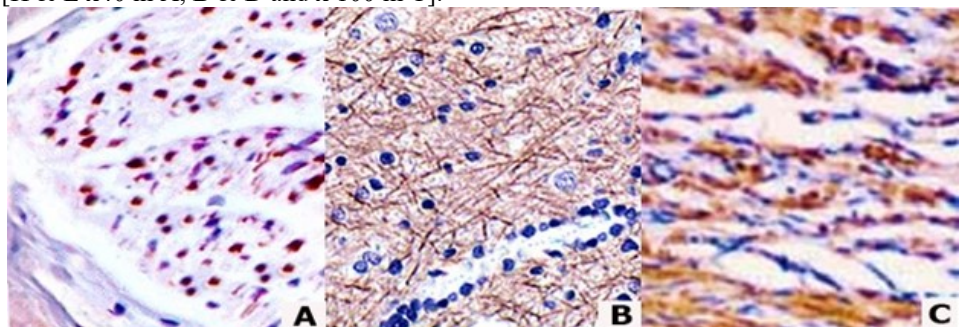

Fig. 3: A photomicrograph of part of normal sciatic nerve fascicle (A) in the sham group showing weak immunopositivity and brown staining for neurofilament protein. Also, a photomicrograph of a crushed nontreated sciatic nerve (B) showed irregularly distributed nerve fibers positively stained for neurofilament protein. The sciatic nerve in either locally or intraperitoneally treated rats (C) showed strong positive staining of the regenerated axons for neurofilament protein [Immunoperoxidase x40 in A and x100 in B & C, with hematoxylin counterstaining in C].

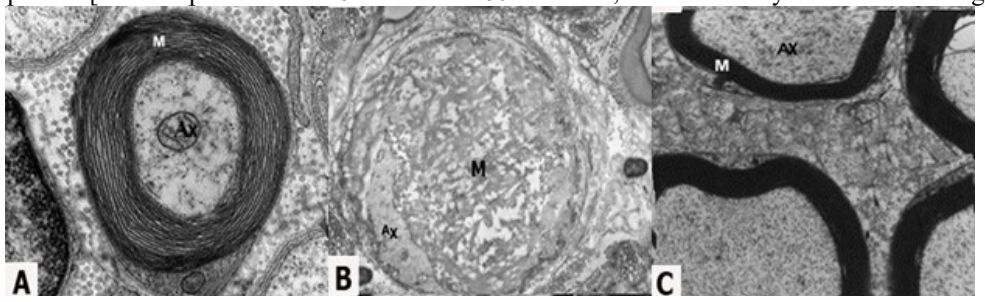

Fig. 4: An electron micrograph (A) of the normal sciatic nerve from a sham animal revealing healthy-appearing axons [Ax] and myelin sheaths [My]. Moreover, an electron micrograph of a crushed nontreated sciatic nerve (B) showed a Schwann tube containing disorganized myelin [My] and a portion of the axon [Ax]. In contrast, an electron micrograph of the treated nerve (C) showed regenerated axons [Ax] and myelin sheath [My] [Transmission electron microscope using uranyl acetate and lead citrate stain x 12,000].

Immunohistochemical observations

Irregularly distributed nerve fibers positively stained for neurofilament protein were found in the sections of the second group (injured nontreated). The treated groups showed strong immuno-positive brown staining indicating regenerative axons (fig. 3).

Electron microscopy observations

The micrographs of the control group showed regularly arranged myofibrils. On the other hand, the results obtained for the crushed nontreated sciatic nerve showed disorganized myelin and axons contained in the Schwann tube. However, the treated groups' results suggested regenerated axons and myelin sheaths (fig. 4).

DISCUSSION

The results revealed that the animals with crush sciatic nerve injury, which were treated with the honey, showed significant improvement in the physiological indicators as well as the structure of the sciatic nerve and target muscles compared with the injured nontreated animals. The locally treated group showed more rapid relief than the intraperitoneally treated one. Our results were consistent with the findings of several recent studies showing that honey can be significantly used for regenerative purposes. Honey has been confirmed to promote tissue regeneration and wound healing (Liyanage and Mawatha 2017, Niaz *et al*., 2017). Several mechanisms have been suggested through which honey actively promotes the process of wound healing. Honey is a hyperosmotic agent that helps draw fluid from the wound bed and underlying circulation. Moreover, increased sugar content, vitamins, amino acids, and minerals tend to provide topical nutrition responsible for promoting healing and tissue growth (Lusby *et al*., 2002, Fox 2002, Ahmed *et al*., 2003). It also reduces the inflammatory response through the inhibition of neutrophil migration (Franchin *et al*., 2013). Honey also can promote tissue regeneration through its immunomodulatory properties (Niaz *et al*., 2017).

Honey has been shown to ameliorate neuronal deficit and protect neuronal cells in different neurotoxicity models (Dar *et al*., 2017, Ali and Kunugi 2019). Propolis, a honeybee product, was proven to effectively treat crush injuries of the sciatic nerve and ameliorates the histopathological changes in the myelinated fibers of the nerve in rat models (Yüce *et al*., 2015, Kassab and Elkaliny 2017). The neuroprotective effect of propolis has been attributed to its anti-apoptotic effect and its capability to enhance the differentiation of the neural stem cells into nerve cells (Swamy *et al*., 2014, Park *et al*., 2020). Also, it may play an important role in suppressing pathogenesis of degenerative disorders of nervous system as a potential source of cholinesterase inhibitors (Baranowska-Wójcik *et al*., 2020).

In the peripheral nerve crush injury, interruption of mechanical transmission and micro-vascularization of the nerve occur followed by reperfusion (Yilmaz *et al*., 2011). The reperfusion leads to accumulation of oxygen and nutrients causing formation of free radicals. These free radicals have destructive effect on tissue and attack tissue lipids leading to lipid peroxidation (Yilmaz *et al*., 2011). The curative effect of honey on the nerve injury has been primary attributed to its powerful antioxidant action due to the presence of phenolic compounds and flavonoids. Honey has inhibitory effects toward enzymes that induce lipid hydroperoxide and free radical formation. It contributes to immune system stimulation and prevention of reperfusion injuries ((Yüce *et al*., 2015). Local treatment by honey through the microenvironment surrounding the injured axons provides topical nutrition responsible for promoting healing and enhances nerve regeneration at a cellular level ((Yüce *et al*., 2015).

Non-surgical conservative management for peripheral nerve crush injury in humans was limited to physical therapies (De Albornoz *et al.,* 2011). However, in animal models, various treatments have been tried. Growth factors have been used locally or added to grafts at the lesion site to enhance regeneration (Rao *et al.,* 2020). Transplantation of neural stem cells into the nerve can promote myelination and axon regeneration and improve functional recovery of the sciatic nerve in rats (Onode *et al.,* 2021). Some protein intervention at the site of lesion was tried (Luria *et al*., 2010). Furthermore, oral administration of some natural products such as propolis (Yüce *et al*., 2015, Kassab and Elkaliny 2017) and curcumin (Yüce *et al*., 2015) was shown to be effective. To this end, it can be inferred that the current study holds significance as it discusses, for the first time, the influence of Yemeni honey on the promotion of peripheral nerve regeneration among rats.

CONCLUSION

Based on the results, it has been concluded that the Yemeni honey has a curative effect on the peripheral nerve crush injury in the rat model. Local application of the honey on the crushed nerve gives rapid ameliorative effect, while systemic administration of the honey induces a gradual curative effect. Further quality human trials are recommended to clarify the honey's neuro-regenerative effect and provide accepted scientific evidence for the clinical practice.

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