Synergistic Effect of Honey and Propolis on Cutaneous Wound Healing in Rats

Nasrin Takzaree[1], Abbas Hadjiakhondi[2], Gholamreza Hassanzadeh[3], Mohammad Reza Rouini[4], and Azadeh Manayi[5]

[1] *Department of Anatomy and Medicinal Plants Research Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Department of Pharmacognosy and Medicinal Plants Research Center, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[4] *Department of Pharmaceutics, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[5] *Medicinal Plants Research Center, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract- Accelerating wound healing is now considered as a principle clinical treatment and increasing the quality and speed of healing which has always been emphasized by the scientists. Propolis and honey are natural bee products with wide range of biological and medicinal properties. This study was aimed to determine the synergistic effect of honey and propolis in wound healing of rat skin. A total of 75 Wistar rats weighing 200-250 gr were placed under general anesthesia and sterile conditions. Then a square shape wound with 1.5*1.5 mm dimension was made on the back of the neck. Animals were randomly divided into control, honey, propolis, combined honey propolis and phenytoin 1% groups, respectively. Rats were randomly divided into the following groups: 4th, 7th and, 14th days of treatment in each period of study. Wound area in the experimental group was covered once daily with a fixed amount of thyme honey, propolis, propolis and honey and phenytoin cream (1%), the control group did not receive any treatment. For histological studies, during the fourth, seventh and fourteenth day's rats were sacrificed and samples were taken from the wound and adjacent skin. After histological staining fibroblast, neutrophils, macrophages and vascular sections were counted in the wound bed. The macroscopic and microscopic evaluations showed that the percentage of wound healing on different days in the experimental and control groups were significant (P<0.05). The macroscopic and microscopic evaluation showed that the percentage of wound healing on different days in combined propolis and honey experimental group was significantly different from the control group (Multivariate ANOVA test) (P<0.05). Combined application of propolis and honey on the open wound healing in rats has a synergistic effect.

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Introduction

Application of natural ingredients and herbs for treating ulcers has been in the history of human life for long times. In recent years, the application of chemical and synthetic drugs has become extremely popular but revealed shortcomings, limitations, and adverse effects in some cases caused inclination to natural ingredients and medicinal plants (1). On the other hand, due to the lack of an effective drug for the treatment of wounds, the effects of medicinal herbs and natural ingredients for healing in the shortest time with the least complication is necessary. Honey was used many years ago for the treatment of infected wounds even before bacteria were discovered (2). The greatest characteristic of honey is its antimicrobial effect. Mechanism of this action is in relation to high osmolarity, acidity, presence of hydrogen peroxide inhibitors, flavonoids, and phenolic acids (3). The concentration of hydrogen peroxide in honey is 1 mmol/L, which is released slowly into wound bed and plays an important role in the elimination of microbial agents. Hydrogen peroxide with its insulin-like effect influences the cells involved in the healing and cause angiogenesis (4-7). Fibroblasts cells are an important part of wound repair because as cells proliferate in the wound, collagen emerges and results in faster wound healing (2,8). Acidic PH of honey along with its osmotic effects stimulates the activity of

Corresponding Author: Gh.R. Hassanzadeh
Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88953008, Fax: +98 21 88953008, E-mail address: Hassanzadeh@tums.ac.ir

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phagocytes and lymphocytes in the wound and increases the other antibacterial components (9,10). Proline in honey with antioxidant activity prevents the formation of free radicals, and it is responsible for anti-inflammatory effects of honey (11,12). Combinations of various properties of honey enhance angiogenesis, granulation, and epithelialization and finally accelerate the wound healing (13-16). Propolis is a bee product and a substance like wax. It is a resin made by bees collected from the nectar of buds, trees gum and various plants (17). Antibacterial and anti-fungal properties of propolis have been proven in various studies (18). Propolis contains high amounts of vitamins, especially vitamin B complex, vitamin C, vitamin E, and provitamin A (19). They also contain mineral elements like copper, iron, calcium, zinc, cobalt, and potassium. The biological activity of Propolis is mainly due to the some substances such as flavonoids, terpenes, caffeic acids, phenolic, and esters (20). Propolis is applied for antimicrobial activities against the widest range of microorganisms. They include bacteria, fungi, and viruses as well. They play an important role for anti-inflammatory activity (21), numbness, healing, as antioxidant, and anti-tumor lesion restoration (22,23). There were reports of synergistic effects between propolis chemistry of antibiotics. Sometimes they will increase antibacterial effect several-fold (24,25). Multiple properties of honey and Propolis like anti-bacterial and healing have been proven in many studies. The aim of this study was to investigate the synergistic effect of honey and Propolis in the open wound healing process in rats.

Materials and Methods

Animals

This experimental study was designed on 75 adult male Wistar rats weighing 200-250 g and ages were between 3-4 months. They were placed in individual cages during the study period. Twelve hours of darkness and 12 hours of light were available, and also, they had ready access to water and food. The animals were randomly divided into control, honey, Propolis, combined honey Propolis and phenytoin cream 1 % (Positive control group), respectively. Rats were randomly divided into the following groups: 4^{th}, 7^{th} and, 14^{th} days of treatment in each period of study.

Surgical method

On the day of surgery, rats were anesthetized with intraperitoneal injection of ketamine hydrochloride (Under the trademark Calypsol, Company's product of Gedeon Richter, Germany) at a dose 50 mg/kg and diazepam (company's product Kimidarou, Iran) at a dose of 4.5mg /kg. Their neck hairs were shaved and incised under sterile conditions. Incisions were made with 1/5*1/5mm dimensions and full-thickness sections of the skin were taken. Surgery day was considered as day zero. After surgery the wound was washed with saline and its surface was covered with a fixed amount of honey thyme, Propolis and the combination of honey propolis and phenytoin cream 1%. The control group did not receive any treatment.

Honey

Mono flower honey was collected from the nectar of thymus plants in late spring from the mountainous region of Damavand in Iran. For isolation of impurity, honey was passed from 0.5 mm Whatman filter at 25-30°C temperature. The high temperature was not used in any way because the high heat causes loss of useful compounds of honey such as proline amino acid. This honey had less than 10% water and more than 90% sugar and was transferred within dark lid dish Pharmacognosy to Faculty of Pharmacy Tehran University research laboratory. Analysis of honey was done at biochemistry lab and revealed TDS 84.5, moisture 15, pH 3.85, glucose 40%, fructose 35.5%, sucrose 3% and contained some amount of Na, Mg, K, Ca, Mn, Cu, and Zn. The purity of the honey was 90 percent. Honey and propolis were mixed by a string with the vortex.

Wound healing assay

Wounds on different days were measured by transparent sheet, and the recovery percentage was evaluated with following formula:

Recovery percentage = Wound surface on the first day – wound surface on day X * 100
Wound surface on the first day

X= day of wound surface measurement

Histological study

After completion of treatment, animals were sacrificed, and tissue samples were prepared. Samples were stained with H & E and specific Masson's trichrome to assess the density of collagen fibers. A light microscope was used CX31-OLYMPUS Japan with magnification field 40*/0.65. Ten areas on the wounds bed were investigated and evaluated. In microscopic examination, fibroblast cells, neutrophils, macrophages and vascular sections in the wound bed were counted using the Image tools 3 software.

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Statistical analysis

The data were analyzed using Multivariate ANOVA test by using SPSS Version 21, and a *P* value<0.05 was significant.

Propolis preparation

In this study, alcohol extraction was performed according to Berretta *et al*., procedure. Firstly large components of propolis were chopped into small pieces and then 25 g of it were mixed with 250 ml of 80% ethanol and shaken for 48 hours at room temperature in a horizontal plane (150 rpm). The alcoholic extract obtained by Whatman filter paper 42 scores, extraction was done twice and with the assistance of smooth rotary alcohol evaporate finally pure alcohol was extracted. Then pure alcoholic extract was obtained by weighing and solution 10% (weight to volume) in alcohol 80 ° (Merck, Germany) they were kept in dark glass containers until preparation time in 4 ° C temperature (20).

Results

Wound healing process was assessed by the microscopic and macroscopic study. In the macroscopic study, sizes of lesions were measured on different days (in the 3rd, 5th, 7th, 9th, 11th and 13th days of study) (Table1).

Table 1. Comparing wound surface and wound recovery in all study groups in the 3rd, 5th, 7th, 9th, 11th and 13th days of study (*P*≤0.05)

Day	Groups	Wound Area Mean±SE	Wound improve Mean±SE
3th Day	Control	3.14±.02	1.95±.42
	Honey	2.40±.01	25.09±.38
	Propolis	2.53±.04	20.87±1.36
	Honey + Propolis	2.15±.02	32.81±.70
	Phenytoin	2.69±.03	15.94±.92
5th Day	Control	3.09±.02	3.43±.77
	Honey	2.07±.03	35.18±.98
	Propolis	2.41±.02	24.55±.66
	Honey + Propolis	1.98±.04	38.00±1.11
	Phenytoin	2.47±.03	22.87±.94
7th Day	Control	3.04±.02	5.00±.77
	Honey	1.90±.02	40.62±.52
	Propolis	2.30±.03	28.16±1.09
	Honey + Propolis	1.78±.01	44.25±.16
	Phenytoin	2.28±.02	28.68±.48
9th Day	Control	2.81±.01	12.06±.19
	Honey	1.60±.03	50.06±.96
	Propolis	2.02±.04	36.81±1.25
	Honey + Propolis	1.30±.00	59.43±.15
	Phenytoin	2.01±.07	37.18±2.16
11th Day	Control	2.58±.04	19.37±1.17
	Honey	1.09±.02	65.86±.73
	Propolis	1.70±.01	47.12±.15
	Honey + Propolis	1.04±.05	67.42±1.62
	Phenytoin	1.69±.01	47.31±.41
13th Day	Control	2.05±.05	35.81±1.44
	Honey	1.06±.04	66.86±1.30
	Propolis	1.50±.01	53.18±.33
	Honey + Propolis	.80±.03	74.93±.84
	Phenytoin	1.55±.04	51.50±1.35
Between-Groups *P*-value		<0.000001	<0.000001
Within-Groups (time) *P*-value		<0.000001	<0.000001
Time*Groups ANOVA *P*-value		<0.000001	<0.000001

In comparison, between lesion surface and percentage of wound healing, there was a significant difference between synergic honey propolis combined experimental group and control group (Figure1 and Table1).

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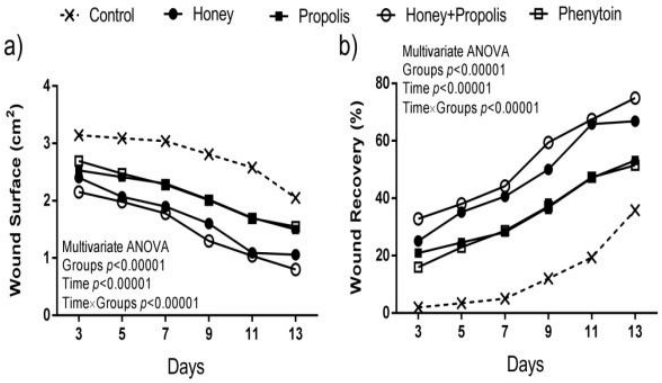

Figure 1. (a) Wound surface size evaluation in the 3rd, 5th, 7th, 9th, 11th and 13th days of study and comparing with Control group. (b) Comparing wound recovery percentage between control and experimental groups in the 3rd, 5th, 7th, 9th, 11th and 13th days of study / *Significant differences between experimental groups and control group (a),(b), ($P \leq 0.05$)

In a microscopic examination of the samples, findings showed that the number of fibroblasts, macrophages, neutrophils and collagen fibers in the honey propolis combined experimental group and control group had a significant difference ($P<0.001$) (Table1). In this study, the number of vessels was significantly ($P<0.05$) higher in the propolis honey combined group (Table2). In the second experimental group, increased collagen and cellular activity (Figure 2). A significant increase of fibroblast cells in the experimental group (Table2) ($P < 0.001$).

Table 2. Histological parameters of wound healing in the Control, Honey, Propolis, Honey+Propolis and Phenytoin groups ($P \leq 0.05$)

Day	Groups	Indices			
		Fibroblast	Macrophages	Neutrophils	Vascular
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
4th Day	Control	70.00 ± 2.02	5.20 ± .37	20.00 ± 1.00	4.20 ± .37
	Honey	85.00 ± 0.71	8.00 ± .32	16.00 ± .71	8.80 ± .58
	Propolis	80.00 ± 1.14	7.00 ± .71	14.00 ± .71	7.00 ± .71
	Honey+Propolis	90.00 ± 0.71	10.00 ± .63	10.00 ± .71	10.00 ± .71
	Phenytoin	80.00 ± 1.45	8.00 ± .32	16.00 ± .32	8.00 ± .32
7th Day	Control	102.00 ± 0.71	4.40 ± .40	19.00 ± .71	8.00 ± .32
	Honey	152.40 ± 2.06	7.20 ± .58	15.00 ± .71	12.80 ± .58
	Propolis	143.00 ± 1.00	6.20 ±.37	12.00 ± .71	11.00 ±.32
	Honey+Propolis	167.80 ± 0.86	7.80 ± .37	8.00 ± .71	14.00 ± .32
	Phenytoin	150.00 ± 1.00	7.40 ± .24	16.00 ± .71	11.80 ± .37
14th Day	Control	98.00 ± 0.32	3.00 ± .32	8.00 ± .71	7.00 ± .32
	Honey	149.00 ± 1.26	2.00 ± .32	4.00 ± .32	12.00 ± .63
	Propolis	139.80 ± 0.80	.80 ± .37	3.00 ± .32	10.20 ± .37
	Honey+Propolis	160.60 ± 0.98	.40 ± .24	2.00 ± .32	12.00 ± .32
	Phenytoin	145.20 ± 0.49	.80 ± .37	4.80 ± .58	10.00 ± .32
Between-Groups *P*-value		<0.000001	0.000002	<0.000001	<0.000001
Within-Groups (time) *P*-value		<0.000001	<0.000001	<0.000001	<0.000001
Time*Groups ANOVA *P*-value		<0.000001	<0.000001	0.003901	0.809110

The average and standard deviation of wound surface during the fourth, seventh and fourteenth days in different experimental groups were recorded in Table1.

Discussion

Wound healing is a process that occurs after skin injury. Wound healing is divided into three phases ofinflammation, proliferation, and remodeling (reconstruction). Immediately after a skin ulcer, cell reaction occurs. Initially blood clotting and degranulation of mast cells occur. Then different chemical mediators are released, and finally inflammation phase occurs (1). In inflammatory phase

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which begins a few hours after injury, primarily neutrophils are released and followed by macrophages, reached to a maximum amount (12). Honey contains moisture absorption properties that can reduce edema of the wound. This results in faster healing and early proliferation phase of inflammation process (10-12). Antibacterial effect of propolis is related to its flavonoids, circular acids, and esters (18). One of propolis feature is its anti-inflammatory properties. Caffeic acid and flavonoids reduce the inflammatory response by inhibiting the production of prostaglandins by blocking the lipoxygenase activity leading to immune cells and phagocytes stimulation and make effective anti-inflammatory and analgesic mechanisms similar to aspirin and with fewer side effects (18,20,21). Bioflavonoids in propolis halt an exodus of inflammatory mediators from mast cells and thereby inhibit the inflammation and allergic reaction (22). Proliferation phase begins after the inflammatory phase and fibroblasts are activated in the wound area. Hydrogen peroxide is produced by honey with its insulin-like properties and causes stimulation, cell proliferation and angiogenesis in the wound bed. Furthermore, it has an important role in the elimination of microbial agents (4-5,23). Lactate synthesis and released mediators from macrophages lead to fibroblasts proliferation, and new blood vessels are formed (16). Angiogenesis is an important factor in wound healing process. The growth of blood vessels in propolis honey combined groups in comparison with control group had an increasing trend to nutrition, oxygenation, cell proliferation and ultimately accelerate wound healing (13-14, 23). Research has shown that honey can accelerate angiogenesis and granulation tissue formation in the wound area (9,15). In this study, the number of vessels was significantly higher in the propolis honey combined group (Table2).

Honey through increasing angiogenesis makes nutrient and oxygen available to fibroblasts, and its acidic PH releases oxygen from hemoglobin and leads to increased activity of fibroblasts and collagen formation (Table2). "As angiogenesis is accelerated so recovery of wound occurs early" (8,10). In the experimental honey propolis combined group, the synergistic effect of this two increased collagen and cellular activity (Figure 2).

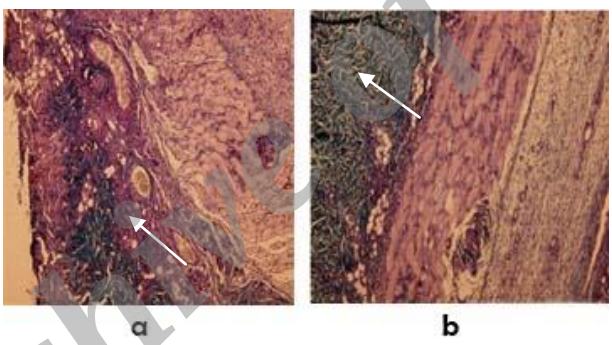

Figure 2. Microscopic view of open cutaneous wounds: (a) Control group, the number of collagen fibers are lesser than the experimental honey propolis combined group in this photomicrograph (b) Combined honey propolis group, the number of collagen fibers are more than the control group in this photomicrograph (specific staining, Masson's trichrome *10)

A significant increase of fibroblast cells in the experimental group showed a positive effect of Honey and propolis on the proliferative phase of wound healing process (Table2). Fibroblasts synthesized part of the extracellular matrix, such as fibronectin and proteoglycans. They provided suitable migration and proliferation of cells (9-14). Fibroblasts were then synthesized collagen, Majtan.*et al*., reported that the proliferation of fibroblasts in the group treated with honey was increased as compared with control group. Similarly, results in we found in this research (16). In the review of the fourth day's samples, the numbers of fibroblasts in experimental groups in comparison with control group indicated that the proliferative phase began earlier in experimental groups while the control group was still in the inflammatory phases (Table2). Increased numbers of fibroblasts caused an increase in collagen which was the main cause to reduce the size of the wound (Table1, Figure1). Propolis is due to vitamin B complex, provitamin A, Arginine and various minerals and also Bioflavonoids, and it contributes to the production of collagen, so wound healing would be faster. Kujumgiev *et al*., showed that the use of propolis with antibiotics improved its antibacterial effect and it seemed that they have had a synergistic effect (18). Flavonoids in honey and propolis stimulate the immune system and increase its antibiotic properties. Reducing the size of the wound and restoring it caused to reducing

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inflammation, infection and activation of fibroblasts to produce collagen fibers. The percentage and rate of wound healing in the combined propolis honey experimental group was significant (Figure1), and there was a synergistic effect between honey and propolis. Application of combined honey and propolis accelerated wound healing process, shortened inflammatory phase, and increased tissue granulation and angiogenesis.

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