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Efficacy of local application of manuka honey for regeneration of critical size calvarial bone defects in rats

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Abstract
Objective: Regeneration of maxillofacial bone defects is clinically challenging. It is believed that Manuka honey can enhance wound healing and decrease inflammation. This study sought to assess the efficacy of Manuka honey for the regeneration of critical size calvarial bone defects in rats.
Methods: This study was conducted on 36 adult male Wistar rats in two groups of 18. Bone defects measuring 8mm in diameter were created in the parietal bone at the sagittal suture. In the test group, defects were filled with Manuka honey while defects remained empty in the control group. Six animals were sacrificed in each group at four, eight, and 12 weeks post-operation for histological and histomorphometric analyses of tissues. The data were analyzed using two-way ANOVA, Independent t-test, and one-way ANOVA ($P< 0.05$).
Results: No significant difference was noted in bone formation between the test and control groups at four weeks (P=0.53) but the amount of newly formed bone in the test group was higher than that in the control group at eight (P=0.004) and 12 ($P< 0.001$) weeks. The amount of newly formed bone in the control group was not significantly different at four, eight, and 12 weeks (P=0.54) while the corresponding values were significantly different in the test group ($P< 0.001$). No inflammation was noted in any group.
Conclusion: Local application of Manuka honey may enhance the regeneration of critical size calvarial bone defects in rats.

Keywords: Bone regeneration, calvarial bone defects, critical size, manuka honey

Introduction
Regeneration of craniofacial bone defects as a result of trauma, congenital anomalies, inflammation or tumor removal is clinically challenging for orthopedists and oral and maxillofacial surgeons [1-3]. Autografts, allografts, and synthetic bone substitutes are often employed for this purpose with varying degrees of effectiveness. However, they all have drawbacks limiting their clinical application [4].
Honey has long been used as a nutrient as well as a medication [5]. Its use for covering the wounds dates back to a thousand years ago [6]. Clinical evidence shows restorative and healing effects of honey owing to its antibacterial, anti-inflammatory, and anti-oxidant activities [5, 7]. When honey is applied to a wound, it produces a moist condition for the wounded tissue while also providing oxygen and nutrients [8]. Moreover, it induces angiogenesis, increases granulation tissue formation, enhances epithelialization, and promotes tissue regeneration as such [5, 9, 10]. It is believed to be an efficient antibacterial agent via mechanisms such as the production of hydrogen peroxide, the presence of flavonoids in its composition, its osmotic properties, and low pH [6, 11]. By its broad-spectrum antimicrobial activity, honey prevents the growth and proliferation of over 80 bacterial species, fungi, protozoa, and viruses [7, 12]. Evidence shows that honey is effective against antibiotic-resistant bacterial strains and can inhibit bacterial proliferation in infected wounds [11, 13].
Honey has anti-inflammatory properties as well, enhances debridement, promotes wound healing, and rapidly decreases pain, edema, and exudate production [5]. In addition to antibacterial and anti-inflammatory properties, phenolic acid and flavonoid antioxidants

present in the composition of honey protect the wound from the adverse effects of free oxygen radicals produced by inflammatory cells and enhance wound healing as such [6, 7, 14]. Manuka is the most common type of honey used in medicine [6, 9]. It is made from the nectar of the Leptospermum Scoparium tree in Australia and New Zealand [6]. It has greater antibacterial action (when compared to other varieties) because of the presence of compounds other than hydrogen peroxide in its composition such as phytochemicals and methylglyoxal and has gained the spotlight due to such optimal properties [6, 15-17].

Considering the above-mentioned unique properties and lack of research on the impact of Manuka honey on the regeneration of bone defects, this study sought to assess the effect of Manuka honey on the regeneration of critical size calvarial bone defects in rats.

Materials and Methods

Preparation of Manuka honey

Manuka honey (MGO™ 100+; Manuka Health New Zealand Ltd., New Zealand) was used in this study. Before use, it was subjected to gamma radiation for sterilization [5]. For this purpose, Manuka honey was poured into a screw-top glass container and sterilized in a Gamma cell (GC-220) with 25kGrey gamma rays [18]. After sterilization, honey was stored away from heat and direct sunlight at 20°C until the experiment.

Surgical procedure on rats

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (ethical code: 93/D/130/1590). A total of 36 adult male Wistar rats weighing 250-300g were used in this study. They had ad libitum access to food and water and were kept under standard conditions (22±5 °C temperature, 12-hour dark/12-hour light cycles, 50±5% humidity). General anesthesia was induced by injection of 60mg/kg 10% ketamine (Alfasan, Woerden, the Netherlands) and 5mg/kg 2% xylazine (Alfasan, Woerden, the Netherlands). After the induction of general anesthesia, 1mg/kg enrofloxacin (5% Enrocin, Razak, Tehran, Iran) was administered prophylactically. After shaving, the surgical site was scrubbed with 7.5% betadine and its 10% solution. The skulls were then fixed in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA). After draping, a longitudinal incision was made sagittally with a 20mm length at the middle of the posterior surface of the skull under aseptic conditions (Figure 1a). The subcutaneous tissue was incised and the periosteum was retracted to expose the skull (Figures 1b, c). Considering the critical size defect in rats' skull, one round bone defect was drilled with 8mm diameter [19] in full-thickness between the two parietal bones in such a way that the sagittal suture split it in half (Figures 1d, e). A trephine bur (XTP8409, Dentium, Korea) was used for this purpose under irrigation with 0.9% saline to prevent over-heating and eliminate bone chips and debris. Care was taken not to traumatize the dura matter. The created 36 bone defects were randomly divided into two groups of negative control (n=18) and Manuka honey (n=18). Bone defects in the control group remained empty and received no intervention. Bone defects in the Manuka honey group were filled with 0.1ml Manuka honey (Figure 1f). The periosteum along with the subcutaneous tissue was sutured with absorbable 4-0 polyglycolic acid sutures (Polyglycolate coated, Supa, Iran) while the skin was sutured with non-absorbable 4-0 nylon sutures (Nylon, Supa, Iran) in two layers (Figures 1g, h). To relieve post-operative pain, 0.1mg/kg tramadol (Chemidarou, Tehran, Iran) was administered. Six rats in the test and six in the control groups were sacrificed at four, eight, and 12 weeks post-treatment by CO_2 inhalation.

Histological analysis

The skulls were immersed in 10% phosphate buffered formalin (pH of 7.0). After fixation in formalin for 48 hours, the skulls were decalcified in 10% nitric acid for 10 days at room temperature. Decalcified skulls were sectioned coronally through the center of defects and embedded in paraffin blocks. Serial sections were then made at the center. A minimum of four sections with 4µm thickness was made by a microtome (Accu-cut SRM 200, Sakura Fihetek, Europe BN, Holland) out of paraffin blocks and stained with hematoxylin and eosin. The specimens were examined under a light microscope (ECLIPSE E400, Nikon, Japan) at ×40, ×100, ×200, and ×400 magnifications by a pathologist blinded to the grouping of specimens. Type of connective tissue (normal bone marrow, fibrous tissue, granulation tissue, necrosis), type of inflammation (acute, chronic), and percentage of inflammation was reported. The percentage of inflammation was scored as follows: [20]

0 (normal): Number of inflammatory cells<10

1 (moderate inflammation): Number of inflammatory cells between 10-30

2 (severe inflammation): Number of inflammatory cells >30 (HPF ×400)

Histomorphometric analysis

Stained sections were evaluated under a light microscope (ECLIPSE E400, Nikon, Japan) equipped with a digital camera (E8400, Nikon, Japan) at ×40 magnification. The obtained images were analyzed on a computer using the IHMMA version 1 (SBMU, Iran) software program. The total size of the defect was considered as the largest diameter between the borders of the defect. The amount of newly formed bone in the four serial sections of each sample was measured and reported as a mean percentage of the total size of the defect.

Statistical analysis

The percentage of newly formed bone was reported as mean and standard deviation. Two-way ANOVA was used to compare the quantity of newly formed bone in different groups and at different time points. Since the interaction effect of these two variables was significant, an Independent t-test was applied to compare the control and test groups at each time point and one-way ANOVA was applied for intra-group comparisons among different time points (four, eight and, 12 weeks). The percentage of inflammation was analyzed using the chi-square test. The data were analyzed using SPSS version 22 (SPSS Inc., Chicago, IL, USA). $P< 0.05$ was considered statistically significant.

Results

Histological assessment revealed bone formation at the margins of defects along with the underlying fibrous connective tissue in all samples at all weeks (Figure 2). Inflammation was not seen in any sample (score 0, <10 inflammatory cells). No inflammatory cells or giant cells were seen in Manuka honey samples.

Table 1 shows the mean percentage of osteogenesis at different time points in the test and control groups. Based on histomorphometric studies, the rate of osteogenesis at four

weeks was not significantly different between the two groups (P=0.53) but the difference between the test and control groups at eight (P=0.004) and 12 weeks (*P< 0*.001) was statistically significant.

Comparison of the amount of newly formed bone in the control group did not show significant differences at four, eight and, 12 weeks (P=0.54). However, significant differences existed in this regard in the Manuka honey group (*P< 0*.001). In other words, a significant amount of new bone was formed in the Manuka honey group.

Discussion

Given the limits of current therapies, discovering an appropriate alternative for the restoration and regeneration of maxillofacial bone abnormalities is a highly disputed issue among researchers [21].

Several methods have been used to assess the efficacy of graft materials used for bone tissue engineering. The creation of calvarial bone defects in rats is a standard and affordable approach to evaluate the osteogenic effects of various materials before the conduction of extensive animal research or human trials [19]. Skull is similar to maxillofacial bone and defects created in the skull [22], similar to those in the femur, do not require fixation [19]. Critical size bone defects were evaluated in this study since spontaneous healing of these defects does not occur. Evaluation of critical size defects provides more reliable results regarding the effect of materials on bone regeneration [4].

Due to its excellent properties, honey has long been used to cover wounds. Human and animal trials have found the optimal efficacy of honey for healing wounds and burns [5, 6]. Antimicrobial, anti-inflammatory, and antioxidant activities are among the most essential factors responsible for enhanced wound healing by the application of honey [5, 6, 9, 14, 23]. Moreover, it has been documented that honey has balancing impacts on the inflammatory, proliferation, and remodeling stages of wound healing [24]. Despite the variability in honey types, Manuka honey is the most commonly used type of honey for medical applications and wound healing [17, 25].

The most important finding of the current study was that local application of Manuka honey in calvarial bone defects of rats enhanced bone regeneration and caused no inflammatory reaction.

In bone fracture, hematoma and inflammation are two immediate responses. Hematoma prevents further bleeding and loss of blood factors. Inflammatory response provides primary stability between the two ends of a broken bone and releases a cascade of signals to enhance healing. After the formation of hematoma, progenitor cells are concentrated in the area. This process is followed by angiogenesis and the accumulation of fibroblasts and other supporting cells. Granulation tissue is then formed between the two broken segments [26]. Angiogenesis is a critical step in bone formation, and impaired angiogenesis is a big obstacle against bone regeneration [4].

Honey has been shown to increase vascular endothelial cell proliferation and angiogenesis at wound sites in animals. Also, it has been reported that the angiogenic potential of honey induces the formation of granulation tissue [5].

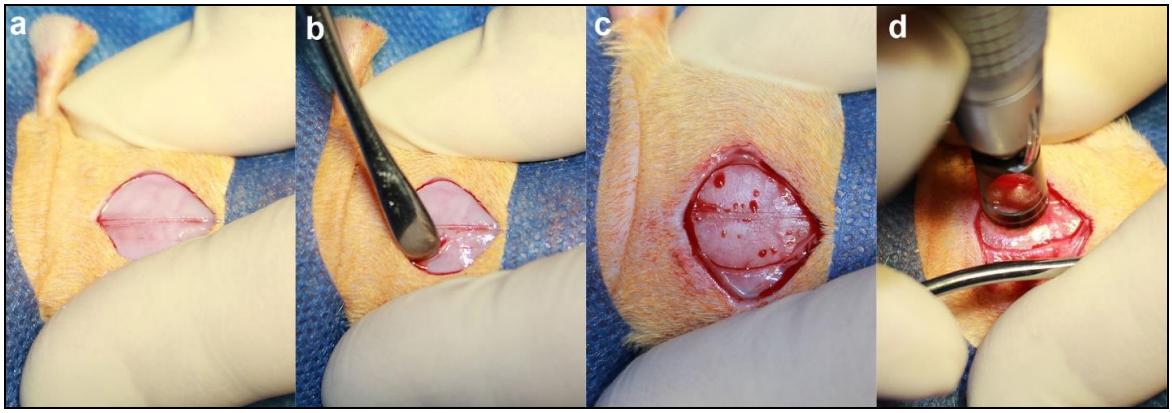
Hydrogen peroxide generated by glucose oxidase, which is contained in honey's formulation, stimulates fibroblast and epithelial cell development and proliferation [27]. On the other hand, the osmotic properties of honey provide nutrients and oxygen required for the injured tissue through the lymphatic system and thus, enhance healing [5]. In the current study, it appeared that honey enhanced the formation of a highly vascular granulation tissue rich in fibroblasts, increased angiogenesis, and enhanced healing as such. Moghazy *et al*. showed that using honey to treat diabetic foot patients was associated with the greater formation of granulation tissue and healing was enhanced as such [11].

Despite greater osteogenesis in the honey group, histological analysis showed bone formation at the margins of defects in both groups. It is assumed that due to the structure of honey and greater angiogenesis, honey was soon removed from the area and did not have adequate time to exert its effects. Because growth factors are ineffective for bone regeneration when injected directly into the defect site due to rapid infusion, they are often combined with a carrier [28]. Similarly, honey may be used with a carrier to exert greater effects on bone regeneration.

Comparison of osteogenesis in different time points in the current study showed a significant increase in the Manuka honey group at eight and 12 weeks. However, the value at four weeks was not significantly different between the test and control groups. It appears that honey had a more significant effect on enhancing osteogenesis from week four on.

The anti-inflammatory properties of honey have been proven in several animals and human research [5, 6, 9, 14]. Histological and biochemical assessments of superficial burns treated with honey showed less inflammation [9]. Subrahmanyam in his study on burns treated with honey showed superior healing and less inflammation [29]. In the current study, defects treated with Manuka honey showed no sign of inflammation.

Honey is an easily accessible and cheap substance compared to the current medications [6, 11, 30,31]. Treatment with honey is cost-effective and enhances the speed of healing and recovery in patients with diabetic foot and decreases their hospitalization period [11]. Also, it is safe and its use has not been associated with any local or systemic conditions [11, 23, 30].



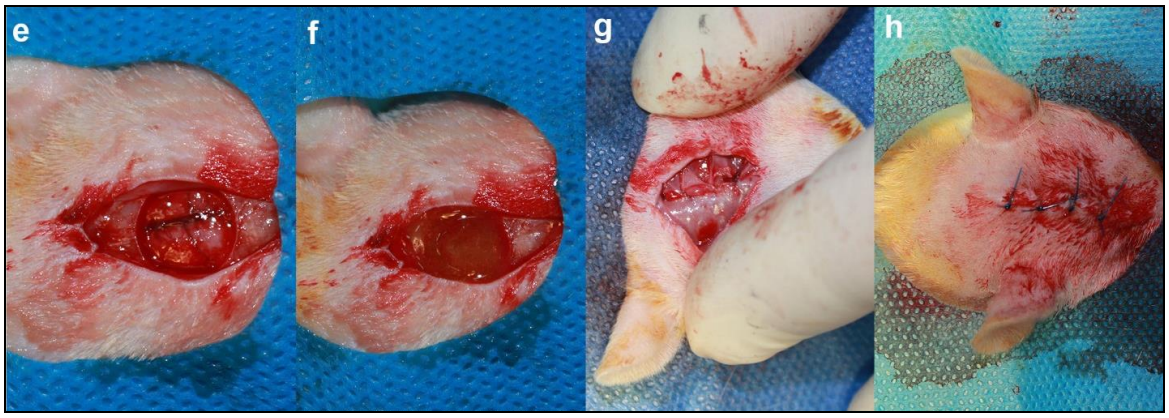


Fig 1: Surgical procedure. (a) A 20mm midline incision through the skin along the sagittal suture of the skull. (b, c) Elevation and reflection of soft tissues and periostea. (d, e) Creation of a critical size, 8mm, full thickness, cranial defect centered over the sagittal suture using a trephine bur without disrupting the underlying dura mater. (f) Filled defect with Manuka honey. (g) Closure of the periosteum. (h) Closure of the overlaying skin.

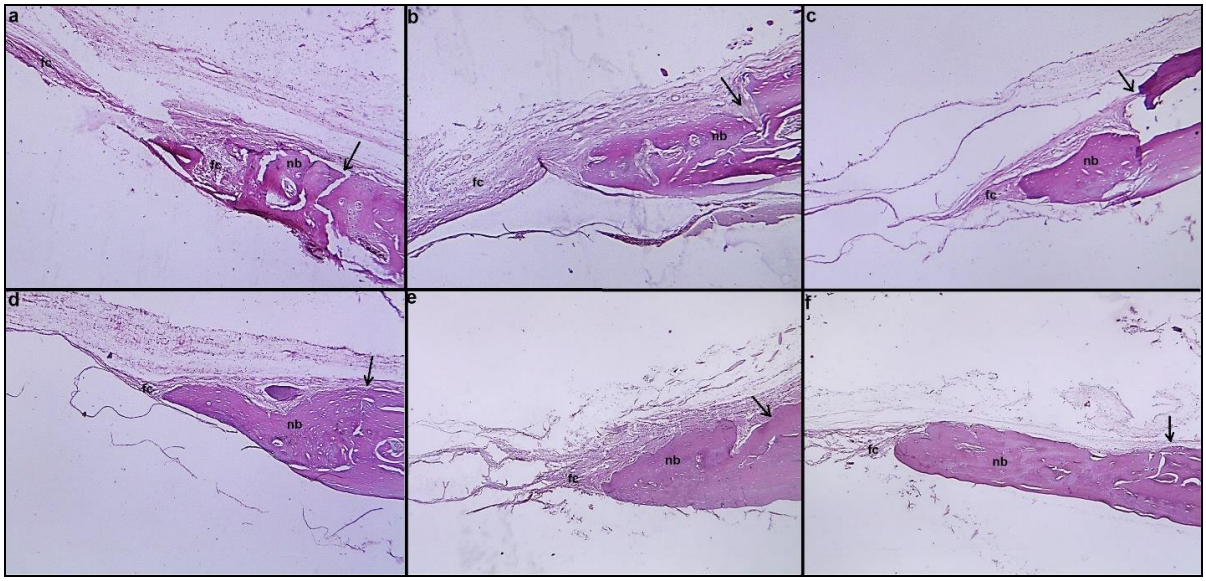


Fig 2: Histological findings in hematoxylin and eosin-stained sections. (a) Control group at 4 weeks. (b) Manuka honey group at 4 weeks. (c) Control group at 8 weeks. (d) Manuka honey group at 8 weeks. (e) Control group at 12 weeks. (f) Manuka honey group at 12 weeks. (Original magnification ×40). Arrows, defect margins; nb, new bone formation; fc, fibrous connective tissue.

Table 1: Evaluation of new bone formation (%) in the Manuka honey and control groups at 4, 8 and 12 weeks

Groups	Week 4		Week 8		Week 12	
	n	Mean ±SD	n	Mean ±SD	n	Mean ±SD
Control	6	16.93±5.83	6	15.89±3.27	6	13.60±6.03
MH	6	15.32±1.65	6	25.54±5.50	6	30.45±4.28
P value*		.53		.004		< .001

Abbreviations: SD, standard deviation; MH, Manuka honey.
*P< 0.05 was considered statistically significant

Limitations

This study has a relatively small sample size and a short follow-up period. In addition, the molecular mechanism of bone healing through honey was not examined.

Conclusion

The results of this study showed that Manuka honey was suitable for the regeneration of calvarial bone defects in rats. However, the impact of honey on bone regeneration will need to be studied more in the future.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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