Research

In vitro activity of Manuka and Trigona honey on fibroblast and keratinocyte cultures

Harim Priyono*, Ratna Dwi Restuti*, Indra Kusuma, Alida Harahap***, Purwantyastuti Ascobat****, Rianto Setiabudy****, Ahmad Sulaeman*****, Lina Lasminingrum******, Pingkan Permata Putri*, Anggi Puspa Nur Hidayati****

*Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo General Hospital, Jakarta

**Department of Physiology, Faculty of Medicine, Universitas YARSI, Jakarta

***Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo General Hospital, Jakarta

****Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Jakarta

*****Department of Community Nutrition, Faculty of Human Ecology, Institut Pertanian Bogor

******Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Universitas Padjadjaran– Hasan Sadikin General Hospital, Bandung

ABSTRACT

Background: Benefits of honey on wound healing is widely reported, but information about its effect on the re-epithelialization of the tympanic membrane (TM) is limited. **Purpose:** To evaluate the effect of Manuka honey (MHn) and Trigona honey (THn) from Indonesia, on TM re-epithelialization through their potential action on proliferation of fibroblasts, keratinocytes, secretion of KFG and basic-FGF. **Method:** This study was conducted at the YARSI University Laboratory. Fibroblast and keratinocyte cultures isolated from chronic suppurative otitis media patients were exposed to MHn and THn with three dilutions: 0.04%, 0.1%, and 0.25%. The cells were then subjected to proliferation assays, KGF and bFGF were also assessed and compared to the control. **Result:** MHn and THn did not increase the number of fibroblasts but shortened the doubling time duration. A significantly higher number of keratinocytes than control was observed in all MH groups and the 0.04% THn group. KGF secretion increased as the number of cells increased. On the 6th and 8th day, KGF secretion was observed to be higher in some intervention groups compared to the control group. Conversely, the level of bFGF secreted by fibroblasts and keratinocytes decreased as the number of cells increased. There was a positive correlation between the duration of both honey exposure and fibroblast proliferation. The duration of 0.04%, 0.1% MHn, and 0.04% THn exposure positively correlated with the number of keratinocytes. **Conclusion:** This research illustrated the positive effects of MHn and THn on fibroblasts and keratinocytes; thus, potential therapeutic properties could be further explored.

Keywords: Manuka honey, Trigona honey, fibroblasts, keratinocytes

ABSTRAK

Latar belakang: *Manfaat madu dalam penyembuhan luka telah banyak diteliti, namun informasi mengenai efeknya pada re-epitelialisasi membran timpani (TM) terbatas.* ***Tujuan:*** *Mengevaluasi efek madu Manuka (MM) dan madu Trigona (MT) dari Indonesia, pada re-epitelisasi membran timpani (MT) melalui potensi aksinya pada proliferasi fibroblas, keratinosit, sekresi KGF, dan basic-FGF.* ***Metode:*** *Penelitian ini dilakukan di Laboratorium Universitas YARSI. Kultur fibroblas dan keratinosit yang diisolasi dari pasien otitis media supuratif kronis terpajan MM dan MT dengan tiga konsentrasi*

yaitu: 0,04%, 0,1%, dan 0,25%. Kemudian dilakukan uji proliferasi, KGF dan bFGF juga dinilai dan dibandingkan dengan kontrol. ***Hasil:*** *MM dan MT tidak meningkatkan jumlah fibroblas tetapi mempersingkat durasi* doubling time*. Jumlah keratinosit lebih tinggi secara bermakna dibanding kontrol pada semua kelompok MH dan TH 0,04%. Sekresi KGF meningkat seiring pertambahan jumlah sel. Pada hari ke-6 dan ke-8, sekresi KGF lebih tinggi pada beberapa kelompok intervensi dibandingkan dengan kontrol. Sebaliknya, kadar bFGF menurun seiring pertambahan sel. Terdapat korelasi positif antara lama pajanan madu dan proliferasi fibroblas. Lama pajanan MM 0,04%, 0,1%, dan MT 0,04% berkorelasi positif dengan jumlah keratinosit. Kesimpulan: Penelitian ini menggambarkan efek positif MM dan MT pada fibroblas dan keratinosit, sehingga potensi terapeutik madu ini dapat diteliti lebih lanjut.*

Kata kunci: *madu Manuka, madu Trigona, fibroblas, keratinosit*

Correspondence address: Harim Priyono. Department of Otorhinolaryngology Head and Neck Surgery, Gedung A lantai 7, Jl. Pangeran Diponegoro No.71, Jakarta Pusat. Email: harimpriyono@yahoo.com

INTRODUCTION

Chronic suppurative otitis media (CSOM) is a health concern with high prevalence in Indonesia. The prevalence was found to be 6.9% at 31- 40 years; which is productive age.[1] Perforation of the tympanic membrane (TM) reduces the ability to hear, hindering academic and work-related activities. CSOM has significant impacts on a person's life, including reduced hearing, restriction from water activities, and risk of fatal complications.[2] One of the therapeutic options for CSOM is tympanoplasty.

Tympanoplasty aims to close the perforated TM and ideally results in a dry ear. However, the failure rate of the procedure is high, reaching 21–33%.[3-6] One reason for procedure failure is poor vascularization of the edges of the perforation due to chronic infection, which reduces the secretion of growth factors that are needed for wound healing.[7] Biomolecular substances known to have mitogenic properties in wound healing include keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF); fibroblasts produce both, and the latter is also produced by keratinocytes.[8]

Previous studies had demonstrated the benefits of honey on wound healing; however, there was little information regarding its effect on TM re-epithelialization. Several studies had reported that honey had a positive effect on cell growth, especially in keratinocytes and fibroblasts, both of which played an essential role in TM perforation closure. Manuka honey (MH) had been the subject of several studies regarding its application at the cellular and biomolecular levels. This research explored some of the effects of Manuka honey (MHn) and Trigona honey (THn) -two types of honey originating from Indonesia- on fibroblasts and keratinocyte cultures from CSOM patients to observe their effects on KGF and bFGF secretion and cell growth.

METHOD

An in vitro study was conducted at YARSI University Integrated Laboratory from September 2021 to August 2022. The honey used in this study was a solution of 527 mg/mL 100% sterile MHn from MANUKApli topical honey (ManukaMed®), and 100% THn, *Heterotrigona itamai.* At the time of the study, medical-grade Trigona honey was not available; therefore, this study did not compare the effects of Manuka and Trigona honey. The honeys were diluted in a culture medium to obtain concentrations of 0.04%,

0.1%, and 0.25% (v/v). There were seven cultures for each fibroblast and keratinocyte experiment (no co-culture): one control group (culture medium without honey) and six intervention groups (culture medium with honey) composed of three MH and three TH groups at concentrations of 0.04%, 0.1%, and 0.25% each.

The research started in June 2021 after ethical approval was obtained from the Research Ethics Committee of the Faculty of Medicine of Universitas Indonesia Dr. Cipto Mangunkusumo Hospital (RSCM), and permission to conduct the study was obtained from the Medical Education and Research Center of the RSCM. Fibroblasts were isolated from an ear canal skin sample of a 30-year-old male who underwent tympanoplasty and had consented to participate in the study. The explant method was used to isolate the fibroblasts. The experiment with fibroblast cultures was performed on passage 1 (P1). Fibroblasts were planted on a 24-well plate with Dulbecco's Modified Eagle Medium (DMEM) (Gibco™), 10% fetal bovine serum (Gibco™), and 1% Antibiotic-Antimycotic (Gibco™) and were incubated at 37°C with 5% CO_2. The mediums were changed every 2–3 days.

Keratinocytes were isolated from a retroauricular skin sample of an 18-year-old female that was obtained while the patient underwent tympanoplasty. Keratinocyte isolation was performed according to the procedure detailed by Kusuma and Hadi,[9] with several modifications. In this experiment, serum-free cultures with collagen extracellular matrix layer were used during keratinocyte culture. Keratinocytes were planted in a 24-well plate coated with 50 µg/cm^2 of type I collagen. The culture medium was defined keratinocyte serum-free medium (DKSFM) (Gibco™), which contained 1% (v/v) Antibiotic-Antimycotic (Gibco™). The keratinocyte culture experiment was performed on the primary culture (P0). The intervention was applied after a 24-hour incubation period to allow time for cell adhesion, and the medium was changed every 2–3 days.

The cells were quantified using a Cell Counting Kit (CCK-8) (Sigma-Aldrich®), which used colorimetric examination to determine the number of viable cells. The CCK-8 solution was added directly to the cells, causing colorless, water-soluble tetrazolium salt (WST-8) bioreduction by cellular dehydrogenase, which resulted in orange-colored formazan proportional to the number of viable cells. The intensity of the produced color was measured via spectrophotometry using a microplate reader to record the absorbance at a wavelength of 450 nm. The number of cells in the fibroblast culture were calculated on days 1, 3, 6, and 8. The number of cells in the keratinocyte culture were calculated on days 1, 3, 5, and 7. All calculations were performed twice.

Evaluation of KGF and bFGF was performed on the fibroblast culture medium on days 1, 6, and 8; bFGF was evaluated in the keratinocyte culture medium on days 1, 5, and 7. The KGF level was measured using a human KGF/FGF-7 enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, catalog number of MBS459312). Measurement of bFGF level was performed using a human bFGF/FGF2 ELISA kit (MyBioSource, catalog number of MBS260698). Measurements were performed twice for fibroblast and thrice for keratinocytes.

Microsoft Excel 2021 and Statistical Package for the Social Sciences version 26 were used for the statistical analyses. Statistical analyses were performed using the paired t-test. The correlation between two numeric variables was evaluated with Pearson correlation test or Spearman's correlation test, as appropriate. Normality was assessed using the coefficient of variation and Shapiro-Wilk test. All hypothesis testing was two-tailed, and P values <0.05 were considered significant

statistically. All graphics were produced using GraphPad Prism 9.1.0.

RESULT

Number of Cells in the Keratinocyte and Fibroblast Cultures

Both the fibroblast and the keratinocyte cultures showed an increased number of cells over time, indicating that cellular mitosis was taking place. The application of honey did not have a cytotoxic effect on the fibroblasts (within 8 days) and keratinocytes (within 7 days), and did not cause changes in osmolarity that might have induced cell death. MHn and THn did not significantly increase the number of fibroblasts compared to the control. However, the control group reached the stationary stage after day 6, while the MHn and THn group exhibited proliferation until day 8, after which the stationary stage set in. Cell proliferation activity could also be measured using doubling time (DT). From day 1 to day 6, there was no significant difference in DT between the control and intervention groups. However, from day 6 to day 8, the MHn and THn intervention groups exhibited significantly shorter DTs than the control group (Fig. 1).

A significant difference in the number of cells between the control and the MHn intervention groups was observable from day 3 to day 7 in the keratinocyte cultures, specifically in the 0.04% and 0.1% groups. The 0.25% MHn group had a significantly higher number of cells compared with the control group starting on day 5, and entered the stationary stage on day 7. A different result was observed in the THn group; only the 0.04% THn group had a positive effect on keratinocyte proliferation, evidenced by the significantly higher number of viable keratinocytes compared with the control group on day 3 and day 5. The application of 0.1% THn did not produce any significant effects, while the 0.25% THn group produced a lower number of viable keratinocytes compared with the control group from day 3 to day 7.

KGF and bFGF Secretion in the Fibroblast Culture

The secretion of KGF by fibroblasts increased with higher cell numbers. On the first day, KGF levels were undetectable in honey-treated groups, unlike the control. However, by culture days 6 and 8, KGF secretion in Mn 0.04%, Tr 0.04%, and Tr 0.25% groups surpassed the control on day 6, and in Mn 0.25%, Tr 0.1%, and Tr 0.25% groups on day 8.

There was no significant difference in bFGF secretion between the control and intervention groups in fibroblast cultures on the first day. By day 6, bFGF secretion decreased significantly across all groups. However, on day 8, there was a resurgence in secretion in the honey-treated groups, with Mn 0.04%, 0.1%, 0.25%, Tr 0.1%, and 0.25% groups showing significantly higher secretion compared to control.

bFGF Secretion in the Keratinocyte Culture

On day 1, the secretion of bFGF in keratinocyte cultures treated with MHn and THn was significantly higher compared to the control. However, by day 5, there was a sharp decrease in bFGF secretion across all groups. Keratinocyte cultures treated with MHn showed significantly lower bFGF secretion than the control on day 5 and day 7, except for MHn 0.04%. Similarly, THn 0.04% also exhibited significantly lower bFGF secretion on days 5 and 7 compared to the control. Conversely, THn 0.25% showed significantly higher bFGF secretion than the control on days 1, 5, and 7.

Correlation Between Honey Exposure Time and Cell Quantity, KGF level, and bFGF level in Fibroblast Cultures

There was a strong positive correlation between the duration of exposure to all honey groups and the cell count in fibroblast cultures. The longer the exposure to MHn and thN, the higher the cell count in fibroblast cultures. The exposure duration of MHn 0.04% correlated strongly and positively with the level of KGF in fibroblast cultures. However, there was no correlation between exposure to other types of honey and the level of KGF in fibroblast cultures. There was no correlation between exposure to all honey groups and the level of bFGF in fibroblast cultures.

Correlation Between Honey Exposure Time, Cell Quantity, and bFGF levels in Keratinocyte Cultures

There was a strong positive correlation between exposure duration to MHn 0.04%, 0.1%, and THn 0.04%, and cell count in keratinocyte cultures. Longer exposure correlated with higher cell count. No correlation was found between other honey types and cell count. There was a strong negative correlation between exposure duration to MHn 0.04%, 0.1%, THn 0.04%, 0.25%, and bFGF secretion in keratinocyte cultures.

Table 1. Keratinocyte size in the 0.25% Manuka honey and 0.25% Trigona honey groups

Days	Cell size (µm²)	
	Manuka honey 0,25%	Trigona honey 0,25%
1	949.4 (401.4-28861)	1450.1 (797.6-3133.9)
3	1558.1 (638.1-2413.3)	1480.5 (340.5-2124.6)
5	786.7 (537.9-1469.9)	1399.4 (619.7-2419.5)
7	441.0 (216.8-677.4)	1597.0 (637.3-7502.6)

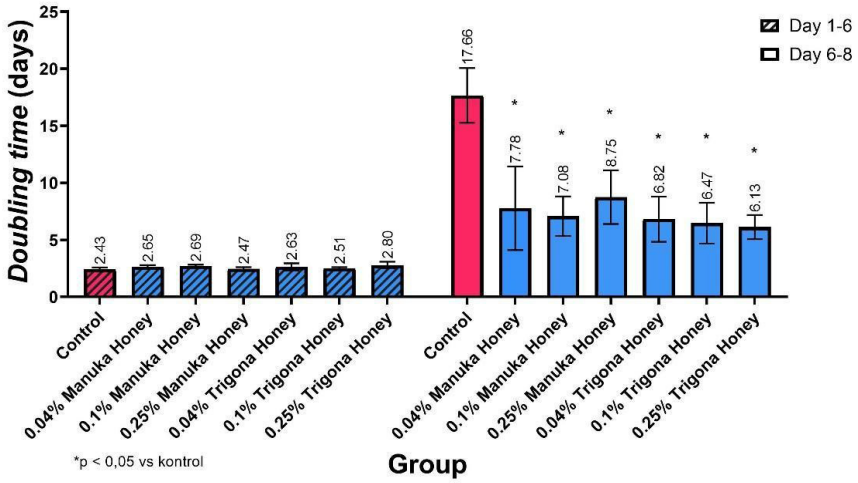

Figure 1. Doubling time of fibroblast culture

Note:
Data were represented as mean ± SD (n=4), *p<0.05 with paired t-test compared with control.

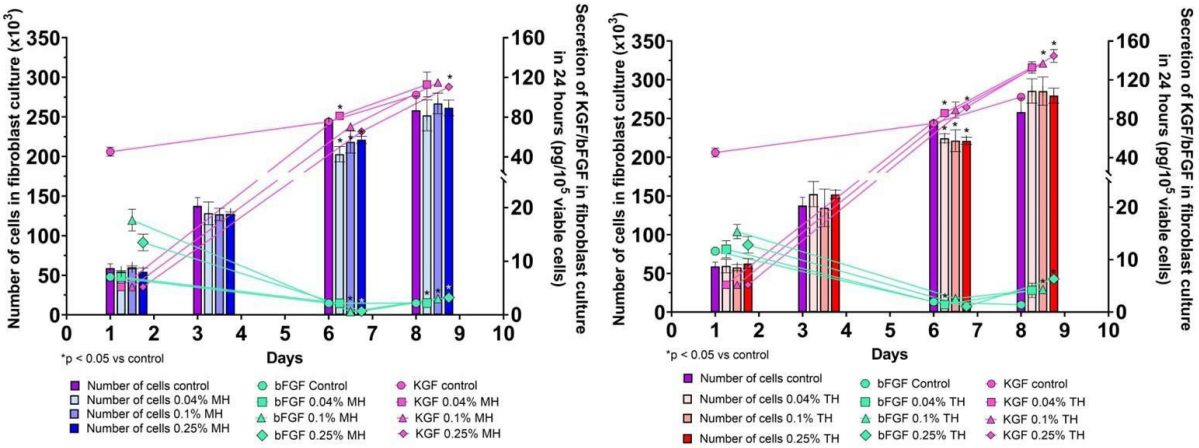

Figure 2. Interactions between the number of cells, bFGF levels, and KGF levels in the fibroblast cultures for the 0.04%, 0.1%, and 0.25% Manuka (left) and Trigona honey (right) intervention group

Note:
Data were represented as mean ± SD (n=4), *p<0.05 with paired t-test compared with control.
On day 1, KGF levels in the intervention groups were not detected, (below 5.2 pg/mL); thus, statistical analyses were not conducted.

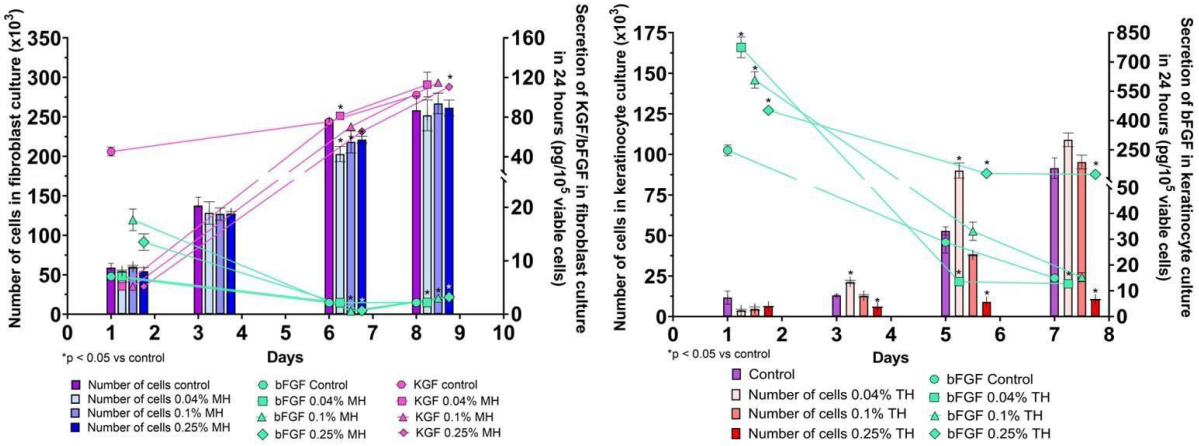

Figure 3. Interactions between the number of cells and bFGF levels in keratinocyte cultures for the 0.04%, 0.1%, and 0.25% Manuka (left) and Trigona honey (right) intervention groups

Note:
Data were represented as mean ± SD (n=3), *p<0.05 with paired t-test compared with control.

DISCUSSION

The results of the honey intervention experiment in fibroblast cultures demonstrated that none of the cell groups experienced cell death, indicating no toxicity from MHn and THn concentrations at 0.04%, 0.1%, and 0.25%. The honey intervention also prolonged cell viability compared to the control group. Observing the increase in cell numbers over culture days, a significant rise was seen in the control group until day 6, while the intervention groups continued to increase significantly until day 8. This prolonged viability facilitated the healing process, allowing healthy cells more time

for proliferation, migration, and growth factor release, particularly bFGF and KGF. The fibroblast-produced KGF also strongly stimulated keratinocyte mitosis. Additionally, honey intervention led to shorter cell doubling times, which benefited wound healing as the shorter the process, the faster re-epithelialization occurred, subsequently reducing the likelihood of disturbances in the wound healing process.

A significant difference in the number of cells between the control and the MHn intervention groups was observable from day 3 to day 7 in the keratinocyte cultures, specifically in the 0.04% and 0.1% groups. The 0.25% MHn group had a significantly higher number of cells compared with the control group starting on day 5 and entered the stationary. The sodium content of 0.25% THn caused the size of keratinocytes to increase in size and inhibits proliferation (by prevention of colony formation) in cultured keratinocytes (Table 1).

The results in Table 1 indicated the different effects of MHn and THn on keratinocytes. One explanation was that the Na^+ content of THn was 15 times higher than MHn;[10] the entry of Na^+ into cells caused an increase in cell volume, and large cells did not form cell colonies as readily as small cells.[11] This could explain the slow growth of keratinocyte cultures in the 0.25% THn group, but not at lower concentrations.

Fibroblast KGF secretion increased as the number of cells increased. On day 6 and day 8, KGF secretion was higher in some of the intervention groups compared with the control group ($p<0.05$). In contrast, fibroblast bFGF secretion decreased as the number of cells increased (Figure 2). Based on this finding, we inferred that the fibroblasts secreted these two growth factors, which explained the increasing level of free KGF in the culture media with an increasing number of fibroblasts. bFGF regulated via paracrine secretion in keratinocytes and via autocrine secretion in fibroblasts; therefore, the free bFGF in the culture medium was consumed by the fibroblasts. bFGF could exhibit a negative modulatory effect (inhibit proliferation and cytokine secretion) on fibroblasts. Therefore, higher bFGF levels could inhibit cytokine secretion. In addition, many studies had indicated that bFGF had a short half-life; hence, its levels were always rapidly decreasing.

Intervention with honey did not increase the bFGF level in the keratinocyte cultures except for in the 0.25% THn group, which produced significantly higher bFGF levels than the control group ($p<0.05$ on day 1, 5, and 7). This might be due to the inhibition of proliferation in the keratinocyte cultures. The Na^+ level in THn was 15 times higher than in MHn.[10] Keratinocytes expressed epithelial sodium channel (ENaC), facilitating Na^+ entry into cells and resulting in a larger cell volume, which consequently inhibited keratinocyte colony formation.[11] The result was a fewer number of cells, which reduced bFGF absorption by keratinocytes (autocrine effect), resulting in a higher level of bFGF in the culture media compared with other groups. The reduction of bFGF secretion in the keratinocyte cultures could be explained in the same manner as bFGF secretion in the fibroblast culture.

The data revealed a positive correlation between the exposure time of all intervention groups and the number of cells in the fibroblast culture ($r =>0.8$, $p <0.05$, $n=4$ for all groups). The 0.04% MHn exposure time showed a strong positive correlation with KGF levels in the fibroblast cultures ($r =1{,}000$, $p =0.004$, $n=3$). These findings indicated that longer exposure time could allow the honey to exert indirect stimulatory effects on fibroblast cells to increase KGF secretion, which had mitogenic properties on keratinocytes. Honey exposure time was not correlated with bFGF levels in the fibroblast cultures ($p >0.05$).

Data analysis revealed that prolonged exposure time to 0.04% ($r = 0.984$, $p = 0.016$, $n=3$), and 0.1% MHn ($r = 0.973$, $p = 0.027$, $n=3$), and 0.04% THn ($r = 0.936$, $p = 0.033$, $n=3$) caused an increase in the number of cells in the keratinocyte cultures. This differed from fibroblast cells, where cell number was strongly correlated with both types of honey at all concentrations ($r = >0.8$, $p < 0.05$, $n=4$ for all groups). A possible explanation for the difference between keratinocytes and fibroblasts might be due to the difference in characteristics and response of keratinocytes and fibroblasts toward MHn and THn exposure.

The interaction between the cells and cytokines in this study could be explained by the following mechanisms. First, a double-loop paracrine mechanism involved keratinocytes, fibroblasts, bFGF, and KGF. In this mechanism, keratinocytes produced bFGF, which had a mitogenic effect on fibroblasts. Then, the fibroblasts responded by producing KGF, which had a mitogenic effect on keratinocytes. The stimulation increased bFGF production by keratinocytes, causing the fibroblasts and keratinocytes to stimulate each other. Second, another double-loop paracrine mechanism involved keratinocytes, fibroblasts, and bFGF, but not KGF. Keratinocytes produced bFGF, which had a mitogenic effect on fibroblasts. Then, fibroblasts responded by producing bFGF, which positively modulated keratinocytes. The keratinocytes also responded by increasing bFGF production, positively modulating the fibroblasts. Third, there was a negative feedback mechanism of bFGF on fibroblasts and keratinocytes in vitro; this negative feedback modulated cell activity toward controlled and physiological activity (Figure 4).

The stimulatory effects of MHn and THn on keratinocyte proliferation and their ability to shorten the fibroblast DT in vitro could be attributed to their glucose content.[12,13] The increased energy requirement by the cells caused adenosine monophosphate-activated protein kinase (AMPK) activation and increased glucose utilization to produce ATP. The glucose was utilized through the following mechanism: first, AMPK increased the expression and transport of glucose; second, AMPK triggered glycolysis under anaerobic conditions; third, AMPK triggered mitochondria formation; and fourth, AMPK inhibited gluconeogenesis in the liver, which used glucose as an energy source.[14] MHn contained 35.9% free glucose, and THn contained 8.1–31% glucose.[9] In addition, the calcium (Ca) in the honey could stimulate keratinocyte and fibroblast proliferation. MHn and THn contained 1.15% and 14.4–20.6% Ca, respectively.[10]

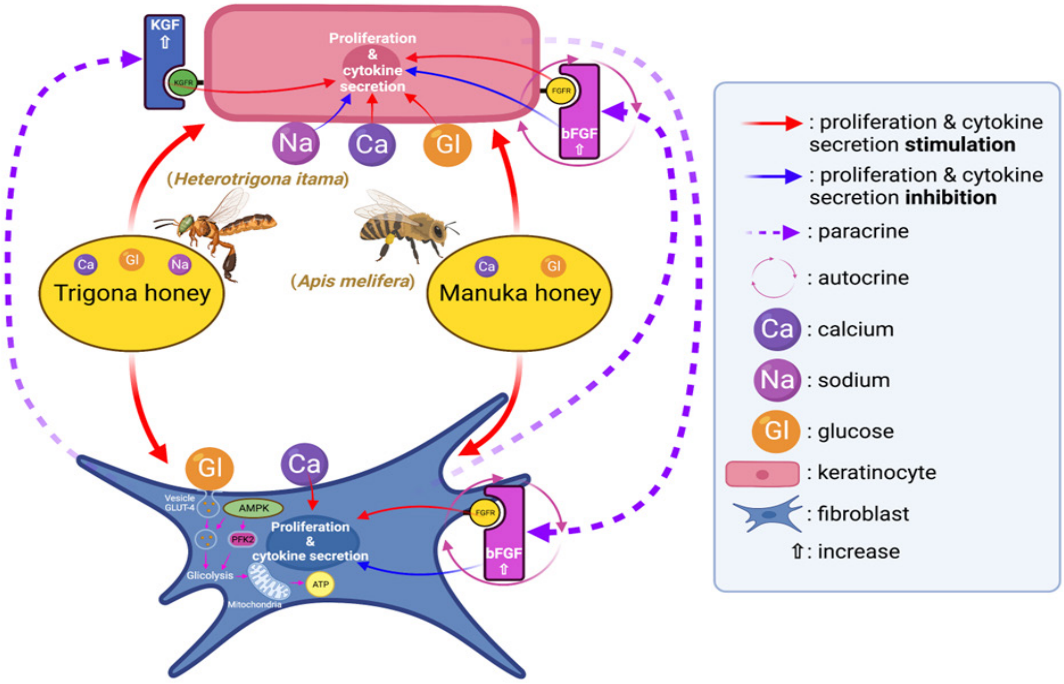

Figure 4. Proposed mechanism of the interventional effect of honey on the in vitro interactions between keratinocytes, fibroblasts, KGF, and bFGF

Note:
AMPK: adenosine monophosphate-activated protein kinase;
ATP: adenosine triphosphate;
bFGF: basic fibroblast growth factor;
FGFR: fibroblast growth factor receptor;
GLUT-4: glucose transporter 4;
KGF: keratinocyte growth factor;
KGFR: keratinocyte growth factor receptor;
PFK-2: phosphofructokinase-2.

In conclusion, this study demonstrated the effects of MHn and THn on stimulating keratinocyte and fibroblast proliferation, and bFGF and KGF secretion. Furthermore, this study described parts of the mechanism and interaction between cells and cytokines that aided in cell growth and proliferation. Findings regarding the exposure duration and its stimulatory effect revealed that further research is needed to validate the benefits of MHn and THn in the field of otology.

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