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Honey stimulates inflammatory cytokine production from monocytes

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Abstract

Clinical observations indicate that honey may initiate or accelerate the healing of chronic wounds and has, therefore, been claimed to have anti-inflammatory properties. The aim of this study was to investigate the effects of honey on the activation state of immunocompetent cells, using the monocytic cell line, MonoMac-6 (MM6), as a model.

We investigated the effect of each of the three honeys (manuka, pasture and jelly bush) on the release of important inflammatory cytokines from MM6 cells. These honeys, together with a sugar syrup control (artificial honey), were incubated with MM6 cells at a concentration of 1% (w/v) for 0–24 h. Cell culture supernatants were tested using specific ELISA assays for tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β and IL-6. All honeys significantly increased the TNF-α, IL-1β and IL-6 release from MM6 cells (and human monocytes) when compared with untreated and artificial-honey-treated cells ($P < 0.001$). Jelly bush honey significantly induced the maximal release of each cytokine compared with manuka, pasture or artificial honeys ($P < 0.001$).

These results suggest that the effect of honey on wound healing may in part be related to the stimulation of inflammatory cytokines from monocytic cells. Such cell types are known to play an important role in healing and tissue repair.

Keywords: Cytokine; Honey; Macrophage; Monocyte; Wound healing

1. Introduction

The use of honey in the treatment of wounds, burns and skin ulcers originated with ancient civilizations [1], but was superseded by modern dressings and antibiotic therapy [2]. However, the emergence of antibiotic-resistant strains of bacteria has confounded the current use of antibiotic therapy leading to the re-examination of former remedies. To date, the evidence to support the efficacy of honey in wound healing is largely anecdotal, but there are many claims that as a dressing on wounds, it reduces inflammation, debrides necrotic tissue, reduces oedema and promotes angiogenesis, granulation and epithelialization [3]. The anti-bacterial activity of honey has been established in previous studies [4,5], but the mechanisms by which further therapeutic effects are achieved, remain incompletely explained.

Normal wound healing is a complex process in which damaged tissue is removed and gradually replaced by restorative tissue during an overlapping series of events that include inflammation, cell proliferation and tissue remodelling [6]. During the early inflammatory phase, neutrophils infiltrate the injured region and remove cell debris, foreign material and bacteria by a combination of phagocytosis and proteolytic action. Later, macrophages dominate and as in phagocytosis, produce a number of specific mediators including reactive oxygen intermediates (ROIs), lipid metabolites and cytokines [7]. Generation of inflammatory mediators by cells present in wounds aid in controlling the intricate processes involved in wound repair [8]. Alterations in the levels of these mediators may lead to extended

[1] The work undertaken by these authors was performed at UWIC; however, their address has subsequently changed.

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inflammation, defective wound matrix and failure to re-epithelialise [9].

We have previously shown that co-culture of Mono-Mac-6 cells (MM6; a monocytic cell line and precursors of macrophages) with various honeys for 2 h reduced ROI production as well as increased the release of the potent pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α) [10]. What is not known is the long-term effect of honey on the release of pro-inflammatory mediators such as TNF-α and interleukin (IL)-1β as well as anti-inflammatory mediators, such as IL-6. IL-1β is an endogenous pyrogen capable of raising body temperature by inducing prostaglandin production. The increase in body temperature may be correlated with various modulatory aspects of the immune system including the acute-phase response [11]. TNF-α potentiates IL-1β production forming an autocrine loop, thereby escalating the levels of these cytokines [12]. TNF-α and IL-1β are, in part, responsible for the pathogenesis of many inflammatory diseases. IL-6 is considered to be an anti-inflammatory cytokine, inhibiting the generation of TNF-α and augmenting the actions of acute-phase proteins and immunoglobulins [13].

MM6 cells were established in the late 1980s, offering many advantages over the then available U-937 and THP-1 cell lines [14]. This monocytic cell line has been proven to be extremely useful because to varying degree, cells, either constitutively or after stimulus, express in vitro many of the properties manifested by their in vivo counterparts, mature peripheral blood monocytes and tissue macrophages.

This study was undertaken to determine the effects of honey on the release of TNF-α, IL-1β and IL-6 from MM6 cells and human monocytes isolated from peripheral blood. Effects on these important pro-and anti-inflammatory responses may serve to explain the observed wound-healing properties of honey.

2. Results

2.1. Cellular viability of cells pretreated with manuka, pasture, jelly bush or artificial honeys

Assessment of cellular viability performed by the exclusion of trypan blue demonstrated that greater than 90% of MM6 cells and human monocytes exposed to 1% (w/v) honey solutions were viable.

2.2. Endotoxin (lipopolysaccharide) content of honeys

Depending on cell type and culture conditions, lipopolysaccharide (LPS) can have a variety of effects on cell function and growth. There is substantial evidence indicating that nanogram concentrations of LPS may affect experimental outcomes by triggering direct responses from cells including the release of bioactive lipids, cytokines and the 'priming' of cells for enhanced function [15]. Therefore, all the honey solutions that were used were quantified for LPS. All the samples contained less than 0.7 ng/ml LPS (Table 1). In addition, control experiments showed that a known amount of LPS reconstituted in the syrup control had recovery levels of LPS similar to the same amount of LPS reconstituted in endotoxin-free water (Table 1). This suggests that the syrup control or honeys were not interfering with the limulus amoebocyte lysate (LAL) assay under these conditions.

Table 1
Quantification of LPS in honey solutions[a]

Samples	LPS (ng/ml)
1% Honey solutions (w/v)	
Manuka	0.056
Pasture	0.340
Jelly bush	0.690
Artificial honey (syrup control)	0.009
Control experiments	
0.15 ng/ml LPS reconstituted in sterile water	0.122
0.15 ng/ml LPS reconstituted in syrup control	0.130

[a] Data shown are representative of honey-batch testing.

2.3. Time-course effect of honeys on TNF-α, IL-1β and IL-6 production

All honeys at concentration 1% (w/v) induced or stimulated the release of TNF-α, IL-1β and IL-6 from MM6 cells when compared with the syrup control (artificial honey) and untreated cells (Figs. 1–3). TNF-α release peaked at 4–6 h following honey stimulation, while the release of IL-1β and IL-6 peaked at approximately 12–18 h. MM6 cells pretreated with jelly bush honey gave significantly higher ($P < 0.001$) cytokine release than the manuka and pasture-honey-treated cells. The control sugar syrup induced only baseline levels of TNF-α, IL-1β and IL-6 production (10 pg/ml), which were the same as those from untreated cells.

2.4. Effect of honey on TNF-α, IL-1β and IL-6 production in human peripheral blood monocytes

To extend the effects of honey on MM6 cells, a monocytic continuous cell line, the honey incubation times shown to stimulate maximal cytokine production were assessed in human monocytes isolated from peripheral blood. Treatment of these cells with honey at a concentration of 1% (w/v) stimulated a significant release of TNF-α, IL-1β and IL-6 (Fig. 4), while artificial honey solution did not stimulate the secretion of these cytokines. This response is similar to that

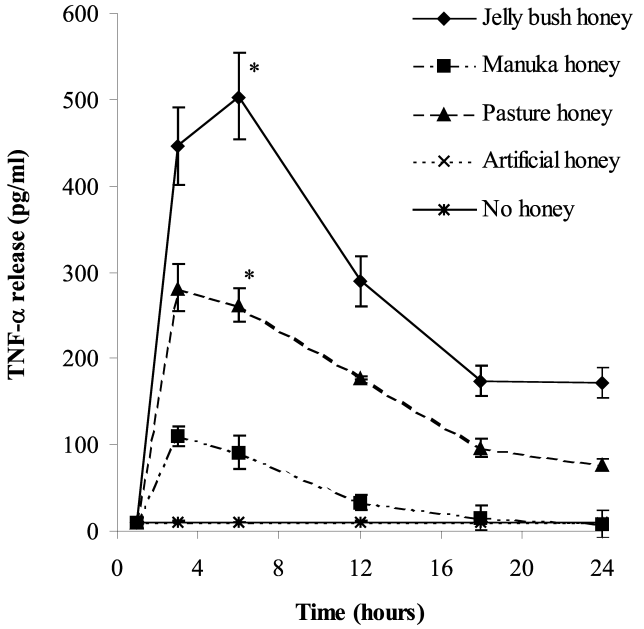

Fig. 1. Time-course effect of 1% (w/v) honeys on TNF-α release from MM6 cells. Results are expressed as mean ±SD. *$P < 0.001$ analysed by ANOVA and Tukey's pair-wise comparisons.

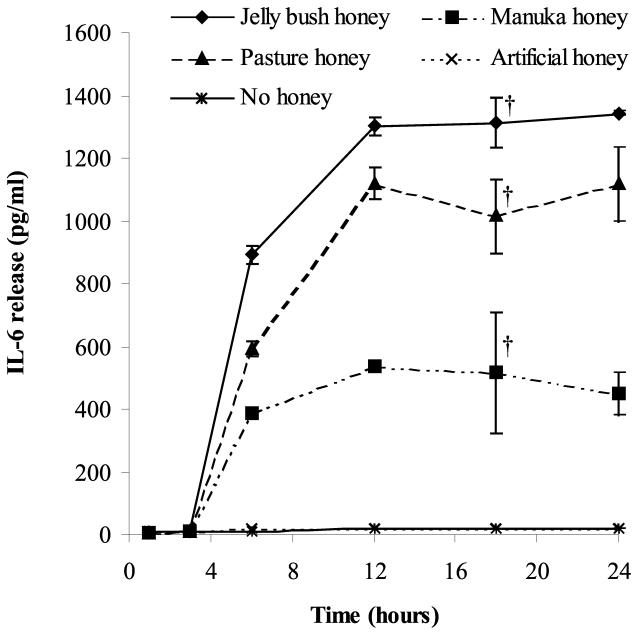

Fig. 3. Time-course effect of 1% (w/v) honeys on IL-6 release from MM6 cells. Results are expressed as mean ±SD. †$P < 0.05$ analysed by ANOVA and Tukey's pair-wise comparisons.

obtained from MM6 cells incubated with honey to stimulate the release of these cytokines.

3. Discussion

The data presented in this study indicate that the natural honey samples that were tested have a stimulatory effect with regard to the production of TNF-α, IL-1β and IL-6 by monocytic cells. The stimulatory effects of honey are unlikely to be an artefact of the MM6-cultured cell line, since the responsiveness appears to be a property endogenous to some inflammatory leukocytes including human primary monocytes isolated from peripheral blood. The cytokines involved are both pro- and anti-inflammatory. These effects are independent of

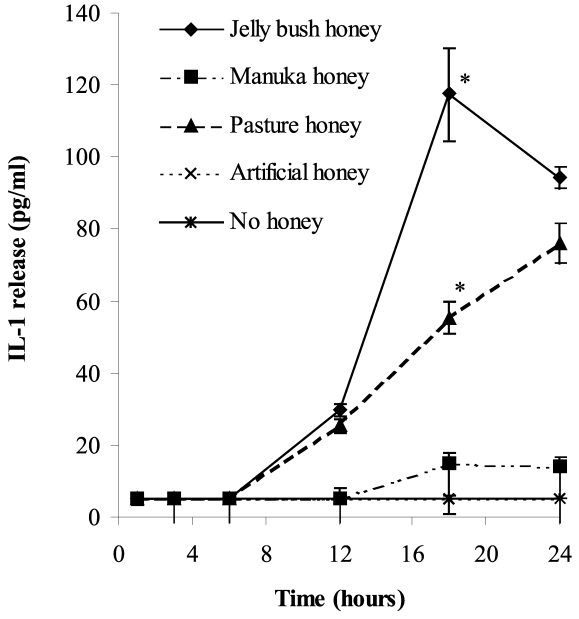

Fig. 2. Time-course effect of 1% (w/v) honeys on IL-1β release from MM6 cells. Results are expressed as mean ±SD. *$P < 0.001$ analysed by ANOVA and Tukey's pair-wise comparisons.

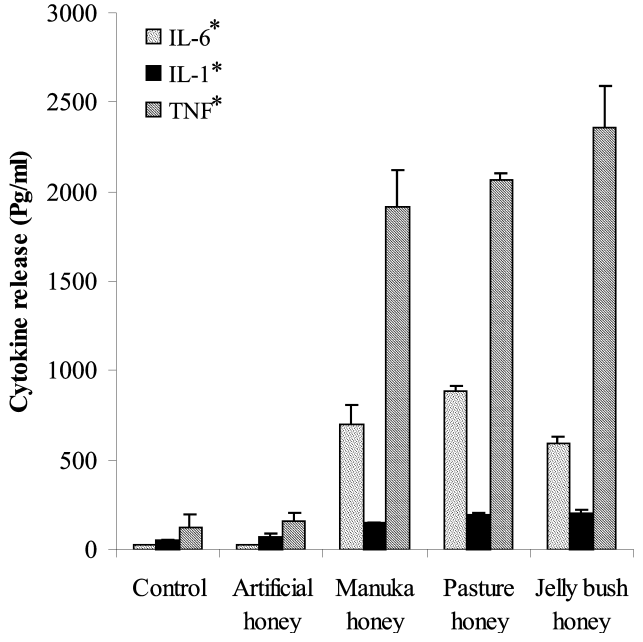

Fig. 4. Effect of 1% (w/v) honeys on TNF-α, IL-1β and IL-6 release from isolated human peripheral blood monocytes. Results are expressed as mean ±SD. *$P < 0.001$ analysed by ANOVA and Tukey's pair-wise comparisons.

endotoxin. The stimulation of monocytic cells within the wound environment is marked by the synthesis of cytokines that modulate the activity of many cell types that are intimately involved in the regeneration of tissue [16]. Following tissue injury, a co-ordinated cascade of inter-related events is initiated that normally culminates in wound closure, but diverse influences can interrupt this dynamic process leading to chronic inflammation and delayed healing [17]. Depletion of both macrophages [16] and T lymphocytes [18] is associated with impaired healing, as well as the presence of macrophages with suppressed activation at the margins of chronic wounds [18,19]. Macrophages are central to the regulation of cutaneous wound healing [8], and dressings that elicit macrophage activation [19,20] may generate a pro-inflammatory stimulus in non-healing wounds, which resolves the chronic inflammatory status towards cell proliferation and progression into the healing phase.

The role of TNF-α in wound healing has been the subject of considerable debate with suggestions by some that it may impair reparative processes and by others that it may serve to augment them [21]. TNF-α is a pleiotropic, pro-inflammatory cytokine, with the ability to affect almost every tissue and organ system. This pluripotent protein is chemotactic for macrophages and promotes macrophage activation. It stimulates angiogenesis [22], fibroblast proliferation [23] and the synthesis of prostaglandins and collagenase by fibroblasts [24]. It has also been suggested that the stimulation of TNF-α production may exert much of its beneficial effects by affecting the increasing levels of IL-6 [25]. IL-6 is a pleiotropic cytokine with significant impact on healing. It is mitogenic for keratinocytes [26,27], and its contribution to epithelialisation has been demonstrated in IL-6-deficient transgenic mice in which the wound-healing rates were retarded threefold compared with wild-type control mice [28]. In humans, decreased synthesis of IL-6 with age interferes with healing [29]. In the dermis, macrophages and fibroblasts produce low levels of IL-1 that functions by induction of KGF a potent keratinocyte mitogen. Interestingly, TNF-α also induces IL-6 production by keratinocytes, indicating an important synergistic relationship between these two cytokines [30]. Furthermore, IL-6 induces KGF-receptor expression in fibroblasts [31]; it also stimulates re-epithelialisation [16]. Both TNF-α and IL-1β stimulate the release of growth factors; these, in particular PDGF and TGF-β, are chemotactic for monocytes and fibroblasts and maintain the activity of these cells within the wound environment [16].

The observed effects on cytokine production were not seen in all honeys that were tested. The greatest effect was seen in the Australian jelly bush honey, with New Zealand manuka and pasture honeys also showing significant increases in cytokine compared with the sugar solution control levels, but at significantly lower levels than those seen in jelly bush. These results would suggest that the regulatory effects of honey are related to components other than the sugars present, although the identity of the actual component(s) that mediate these effects are as yet unknown. Further studies, on the isolation and characterisation of these agents, are required to further elucidate the mechanisms by which they exert their actions.

This study has demonstrated that the activity of monocytic cells intimately involved in the repair of wounded tissue is modulated by honey. The mechanisms by which honey affects the release of anti-inflammatory agents and growth factors from monocytic cells are as yet unclear, and this represents an area for further study. Whether honey affects other cell types, particularly endothelial cells and fibroblasts, involved in wound healing also needs to be clarified.

4. Materials and methods

4.1. Cell culture

The human monocytic cell line MM6 was obtained from the German collection of micro-organisms and cell cultures (DSM; Braunschweig, Germany). MM6 cells were maintained in RPMI 1640 medium without L-glutamine (Sigma-Aldrich Co. Ltd, Dorset, UK). Media were supplemented with 1% bovine insulin, 10% heat inactivated foetal bovine serum (FBS), 1% 2 mM L-glutamine, 1% non-essential amino acids, 1% penicillin (50 IU/ml)/streptomycin (100 μg/ml) and 1% sodium pyruvate (purchased from Gibco, Paisley, UK) at 37 °C in 5% CO_2-humidified atmosphere. Cells were subcultured every 3 days at a density of 0.4×10^6 cells/ml.

Human peripheral blood monocytes were isolated, purified and cultured as previously described [32]. Briefly, monocytes were isolated from buffy coats from normal donors whose blood were collected in sodium citrate test tubes. Buffy coats were mixed with Hanks Balanced Salt Solution (HBSS) at 1 : 1 ratio followed by Ficoll-hypaque centrifugation, $600 \times g$ for 40 min at RT (Sigma). The leukocyte-rich fraction (settled at the Ficoll-hypaque surface) was removed, washed twice in HBSS and finally re-suspended at 1×10^6 cells/ml in supplemented RPMI medium. Suspensions of 2 ml were plated in 35 mm Petri dishes (Falcon, BD, UK). Monocytes were purified by washing off the non-adherent cells after incubation for 120 min, at 37 °C in a 5% CO_2-humidified atmosphere. Monocytes were then cultured in supplemented RPMI media as MM6 cells.

4.2. Preparation of honey media

Two New Zealand honeys and one Australian honey were used: manuka honey (airborne f/d), a pasture

honey (Lorimers) and jelly bush honey. These were sampled from different batches of honey from known floral sources selected for their anti-microbial activity by a *Staphylococcus aureus* (ATCC 25923) inhibition assay; activity was equivalent to 18.0% (w/v), 13.7% (w/v) and 16% (w/v) phenol, respectively [33]. Artificial honey (100 g) was prepared by dissolving 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose in 17 ml of endotoxin-free distilled water. The desired amounts of manuka, pasture, jelly bush and artificial honey (w/v) were weighed and diluted in supplemented media. The honey solutions were made up to 1% (w/v) and rendered sterile by filtration (0.2 μm).

4.3. Endotoxin content of honeys

All the honeys and test samples used in this study were assayed for endotoxin content using the kinetic LAL assay (KQCL), purchased from Biowhittaker Ltd, Wokingham, UK. Care was taken at every stage of this assay to avoid contamination with endotoxin, i.e. endotoxin-free materials were used. The assay was performed according to the manufacturer's instruction. Briefly, endotoxin standard was reconstituted in pyrogen-free water and serially diluted. Each standard and test (50 μl) were performed in duplicate; pipetted into wells of a microtiter plate. The plate was placed in the KQCL reader and set to warm up to 37 °C. The LAL reagent was reconstituted by adding 2.6 ml of pyrogen-free water and to each well 50 μl of reconstituted LAL reagent was added. Readings were taken for 100 min. Unknowns were determined from the standard curve. A control experiment was also performed to determine whether the syrup control interfered directly with the LAL assay. A known amount of endotoxin (0.15 ng/ml) was reconstituted in endotoxin-free water or syrup control (see the preparation of artificial honey in Section 4.2) and the concentration of LPS was determined.

4.4. Cellular viability of cells pretreated with honey

MM6 cells at a density of 1×10^6 cells/ml were preincubated with 1% (w/v) of each honey solution for 0–24 h. The cells were washed in PBS (×3) and re-suspended in 1 ml of fresh media. Assessment of cellular viability was performed by exclusion of the vital dye, trypan blue.

4.5. Measurement of TNF-α, IL-1β and IL-6 release from MM6

To determine the effect of honey alone on cytokine release, MM6 cells prepared at a concentration of 1×10^6/ml, were incubated with manuka, pasture, jelly bush or artificial honey solutions of 1% (w/v) for 0–24 h at 37 °C in 5% CO_2 atmosphere. Following honey incubation, supernatants were collected. TNF-α, IL-1β and IL-6 in MM6 cell culture supernatants were quantified by a commercially available ELISA kits (Diaclone Research, France) according to the manufacturer's instruction. The assay was replicated five times.

4.6. Measurement of TNF-α, IL-1β and IL-6 release from human peripheral blood monocytes

Isolated human peripheral blood monocytes were incubated with manuka, pasture, jelly bush or artificial honey solutions of 1% (w/v) for 6 h (TNF-α release) or 18 h (for IL-1 and IL-6 release) at 37 °C in 5% CO_2 atmosphere. Cell culture supernatants were quantified as previously described. The assay was replicated three times.

4.7. Statistics

For multiple group comparisons, the data were subjected to one-way analysis of variance (ANOVA) to determine overall difference between the group means and Tukey's honestly significant difference for pair-wise differences for within group comparisons. Minitab software version 12.0 (Minitab Inc.) was used for all analyses.

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