Original Article

Quantitative analysis of dermal scars in deep skin burns treated with Ulmo honey supplemented with ascorbic acid

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Abstract: Successful repair of burn wounds requires not only the participation of cells, such as granulocytes and fibroblasts, but also non-cellular elements including collagen, which plays a crucial role as a structural and regulatory molecule of scar tissue. Given that honey and ascorbic acid have presented great therapeutic potential at a cellular and structural level, experimental studies have proposed to combine them for the treatment of wounds. The aim of this study was to evaluate the morphoquantitative characteristics of polymorphonuclear cells (PMN), fibroblasts and types I and III collagen in burn wounds treated with Ulmo honey (Eucryphia cordifolia) supplemented with ascorbic acid. Fifteen healthy adult guinea pigs (Cavia porcellus) were divided into three groups: Positive Control (C+), experimental non-supplemented (H), and experimental supplemented (SH) Ulmo honey. The animals were treated daily until biopsies were performed on day 10 of treatment. Number density per area (N_A = mm^-2) and volume density (V_V = %) of PMN; N_A, V_V and surface density (S_S = mm^-1) of fibroblasts, and types I and III collagen were evaluated. A one-way ANOVA was used (P < 0.05). Although all groups were in a proliferative stage, the SH group showed morphoquantitative characteristics suggestive of more advanced healing processes with lower stereological PMN values (N_A = 228.823±174.883 and V_V = 1.050±0.940). However, N_A (4252.025±1041.751), V_V (20.400±5.897) and S_S (100.876±29.431) of fibroblasts and types I and III collagen content were higher in this group (P < 0.001). We conclude that burn wounds treated with Ulmo honey supplemented with ascorbic acid show a more effective healing process than non-supplemented honey.

Keywords: Ascorbic acid, collagen content, healing, Ulmo honey, stereology

Introduction

Wound healing is a physiological response to injury, where damaged structures are replaced and skin integrity is restored. Generally, the healing process can be divided into three stages: inflammatory, proliferative, and remodeling. Burn wounds clearly exhibit these stages and are thus an excellent experimental model [1].

Skin repair is a complex process that requires the highly coordinated activity of various cells, including polymorphonuclear cells (PMN), macrophages, lymphocytes, fibroblasts, endothelial cells and keratinocytes. The inflammatory cell stage begins with the appearance of neutrophils, as a main source of TNF-α and interleukins. These cells are replaced by macrophages and together with fibroblasts and keratinocytes are considered key in the progression from the inflammatory to the proliferative stage [2]. The proliferative stage is characterized by the formation of new blood vessels and dermal fibroplasia, as well as the beginning of wound epithelialization. During the remodeling stage, type III collagen, present during proliferation, degrades and the more resistant type I collagen is deposited [3, 4].

Collagen is critical in the healing process for scar tissue maintenance, elasticity, and tensile strength, whereas collagen types I and III serve...
Evaluation of dermal scars in deep skin burns treated with supplemented honey

As structural and regulatory molecules [5]. Type III collagen that has accumulated in the wound bed influences both migration and epithelial proliferation during the repair process [2], as well as participates in hemostasis in early tissue granulation [6], thereby influencing platelet aggregation and signaling activity [7]. In contrast, fibroblasts are essential for the production of collagen fibers and extracellular connective tissue components.

To achieve rapid establishment of damaged tissue integrity and reduce the development of fibrosis and hypertrophic scars, application of new therapeutic agents to modulate the healing process, including those which are naturally occurring, are of the utmost importance. In this context, numerous studies have focused on demonstrating that the biological and physical properties of honey are highly effective in treating wounds of various etiologies [8]. These properties act at all healing stages [9]. However, its effect manifests significantly in the first two stages due to its antioxidant, anti-inflammatory, and antibacterial properties [10], revealing its ability to debride necrotic tissue, reduce swelling, and promote angiogenesis, granulation, and epithelialization [11]. The therapeutic potential of honey has been observed in immunohistochemical and molecular expression studies, involving traumatic wound biopsies that fail to respond to conventional topical antibiotics. Gradual increases in p63 proteins (keratinocyte marker) and E-cadherin (cell surface glycoprotein responsible for cell-to-cell adhesion) demonstrate how honey promotes healing. It also assists in the deposition of collagen in the dermis during healing, causing the ratio of types I and III collagen to be similar to that observed in healthy biopsies [12]. Furthermore, it has been shown that honey is capable of stimulating fibroblast growth in culture due to the principal content of sugar (fructose and glucose).

Ascorbic acid also contains properties that contribute significantly to wound healing processes, showing great benefits in burn wounds that produce intense oxidative stress [13]. Studies have shown that ascorbic acid accelerates healing, stimulates fibroblast proliferation, develops the epidermal base membrane, and improves wound contraction and scar tissue mechanical traction strength [14]. On the other hand, ascorbic acid is known for its activity in the collagen biosynthetic pathway [15]. Recent experimental animal studies have suggested that enhancing the healing effect of honey can be achieved by combining it with ascorbic acid. The effectiveness of topical Ulmo honey and oral ascorbic acid in the treatment of venous ulcers has also been reported in humans [16], and has succeeded in strengthening the healing and contraction effects in burn wounds compared to non-supplemented honey [17].

If it is considered that fibroblasts and PMN are important to skin repair, and types I and III collagen are the major dermis collagen expressed, this study aims to evaluate the morphoquantitative characteristics of PMN, fibroblasts and types I and III collagen content in the scar tissue of burn wounds treated with Ulmo honey (Eucryphia cordifolia) supplemented with ascorbic acid. This study also compared its effect with non-supplemented Ulmo honey on guinea pigs (Cavia porcellus).

Material and methods

Ulmo honey

Samples of Ulmo honey were harvested from various apiaries in the Valdivian forest of Southern Chile. The honey was centrifuged and stored in high density polyethylene (PE-HD) amber bottles to preserve their biological properties and stored at 4°C. The monofloral Ulmo honey was identified by the melissopalynology method [18], using light microscopy analysis as per NCh2981. The honey was then sterilized by gamma irradiation at 25 kGy in the Laboratory of Radio-sterilized Biological Tissues Processing (LPTR) at the Chilean Commission of Nuclear Energy (CCHEN). This selected Ulmo honey, supplemented with ascorbic acid, is under a license, so the formulation cannot be disclosed here. The supplemented Ulmo honey will take the name of the Ulmoplus® prototype, developed within the CORFO project 13IDL2-23290, from the Corporation for the Promotion of Production, Ministry of Economy, Chile. The physical and chemical properties of Ulmo honey and supplemented Ulmo honey used in this study have been described previously [17]. The chemical formulation of supplemented Ulmo honey is licensed as Ulmoplus® developed within the project CORFO 13IDL2-23290, from the Corporación de Fomento de la Producción (CORFO), Ministerio de Economía, Gobierno de Chile.
Evaluation of dermal scars in deep skin burns treated with supplemented honey

Animals

Guinea pigs (Cavia porcellus) were used as animal models, as their metabolism is dependent on ascorbic acid [19] and they are indicated as experimental animals to evaluate burn wounds. These animals maintain consistent skin thickness, ideal for experimental studies on healing burn wounds [20].

Fifteen healthy adult animals were used, of both sexes, averaging 450 g in weight and acquired from the Center of Excellence in Morphological and Surgical Studies (CEMyQ) at the Universidad de La Frontera, Temuco, Chile. Subjects were randomly divided into three groups: positive control (C+), treated with active advanced dressings of Hydrogel-Tulle [21], non-supplemented Ulmo honey (H), and Ulmo honey supplemented with ascorbic acid (SH). Their diet was pellets supplemented with ascorbic acid and water ad libitum under controlled temperature conditions and controlled noise and light cycles (12:12 hr light-darkness). Experiments were carried out in accordance with the Protocol for the Daily Supervision of Animals from the Guide to Bioethical Aspects of Animal Experiments [22]. The Scientific Ethics Committee of the Universidad de La Frontera approved the experimental protocol.

Wound model

For the wound healing study, each animal was burned according to the protocol described by Schencke et al., [17] under intraperitoneal anesthesia, applying a mixture of ketamine (40 mg/kg), xylazine (5 mg/kg), and atropine (0.05 mg/kg). The burns were treated by applying tepid physiological saline solution by syringe at a distance of 10 cm from the lesion; gauze with Hydrogel and Tulle dressings were applied to the C+ group, gauze with non-supplemented Ulmo honey to the H group, and gauze with Ulmo honey supplemented with ascorbic acid to the SH group. All animals were treated and evaluated daily until biopsies were performed on day 10 of treatment. This day was selected as it represents the proliferative stage of wound healing in previous studies, in which all analyzed elements can be observed [17].

Biopsies and staining

Biopsies were performed with a puncher 1 cm in diameter (for healthy wound margins) to reach the deep dermis. The biopsies were washed with NaCl 0.9%, fixed in buffered formalin (1.27 mol/L of formaldehyde in a phosphate 0.1 M buffer pH 7.2) at 10% for 48 hours, dehydrated and soaked in Paraplast Plus (Sigma-Aldrich Co., St. Louis, MO, USA). Once the blocks were obtained, a series of cuts 5 µm thick were made in each block. Five cuts were made at random (Leica® RM2255 microtome), stained with H&E for subsequent stereological analysis, and Sirius Red for evaluation of collagen fiber content. For the Sirius Red method, type I collagen was observed as thick fibers, strongly birefringent, red or yellow, while type III collagen was observed as thin fibers, weakly birefringent and greenish [23].

Stereological analysis

Five fields were observed for each cut; 125 fields in total per group [24]. Slides were observed through a Leica® DM750 optical microscope 100X objective zoom lens, recorded with a digital camera (Leica® ICC50 HD) and displayed on a View Sonic® LCD monitor. The M42 multipurpose testing system was used for stereology. The parameters measured were number density per area (N), and volume density (V), of polymorphonuclear cells (PMN); N, V, and surface density (S) of fibroblasts on scar tissue. The number density per area of PMN (NPMN) was determined according to the following equation: NPMN = N/A, where N is the number of observations in a given area considering the forbidden lines, A is the total system area (36.36 x d²) and d is known length of the test line system. The volume density of PMN (VPMN) was estimated using the following equation: VPMN = PPMN/Pt (100%), where PPMN is the number of points touching PMN and Pt the total number of points in the system. In addition, the number density per area (NFibroblasts) and the volume density of fibroblasts (Vfibroblasts) were determined as described prior. The surface density of fibroblasts (Sfibroblasts) was evaluated according to the equation Sfibroblasts = 2 × I/L, where: I is the number of intersections that touch the structure, and L is the total line length of the M42 testing system [25].

Collagen fiber content evaluation

Five fields were observed for each cut; 125 fields in total per group [24]. The lower filter (Leica, Wetzlar, Germany) was placed above the microscope’s field iris diaphragm ring, while
Evaluation of dermal scars in deep skin burns treated with supplemented honey

Table 1. Stereological analysis on dermis treated with Hydrogel-Tulle (C+), non-supplemented honey (H) and supplemented honey (SH)

<table>
<thead>
<tr>
<th>Variable</th>
<th>C+</th>
<th>H</th>
<th>SH</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nₐ PMN (mm²)</td>
<td>211.22±160.100</td>
<td>756.87±196.620</td>
<td>228.82±174.883</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Vᵥ PMN (%)</td>
<td>0.61±0.290</td>
<td>2.70±1.370</td>
<td>1.05±0.940</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nₐ fibroblasts (mm²)</td>
<td>3599.56±764.461</td>
<td>3355.33±699.443</td>
<td>4253.02±1041.751</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Vᵥ fibroblasts (%)</td>
<td>16.32±7.719</td>
<td>17.48±6.041</td>
<td>20.40±5.897</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sᵥ fibroblasts (mm⁻¹)</td>
<td>81.67±28.883</td>
<td>81.67±29.781</td>
<td>100.87±29.431</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Nₐ, number density per area; Vᵥ, volume density; Sᵥ, surface density; PMN, polymorphonuclear cells. aSignificance (P < 0.05) different from Group C+. bSignificance (P < 0.05) different from Group H.

Figure 1. Presence of type I and III collagen fibers in dermal scar. Type I (red and yellow) and III (green) collagen fibers in the dermal scar of burn wounds treated with (A) Group C+, Hydrogel-Tulle; (B) Group H, non-supplemented Ulmo honey; (C) Group SH, supplemented Ulmo honey, in guinea pigs (Cavia porcellus) on day 10 of treatment. Sirius red.

the upper filter was constructed from a combination of quarter-wave plates (Leica, Wetzlar, Germany) placed below a linear polarizer, aligned with the transmission axis at 45° to the fast axis of the wave plate [26]. These two filters were aligned to maximize darkness in the background (i.e., the filters were crossed). Tissue images were obtained with a Leica® DM750 microscope 100X objective lens, recorded on a digital camera (Leica® ICC50 HD), and analyzed using Image-Pro Premier 9.1 (Media Cybernetics, Warrendale, PA, USA) software. Collagen content was calculated from each image as the area (A) occupied by types I and III collagen fibers on scar tissue (expressed in µm²).

Statistical analysis

The statistical analysis was performed using IBM’s SPSS Statistic 21© software and assumptions were verified through the Kolmogorov-Smirnov test (data normality test) and Levene’s test (homoscedasticity analysis). A one-way analysis of variance (ANOVA) and Tukey’s HSD or Dunnnett’s T3 post hoc tests were used to analyze the differences between groups (P < 0.05). The P < 0.05 (*) were considered significant and P < 0.025 (**) very significant.

Results

Stereological analysis

In the scar dermis, C+ and SH groups showed an advanced proliferative phase, and the H group showed an initial proliferative phase. The values of Nₐ, Vᵥ for PMN were higher in the H group compared to the SH and C+ groups. The C+ group obtained the lowest values of Vᵥ and Sᵥ of fibroblasts (Table 1). The ANOVA of the stereological analysis on the PMN and fibroblasts performed on scar tissue of the burn wounds of the C+, H and SH groups showed at least one group which differed from the others (P < 0.05). The post hoc tests showed statistically significant differences between the C+ and the SH groups (P < 0.05).

Quantification of collagen fibers

The presence of types I and III collagen fibers in the dermal scar can be observed in Figure 1.
and the collagen fiber content types are presented in Figure 2.

The results show variation in the collagen fiber content of types I and III for each group ($P < 0.05$). The content of type I collagen in the dermal scar was higher in the SH group compared to the H group ($P < 0.001$); however, the highest value was observed in group C+ ($P < 0.001$). The content of type III collagen was higher for the SH group showing differences with groups H and C+ ($P < 0.001$).

Discussion

Burn wounds treated with honey have been evaluated in various researches for both human [8, 16] and animal subjects [9, 11]. Most of these studies have focused on performing descriptive histological analyses where the therapeutic properties of honey on tissue repair are evaluated during the healing stages. However, there are few morphoquantitative studies with accurate results with respect to changes generated in tissue repair components. The application of quantitative methods, such as stereology, is advantageous in this type of study. Stereological analyses of PMN and fibroblasts in scar tissue showed an important correlation with the histopathological characteristics observed previously [17], facilitating the understanding of the observations. On day 10 of treatment, PMN presented higher $N_{a}$ (756.876±196.620 mm$^2$) and $V_{v}$ (2.700±1.370%) values in the H group ($P < 0.001$), correlated with proximity to the inflammatory stage, because at this stage PMN are one of the most abundant cells in the wound area.

Studies have validated the properties of honey [8, 11], ascorbic acid [3, 13, 14] and their combination [16] in wound healing. Honey accelerates the healing process, therefore the presence of PMN that are necessary in the inflammatory stage, rapidly decreases, leading to the proliferation of fibroblasts and epithelial cells at the wound site [27]. Our results show that supplemented honey accelerated the healing process more efficiently than non-supplemented honey. Possible explanations are related to the inflammatory phase, where ascorbic acid is also required for timely neutrophil apoptosis and clearance [28] and during the proliferative phase, where it interacts with the integral processes of collagen synthesis, maturation, and degradation [29]. Along with this, a daily dose of ascorbic acid is not enough for the requirements of burn wound healing due to severe oxidative stress. Consequently, the literature indicates that this vitamin is oxidized by active PMN presenting rapid expenditure during infection and phagocytosis during the inflammatory response [30]. In this context, clinical and experimental evidence shows benefits of high doses of ascorbic acid for the restoration of endothelial function in patients with severe burns [13] and can ensure adequate levels of antioxidants to protect cells and tissues from oxidative damage produced in the inflammatory phase of wound healing [31]. Moreover, researchers have reported that concentrations 10 to 50 times higher than the normal ascorbic acid level then stimulate random migration and in vitro chemotaxis of human PMN without influencing phagocytic capacities [32].

In terms of collagen fiber content, the literature indicates that the increase may be attributed to
honey containing essential amino acids such as arginine and glutamic acid. These materials help supply the proline precursor for collagen synthesis, and also contain sugars that provide the necessary energy for fibroblast metabolism and collagen synthesis. Also present are iron, copper, and ascorbic acid, which are essential for prolyl and lysyl hydroxylase enzymes, promoting hydroxylation and fiber crossing [33].

Furthermore, ascorbic acid improves wound healing as it significantly increases hydroxyproline tissue levels, promotes neovascularization, fibroblast maturation, and collagen deposition and improves the conversion of procollagen to collagen [34]. Ascorbic acid is responsible for the activation of the prolyl hydroxylase enzyme that catalyzes the hydroxylation of proline, a necessary component in stabilizing the triple helix of the collagen molecule [35]. In addition, it enhances the mRNA level of collagens I and III in fibroblast [36].

Our results positively correlate with the above, since the evaluation of collagen fiber content in the SH group demonstrated a significant increase for type III collagen fiber production, when compared with the C+ and H groups. Also, this group showed higher values in the content of type I collagen fibers compared to the H group. These results suggest that Ulmo honey supplemented with ascorbic acid have synergistic effects on the production of dermal collagen fibers. These results become relevant, not only because wounds heal more quickly when treated with supplemented honey, but also because the development of fibrosis and hypertrophic scars is reduced. The intense production of type III collagen observed in the SH group is a necessary step for cell migration and the regulation of type I collagen [7]. These results are positively correlated with recent studies which showed that supplemented Ulmo honey with ascorbic acid enhanced the healing effect and contraction of wounds caused by burns, validating the synergy between the two compounds [17]. Additionally, the greater presence of fibroblasts in this group could favor TGF-β action, creating greater collagen fiber alignment [37], which could be demonstrated in later studies that measure the presence of TGF-β activity in healed dermis treated with supplemented honey.

Moreover, it is worth mentioning that honey contains phenols such as flavonoids, which can protect ascorbic acids from oxidation [38]. This protective ability is important as ascorbic acid is highly unstable when manipulated and requires additional care. In this context, the qualities of honey make it an excellent receptor of this vitamin, allowing proper synergy between the two substances, and achieving excellent results in the wound healing process.

It is important to consider that the process of healing burn wounds requires an adequately moist environment, which is provided by honey as a topical treatment [39]. However, this ideal environment appears not to be provided by the topical use of ascorbic acid alone, as the vast majority of studies that used this vitamin to treat burn wounds administered it oral or intravenous way [4].

Conclusions

In summary, we conclude that in burn wounds, Ulmo honey supplemented with ascorbic acid can accelerate the healing process more efficiently than non-supplemented Ulmo honey. Adjuvant properties of ascorbic acid added to the Ulmo honey are reflected in lower PMN values and higher values of fibroblasts and types I and III collagen fiber contents in the scar tissue.

Morphoquantitative studies like ours are needed to understand more advanced stages of the healing process when considering the therapeutic properties of honey and ascorbic acid in relation to the quality of the formed scar. Hypertrophic burn scars are the most common complication presented in burn wounds and can limit the patient’s ability to function while also affecting self-image.

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Disclosure of conflict of interest

None.
Evaluation of dermal scars in deep skin burns treated with supplemented honey

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Evaluation of dermal scars in deep skin burns treated with supplemented honey


