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Ulmoplus® Increases FGF-2 Expression and Promote Burn Wound Healing

Ulmoplus® Incrementa la Expresión de FGF-2 y Promueve la Cicatrización de Heridas por Quemadura

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SUMMARY: The role of fibroblast growth factor (FGF) in the wound repair process has been described, particularly during the stimulation of angiogenesis in the proliferative phase. Ulmo honey (*Eucryphia cordifolia*) has demonstrated important bactericidal, fungicidal, antioxidant and healing properties. Ulmoplus®, a honey-based medicinal product, accelerates healing time and promotes fibroblast activation and collagen fiber production. The aim of this study was to evaluate the immunohistochemical expression of endogenous FGF-2 in burns treated with Ulmoplus® *vs.* the gold standard (hydrogel-tull), and Ulmo honey as the first step to clarifying the regulatory mechanism of this therapeutic option. 15 adult guinea pigs (*Cavia porcellus*) were used, divided into three groups: C+: positive control treated with hydrogel-tull (gold standard); E1: experimental group treated with Ulmo; and E2: experimental group treated with Ulmoplus®. A deep uniform burn was made under anesthetic that covered 1 cm^2 of the skin on the back of each animal. The daily treatments were administered with hydrogel-tull, Ulmo honey or Ulmoplus®, where appropriate. On treatment day 10 the biopsies were taken and processed for histological and immunohistochemical analysis. The immunolabeling was quantified through an integrated optical density (IOD) analysis, expressed as lum/μm^2 using the Image-ProPremier 9.1 software. The endogenous expression of FGF-2 was consistent with the stage of healing presented by the study groups, varying in its localization and IOD intensity. The E2 group presented a significantly higher IOD (60053.55 ± 59877.84 lum/μm^2) than the C+ ($p = 0.001$) and E1 ($p = 0.001$) groups. Our findings indicated that the medicinal Ulmoplus® honey is effective at facilitating wound closing, and the promoter effect of FGF-2 expression accelerated the healing process compared to the treatments with hydrogel-tull (gold standard) and Ulmo honey.

KEY WORDS: Fibroblast growth factor; Burns, Honey; Wound healing.

INTRODUCTION

Honey has been used in folk medicine since ancient times (Kattan *et al*., 2016), and its use in wound treatment is being rediscovered (Yaghoobi & Kazerouni, 2013). Clinical observations indicate that the use of honey is not limited only its antibacterial action (Almasaudi *et al*., 2017), but also to its anti-inflammatory (Devasvaran & Yong, 2016), antioxidant (Ahmed *et al*., 2018) and regenerative capacities, accelerating the wound healing process (Martinotti *et al*., 2019).

The wound healing process involves coordinated efforts of several cell types, including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets, being executed and regulated by cytokines and a network of growth factors, such as: platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Mast & Schultz, 1996). This last one includes 23 different isotypes; however, after a skin injury only FGF 1, 2, 7, 10 and 22 are expressed (Traversa & Sussman, 2001; Barrientos *et al*., 2008).

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The molecular, cellular, and biochemical events involved in the healing process have been conveniently divided into three phases that continuously and temporarily overlap: the inflammatory phase, the proliferative phase, and the remodeling phase (Ganapathy *et al*., 2012; Ozgok Kangal & Regan, 2021). FGF-2, also known as basic fibroblast growth factor (bFGF) is expressed in all the healing phases (Desmouliere *et al*., 1995; Akasaka *et al*., 2004; Demidova-Rice *et al*., 2012a) and plays a crucial role in the healing process by promoting the proliferation of fibroblasts, inducing neovascularization, increasing collagenase synthesis and contributing to the synthesis and remodeling of the extracellular matrix (Hayek *et al*., 1987; Tsuboi & Rifkin, 1990; Gospodarowicz, 1991; Tassi *et al*., 2001; Werner & Grose, 2003; Braun *et al*., 2004; Xie *et al*., 2008; Demidova-Rice *et al*., 2012b).

To gain a good healing action, the selection of honey according to its botanical origin has enabled the development of certified and authorized medical products for the treatment of wounds in Europe and Australia, producing Bio18+ Manuka Honey, Medihoney® and L-Mesitran®, among others (Lee *et al*., 2011). Ulmo honey (*Eucryphia cordifolia*) from southern Chile has demonstrated significant bactericidal, fungicidal, antioxidant (Montenegro *et al*., 2013; Acevedo *et al*. 2017) and healing properties (Schencke *et al*., 2016), reducing the risk of infection and swelling (Sherlock *et al*., 2010). Its effect on wound healing manifests significantly in the two first stages of healing due to its antioxidant, anti-inflammatory and antibacterial properties, to its capacity to debride necrotic tissue, and to its promotion of angiogenesis, granulation and epithelization (Schencke *et al*., 2013). This is reigniting interest in its use in the clinical care of venous ulcers (del Sol Calderón *et al*., 2015).

Supported by previous studies (Subrahmanyam, 1996; Schencke *et al*., 2013; del Sol Calderón *et al*.), a medicinal product use based on Ulmo honey supplemented with ascorbic acid was developed, called Ulmoplus®, the results of which surpassed the gold standard treatment (hydrogel-tull), accelerating healing time as it promotes fibroblast activation, collagen fiber production, and the formation of basement membrane and granulation tissue (Schencke *et al*., 2015, 2016; Muñoz *et al*., 2018).

Considering the latest advances in research on the effects of native honey in the wound healing process, the aim of the present study was to evaluate the immunohistochemical expression of endogenous FGF-2 in burns treated with Ulmoplus® *vs.* the gold standard treatment (hydrogel-tull) and Ulmo honey as the first step to explaining the regulatory mechanism of this therapeutic option.

MATERIAL AND METHOD

Native Honey. Samples of native Ulmo honey (*Eucryphia cordifolia*) were collected from hives in the Valdivian Forest of southern Chile. Then, the samples were centrifuged and stored at 4 °C in high-density polyethylene (HDPE) amber bottles to preserve their properties. Pollen was identified by melissopalynological analysis (Montenegro *et al*., 2008) using optical microscopy as per standard Nch2981-Of2005. The selected honey had the NHF (Native Honey Factor) seal, which certifies significant levels of antibacterial activity (Fredes *et al*., 2013). Finally, it was supplemented with acid ascorbic and sterilized by gamma irradiation at 25 kGy in the Biological Tissue Processing Laboratory of the Chilean Nuclear Energy Commission (CCHEN). This honey for medicinal use is licensed under Ulmoplus®, being developed with the support of the Chilean Production Development Corporation, CORFO, (13IDL2-23290), and protected by trade secret (RS N°3654).

Animals. For the in vivo study, 15 healthy adult guinea pigs (*Cavia porcellus*) of both sexes were used, with an average weight of 450 g, taken from the Center of Excellence in Morphological and Surgical Studies (CEMyQ) at the Universidad de La Frontera, Temuco, Chile. Guinea pigs were used due to their ascorbic acid-dependent metabolism (Krámer *et al*., 1979), and because they are a good model for experimental studies on wound healing since their skin maintains a constant thickness (Kaufman *et al*., 1990; Andrades *et al*., 2004). The animals were divided randomly into three groups (n=5): (C+) positive control treated with active advanced hydrogel-tull dressings (gold standard); (E1) experimental group treated with selected Ulmo honey that maintained the same standard as that used in the formulation; and (E2) experimental group treated with the Ulmoplus® formulation. The animals were housed at the Animal Facility and maintained under a 12-h light-dark cycle (08:00 am to 08:00 pm), with a pellet-based diet supplemented with ascorbic acid and water *ad libitum*, and a constant room temperature (23 ± 1 °C). This experimental protocol was approved by the Scientific Ethics Committee of the Universidad de La Frontera, Temuco, Chile (DI13-0044).

Experimental model. For the study of wound healing in an animal model, a burn injury was produced according to the protocol described by (Schencke *et al*., 2016). The work was done under intraperitoneal anesthesia, applying a mixture of ketamine (40 mg/kg), xylazine (5 mg/kg) and atropine (0.05 mg/kg). The animals were treated daily with therapies that consisted of a cleaning with lukewarm physiological serum via a syringe 10 cm from the wound

and the application of a gauze impregnated with hydrogel-tull (C+), honey (E1) or Ulmoplus® (E2), where appropriate. Daily evaluations were done until the extraction of the biopsies on treatment day 10. This day was selected because it represents a proliferative stage of wound healing, ideal for the analysis of the growth factor being studied (Robson *et al*., 1998; Cañedo-Dorantes & Cañedo-Ayala, 2019).

Biopsy and histological processing. Punch biopsies of 1 cm^2 were obtained, until the deep dermis was reached. The samples were washed with NaCl 0.9 %, fixed in buffered formalin (1.27 mol/L of formaldehyde in phosphate buffer 0.1 M; pH 7.2) at 10 % for 48 hours, dehydrated and embedded in Paraplast-Plus (Sigma-Aldrich Co., St. Louis, MO, USA). Serial sections 5 µm thick were made (Leica® RM2255) which were mounted on slides with adherent. For the histological analysis, the sections were stained with Van Gieson's collagen staining. The slides were viewed under an optical microscope (Leica® DM 750) and photographed with a digital camera (Leica® ICC50 HD).

Immunohistochemistry. For each histological section, antigenic recovery was performed with HistoReveal (ab103720, Abcam, Cambridge, UK) for 20 minutes at room temperature, and then they were treated with 3% H_2O_2 (v/v) (107209, Merck, Darmstadt, Germany) in PBS for 30 minutes to block the activity of endogenous peroxidase. Each washing was done with PBS. First, the sections were incubated with anti-FGF-2 IgG sheep primary antibody (ab64207, Abcam, Cambridge, UK), dilution 1:200 in PBS for 90 minutes at 37 °C. After washing with PBS, the sections were incubated with anti-sheep IgG rabbit secondary antibody (ab6746, Abcam, Cambridge, UK), dilution 1:500 in PBS. Then, the sections were incubated with streptavidin peroxidase (ab64269, Abcam, Cambridge, UK) for an hour at room temperature. Finally, diaminobenzidine-peroxidase (ab94665, Abcam, Cambridge, UK) was used for the visualization, and nuclear counterstain was done with Harris hematoxylin. The slides were observed under a light microscope (Leica® LED750) and photographed (Leica® ICC50W). For each immunohistochemical reaction, negative controls were used, which were incubated in PBS, omitting the primary antibody (Cell MarqueTM, Rocklin, CA, USA). The immunolabeling was quantified through an integrated optical density (IOD) analysis, expressed as lum/μm^2, using the Image-ProPremier 9.1 software (Media Cybernetics, Warrendale, PA, USA).

Statistical analysis. The quantitative differences in immunolabeling were evaluated using the Kolmogorov-Smirnov test (analysis of normality of the data) and Levene's test (homoscedasticity of the variances). The differences between the groups were analyzed with a one-way ANOVA, followed by Tukey's post hoc HSD test or Dunnett's T3 test, as appropriate. The value $p < 0.05$ (*) was considered statistically significant, whereas $p < 0.025$ (**) was considered very significant (IBM SPSS Statistics, Version 21, IBM Corp., Armonk, NY, USA).

RESULTS

Histology. The C+ group presented an initial proliferative phase, with fibroblastic reaction on treatment day 10. The epidermis regenerated in 50 % of the cases, being in a state

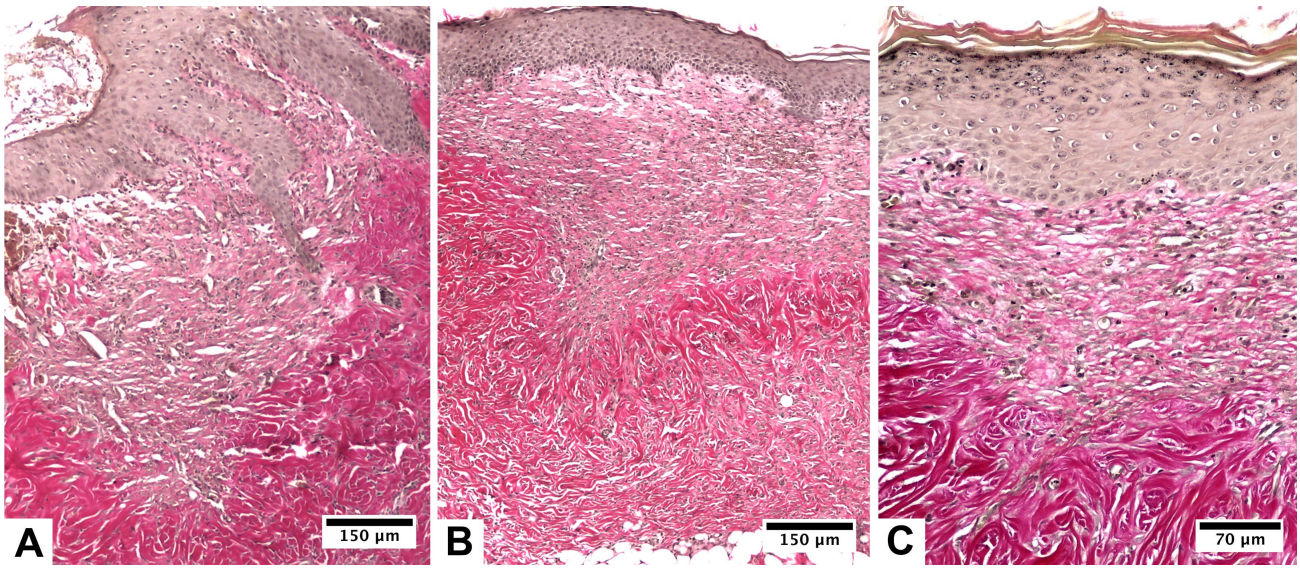

Fig. 1. Biopsies of the burn wound healing area in guinea pig skin (*Cavia porcellus*) on treatment day 10. (A) C+ group: hydrogel-tull. (B) E1 group: native Ulmo honey. (C) E2 group: Ulmoplus® medicinal honey. Van Gieson's collagen staining.

of differentiation. In the healing dermis, loose cellular connective tissue was observed with collagen fibers arranged irregularly and new formation of blood vessels (Fig. 1A). The biopsies from the E1 group showed an initial proliferative phase on treatment day 10, with epidermal regeneration in 60 % of the biopsies. In the healing dermis, a fibroblastic reaction with abundant collagen fibers and new formation of blood vessels was observed (Fig. 1B). In the E2 group, the biopsies of the injuries were in an advanced proliferative phase. The epidermis was regenerated in 70 %, with the presence of keratinization. Some layers were differentiated: the stratum corneum and stratum granulosum were reduced, the stratum spinosum was evident and the stratum basale was well developed on a basal lamina in formation. In the healing dermis, the connective tissue was loose. On the surface, a new formation of blood vessels, fibroblastic reaction and abundant collagen fibers were observed, with an absence of hair follicles and sebaceous glands (Fig. 1C).

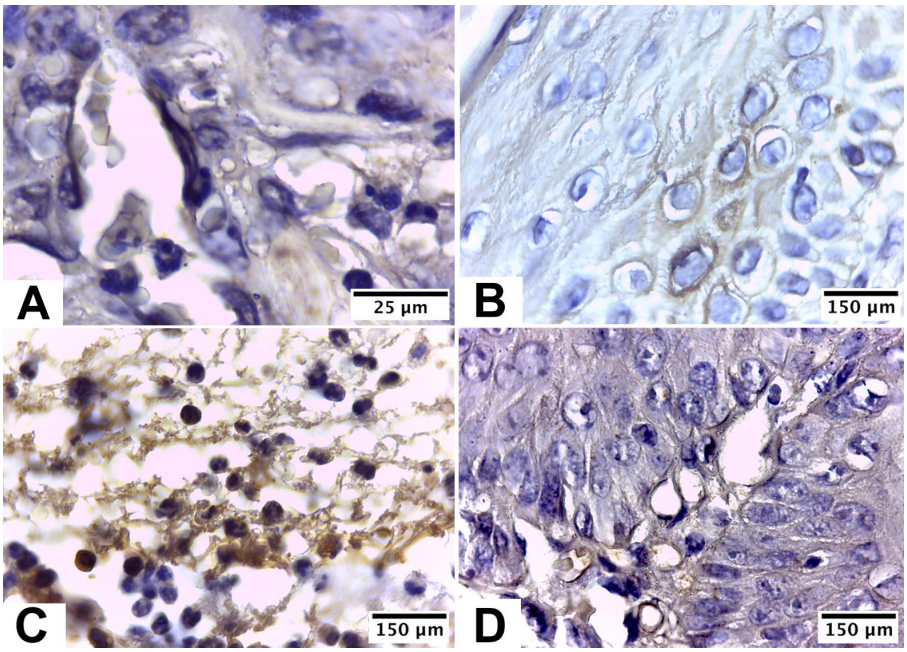

Fig. 2. Immunolocalization of endogenous FGF-2 expressed in the healing of burns on guinea pig skin on treatment day 10 with hydrogel-tull (C+). (A) Negative control. (B) Epidermal basement layer with positive immunolabeling. (C) Cumulus of lymphocyte cells. (D) Blood vessels with immunolabeling on the endothelial level.

Immunostaining. The immunohistochemical localization of FGF-2 was detected in the burn healing in guinea pig skin on treatment day 10 with hydrogel-tull (C+), Ulmo honey (E1) and Ulmoplus® (E2). The biopsies of the analyzed groups presented similar immunolabeling patterns, but with different intensities. The localization was observed at the level of the epidermis, dermis, blood vessels and fibroblasts (Figs. 2, 3 and 4). In the re-epithelialized area, keratinocytes were marked positively with anti-FGF-2 antibody in all the groups. However, none of the groups presented immunolabeling in the upper part of the epidermis (stratum corneum and stratum granulosum).

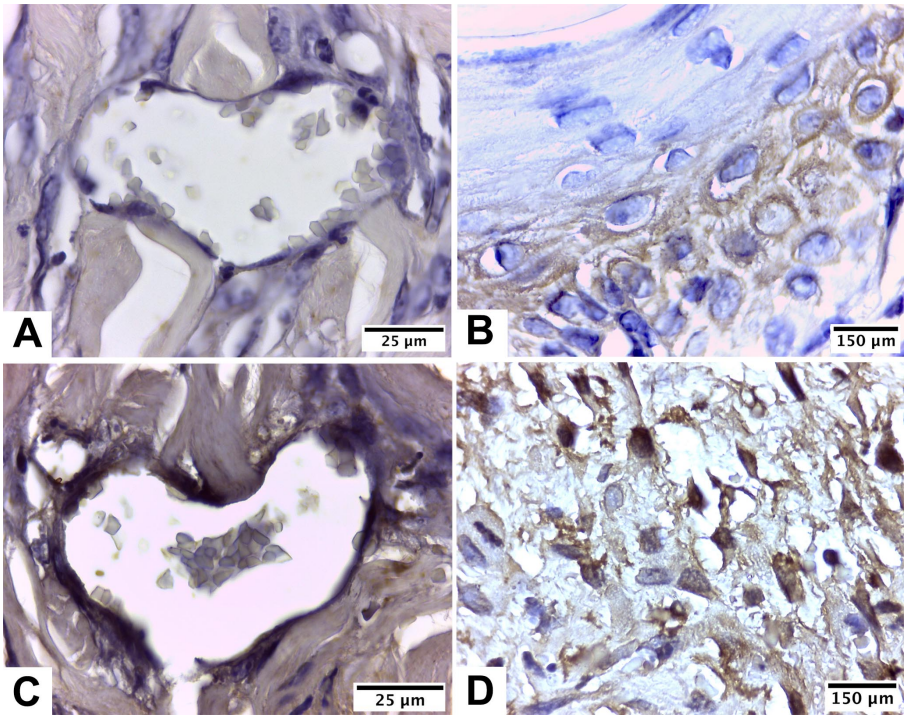

Fig. 3. Immunolocalization of endogenous FGF-2 expressed in the healing of burns on guinea pig skin on treatment day 10 with unsupplemented native Ulmo honey (E1). (A) Negative control. (B) Epidermis with immunolabeled keratinocytes at the cytoplasmic level. (C) Blood vessel with endothelial lining intensely marked. (D) Dermal fibroblasts of treatment area with positive immunolabeling.

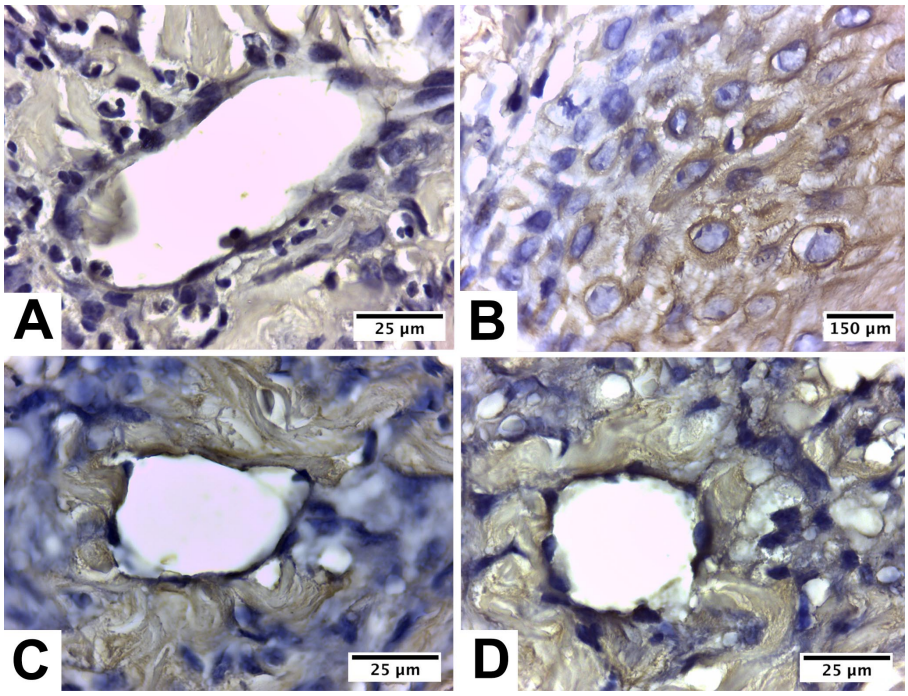

Fig. 4. Immunolocalization of endogenous FGF-2 expressed in the healing of burns on guinea pig skin on treatment day 10 with native Ulmo honey supplemented with ascorbic acid (Ulmoplus®) (E2). (A) Negative control. (B) Epidermis with intense positive immunolabeling. (C, D) Blood vessels with immunolabeling on the endothelial level.

In the C+ group the epidermis presented slight intensity, with positive immunolabeling only at the level of the stratum basale (Fig- 2B). Labeling of the cumulus of lymphocyte cells (Fig. 2C) and at endothelial level (Fig. 2D) was observed. Immunoreactivity of FGF-2 was not detected in the extracellular spaces. The E1 group showed greater immunostaining intensity, presenting several layers of keratinocytes with positive immunolabeling at the stratum basale and part of the stratum spinosum (Fig. 3B). The labeling distribution at endothelial level was very intense (Fig. 3C). In addition, a strong stain was noted at fibroblast level of the healing dermis (Fig. 3D). The E2 group presented immunolabeling in several layers of keratinocytes, particularly in the stratum corneum (Fig. 4B). The immunolabeling of FGF-2 at endothelial level was intense, with noticeable blood vessels and staining in capillary bulbs at the edge of the wound (Figs. 4C, 4D). Immunoreactivity of FGF-2 was not detected in the extracellular spaces.

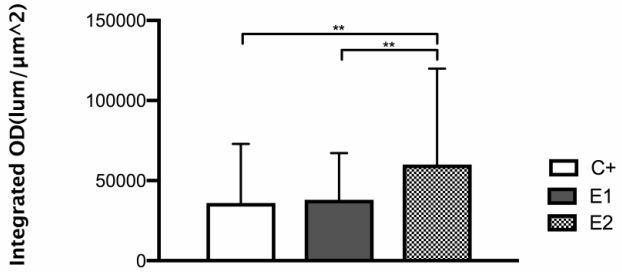

Fig. 5. Analysis by integrated optical density (IOD) of the expression of endogenous FGF-2 in burns treated with dressings with hydrogel-tull (C+), Ulmo honey (E1) and Ulmoplus® (E2).

The expression of FGF-2 was analyzed through IOD to assess the effect of the treatment with active advanced dressings of hydrogel-tull (C+), native Ulmo honey (E1) and Ulmoplus® (E2) in burn wounds. The results demonstrated that at least one group differs from another ($p < 0.001$) (Fig. 5). The E2 group presented an IOD significantly greater than the C+ ($p = 0.001$) and E1 ($p = 0.001$) groups.

DISCUSSION

This study used histological and immunochemical analyses to assess the healing capacity and distribution pattern of endogenous FGF-2 in skin tissue of burns treated with active advanced dressings of hydrogel-tull, native Ulmo honey and Ulmoplus®.

Endogenous FGF-2 was expressed positively in the wound tissues from all the study groups, which demonstrates the significant performance of this factor in healing (Takamiya *et al.*, 2002). An immunohistochemical localization pattern of endogenous FGF-2 was observed in keratinocytes of regenerated epidermis, endothelium of dermal capillaries, fibroblasts and infiltrated cells of the granulation tissue. The expression and distribution of endogenous FGF-2 in burn wound healing observed in this study is consistent with reports from other investigators. Oda *et al.* (2004) and Tepper *et al.* (2004) provided evidence of FGF-2 expression at endothelial level, where they suggested the effect on acceleration of angiogenesis could be related to the mitogenic, angiogenic and chemiotactic capacity that FGF-2 has for vascular endothelial cells, where the predominant mechanism for healing and angiogenesis depends on the significant increase of this factor. For their

part, Kurita *et al*. (1992) described endogenous FGF-2 in re-epithelialized epidermis, indicating that it might be due the keratinocytes, being mitogenic, being essential in covering the defect with the wound; hence, FGF-2 expression manifests its function as leader cells in covering the wound.

Previous studies describe endogenous FGF-2 expressed in skin cells and keratinocytes as acting in an autocrine and paracrine manner by regulating cell proliferation and re-epithelization in wound healing at dermic level (Akimoto *et al*., 2002). FGF-2, a member of the FGF family, is essential for the morphogenesis of suprabasal keratinocytes. Indeed, the findings of Kurita *et al*., showed that germinative keratinocytes express FGF-2 with a noticeable increase in post-burn samples, with constant immunoreactive patterns and intensities throughout the wound repair period.

Our results were correlated positively with previous studies (Schencke *et al*., 2016), where treatment with Ulmo honey supplemented with ascorbic acid presented a greater dermal proliferation area, global healing index and global contraction index in burns, validating the synergy of its compounds. The healing process of the wounds observed in the histological analysis was parallel to the expression of this factor in all the study groups. Nevertheless, the significantly greater expression of FGF-2 in the E2 experimental group could be related to the acceleration of healing, since histologically it was found in a more advanced stage. In this study, all the groups presented a proliferative healing stage on treatment day 10. However, during this stage histological differences were observed, where the C+ and E1 groups were in an initial proliferative stage with some inflammatory signs, whereas the E2 group (Ulmoplus®) presented an advanced proliferative stage, with no signs of inflammation, achieving a better effect in the dermal collagen and fibroblast activation, which could be reflected in the quality of the final scar. These results agree with previous studies, with the same wound model, where a larger number of active fibroblasts and greater collagen fiber concentration were observed in the groups treated with Ulmo honey supplemented with ascorbic acid (Schencke *et al*., 2018).

The growth factors are very promising molecules for the treatment of skin wounds. In vitro studies have demonstrated the effects of FGF-2 on the morphology, proliferation and differentiation of cells involved in wound healing. The results of Gibran *et al*. (1994) indicated that in the extracellular matrix (ECM) and in the cytoplasm of normal, undamaged cells, FGF-2 did not present an immunoreaction, suggesting that its release occurs only in the event of damage, with a significant synthesis during the wound healing period. Their data involve the role of FGF-2 as a pre-synthesizer and local cell mediator in the damaged area, suggesting that inducing a gradual increase in FGF-2 release or the exogenous application of FGF-2 could be beneficial to improving the tissue wound healing time. In addition, it has been described that the exogenous application of this factor acts as a potent inducer of angiogenesis and formation of granulation tissue, stimulating wound healing in animal models (Liu *et al*., 2007). However, their clinical use has been seriously limited by problems related to safety and performance. These problems can arise from the fact that the growth factors are used in supraphysiological levels without optimized management systems (Rizzi *et al*., 2010). Therefore, it is likely that when stimulating the endogenous release of this factor at suitable levels, the healing process of a full thickness burn is accelerated. The expression of endogenous FGF-2 in experimental group E2 (60053.55 ± 59877.84 lum/μm^2) was greater than that in E1 (38100.09 ± 29112.43 lum/μm^2; p=0.001) and C+ (36034.73 ± 36984.52 lum/μm^2; p=0.001), which suggests that Ulmoplus® can induce FGF-2 secretion and promote its expression, participating in wound healing as a positive regulatory factor.

Honey can trigger the sequence of events necessary to improve angiogenesis and the proliferation of fibroblasts (Molan, 2006) and epithelial cells (Tonks *et al*., 2003) when the production of certain growth factors is induced. Studies have shown how honey improves the bioactivity of platelet-rich plasma in skin regeneration, accelerating wound closing and healing (Sell *et al*., 2012). The identification of these individual compounds and their contributions to the treatment of wounds is crucial to a better understanding of the mechanisms behind the healing mediated by honey in normal or chronic wounds, especially at ECM level. In order to obtain a good coordination of the signaling of the growth factor in question, the ECM has a fundamental role (Briquez *et al*., 2015); therefore, understanding the mechanisms by which the ECM modulates the activity of this growth factor is key to designing efficient therapies.

CONCLUSIONS

This study demonstrated that the topical treatment with Ulmoplus® was more efficient than the gold standard treatment (hydrogel-tull), since it accelerated the healing of a burn, positively regulating angiogenesis and re-epithelization mediated by FGF-2.

Finally, it is important to indicate that there are no studies that demonstrate the action at cellular level of native Ulmo honey from southern Chile and Ulmoplus® in the

wound healing process. In future studies it will be important to investigate the mechanisms of action of this honey in the treatment of skin wounds and its essential processes, such as the stimulation of non-invasive angiogenesis and the timely and accelerated proliferation of keratinocytes.

SCHENCKE, C.; VÁSQUEZ, B.; SANDOVAL, C. & DEL SOL, M. Ulmoplus® incrementa la expresión de FGF-2 y promueve la cicatrización de heridas por quemadura. *Int. J. Morphol., 39(6)*:1701-1708, 2021.

RESUMEN: Se ha descrito el papel del factor de crecimiento fibroblaástico (FGF) en el proceso de reparación de heridas, particularmente durante la estimulación de la angiogénesis en la fase proliferativa. La miel de Ulmo (*Eucryphia cordifolia*) ha demostrado importantes propiedades bactericidas, fungicidas, antioxidantes y cicatrizantes. Ulmoplus®, un medicamento a base de miel, acelera el tiempo de cicatrización y promueve la activación de fibroblastos y la producción de fibras colágenas. El objetivo de este estudio fue evaluar la expresión inmunohistoquímica del FGF-2 endógeno en quemaduras tratadas con Ulmoplus® frente al gold standard (hidrogel-tull) y miel de Ulmo como primer paso para esclarecer el mecanismo regulador de esta opción terapéutica. Se utilizaron 15 cobayas adultos (*Cavia porcellus*), divididos en tres grupos: C +: control positivo tratado con hidrogel-tull (gold standard); E1: grupo experimental tratado con Ulmo; y E2: grupo experimental tratado con Ulmoplus®. Se realizó una quemadura profunda uniforme bajo anestesia que cubrió 1 cm^2 de la piel del lomo de cada animal. Los tratamientos diarios se administraron con hidrogel-tull, miel de Ulmo o Ulmoplus®, en cada caso. El día 10 de tratamiento se tomaron biopsias y se procesaron para análisis histológico e inmunohistoquímico. El inmunomarcaje se cuantificó mediante análisis de densidad óptica integrado (DOI), expresado como lum/μm^2 utilizando el software Image-ProPremier 9.1. La expresión endógena de FGF-2 fue consistente con la etapa de cicatrización presentada por los grupos de estudio, variando en su localización e intensidad de DOI. El grupo E2 presentó una DOI significativamente mayor (60053,55 ± 59877,84 lum/μm^2) que los grupos C + ($p = 0,001$) y E1 ($p = 0,001$). Nuestros hallazgos indicaron que la miel de uso médico Ulmoplus® es eficaz para facilitar el cierre de heridas, y el efecto promotor de la expresión de FGF-2 aceleró el proceso de curación en comparación con los tratamientos con hidrogel-tull (gold standard) y miel de Ulmo.

PALABRAS CLAVE: Factor de crecimiento fibroblástico; Quemaduras, Miel; Cicatrización de herida.

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