

TOPICAL TREATMENT WITH PROPOLIS DRESSINGS OF POOR HEALING FOOT ULCERS IN DIABETIC PATIENTS*

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Poor healing foot ulcers affect 15% of diabetic patients, precede 84% of all diabetes-related lower leg amputations⁽¹⁾ and, compared with diabetic patients without ulcers, the mortality at 3 years is 15% higher⁽²⁾. Due to impairment in the physiological synchronization of events that lead to a rapid healing, foot ulcers do not follow an orderly and reliable wound healing process^(3,4).

Surprisingly, although propolis has been used to cure wounds since ancient times, documented clinical experiences have scarcely been published. For this reason, we present the results of a small historic observational study performed in 1983 in 23 ambulatory diabetic patients (13 type I, 10 type II) with foot ulcers who received topical treatment with 8% propolis of Uruguayan origin in hydro soluble vehicle dressings. Quantitative analysis of polyphenols in Uruguayan EEP using high performance liquid chromatography – mass spectrometry showed a mean value of 457mg/g, with a high content of flavonoids⁽⁵⁾. On day 6 of treatment, two patients were withdrawn from the study and excluded for efficacy analysis due to perilesional allergic reaction, which cleared after 3 days' treatment with topical corticosteroids. A study carried out in 1997 to compare the efficacy and safety of the treatment with 8% versus 2% propolis dressings from Uruguay in patients with burns (n=76), wounds (n=122) or ulcers (n=31) showed no differences as to efficacy, but a significant reduction in local allergic events⁽⁶⁾.

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Abbreviations used: EEP, ethanol extract of propolis; TNF- α , tumor necrosis factor-alpha; IL, interleukin; MCP-1, monocyte chemo-attractant protein-1; PGE₂, prostaglandin E₂; LTB₄ and LTC₄, leukotriene B₄ and C₄; ROS, reactive oxygen species; RNS, reactive nitrogen species; MMPs, matrix metalloproteinases; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; TGF- β ₁, transforming growth factor-beta₁; NF- κ B, nuclear (transcription) factor-kappa B; AMPs, antimicrobial peptides; BK_{Ca}, large conductance calcium-activated potassium channels; CAPE, caffeic acid phenethyl ester; PLA₂, phospholipase-A₂; COX-2, cyclooxygenase-2; LOX-5, lipooxygenase-5; NO, nitric oxide; XO, xanthine oxidase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate.

Characteristics of the 21 patients before treatment were as follows: mean age 48 ± 18.7 years (range 17-77); mean diabetes duration 13.5 ± 7.5 years (range 3-33); mean wound duration 77 ± 27.7 days (range 30-120). Eleven patients had neuropathic ulcers (2 with underlying lipoidic necrobiosis; 4 with bilateral ulcers); 5 had neuroischemic ulcers, 3 had traumatic wounds and 2 had venous ulcers. For treatment purposes, wounds were fully covered with propolis occlusive dressings (plus a 8% propolis solution in four deep wounds) and replaced every 3 days; surgical debridement was performed as required. Fifteen patients received concomitant antibiotics. Seventeen wounds from 13 patients, documented photographically before and after treatment are shown below (at the top, patients initials, gender, age and diabetes duration are indicated; at the bottom, number of days before and after mean wound duration and time of treatment, respectively). After a mean treatment time of 40.5 ± 16 days (range 18-75), wounds completely healed in 20 patients and significantly improved in one. Propolis dressings provided for a moist wound environment, facilitated autolytic debridement and healthy granulation tissue formation; were painless and easy to use and to remove without trauma to the wound. In conclusion, propolis dressings were effective, safe and inexpensive to treat poor healing diabetic wounds. It should be noted that in the U.S.A. the costs of treatment of diabetic foot ulcers totaled almost \$ 40 billion in 2007⁽⁷⁾.

A large amount of work was done to elucidate the molecular and cellular milieu of chronic wounds, with the ultimate goal of removing the main barriers that delay healing⁽⁸⁻¹⁰⁾. These barriers are: 1) bacterial burden and a noteworthy biofilm formation; polymicrobial aggregates that developed a successful strategy for increasing virulence, resistance to antibiotics (up to 1000-folds) and phagocytosis, all of which are present in up to 80% of chronic wounds⁽¹¹⁾; 2) ischemia; 3) elevated and prolonged inflammation due to the high numbers of infiltrating neutrophils and macrophages, high levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6) and chemokines (IL-8, MCP-1) and to the increased synthesis of lipid inflammatory mediators (PGE₂, LTB₄, LTC₄); 4) large quantities of toxic ROS and RNS (particularly superoxide anion and peroxynitrite) that increase inflammatory response and tissue damage; 5) high levels of proteases (MMPs, neutrophil elastase) that increase ECM degradation and impair angiogenesis and re-epithelialization resulting from endothelial injury⁽¹²⁾, and inactivation of peptide growth factors (VEGF, TGF- β ₁ and others) and/or its receptors^(9, 13). Collectively, these events are capable of suppressing cell migration and proliferation, thereby impairing an adequate process of repair. Apparently, in chronic wounds, the reestablishment of a normal repair pattern by topical propolis is mainly related to the ability of its polyphenols to down regulate the activation of NF- κ B - the master key of the genetic regulation of immunity and inflammation⁽¹⁴⁾ - induced by bacterial molecules, inflammatory mediators and ROS/RNS; and with its iron chelating capability⁽¹⁵⁾. In this context, the efficacy of propolis is supported by its antibacterial/antibiofilm, antiinflammatory, antioxidant and immunomodulating properties.

The amphipatic structure of flavonoids facilitates interactions with bacterial cell membranes and the formation of channels that enable ion leakage, thereby reducing membrane potential and bacterial viability^(16, 17). As virtually all bacterial pathogens require iron to survive and develop virulence factors, reducing its availability by chelating is a valid antipathogenic strategy, particularly against *Staphylococcus aureus* and *Pseudomonas aeruginosa*^(18, 19), bacteria which are frequently isolated from chronic wounds. Indeed, the ability of propolis as an iron chelator seems to be

the leading cause for significantly reducing biofilm formation by the above-mentioned bacteria⁽²⁰⁻²²⁾. In turn, this reduction improves wound healing outcomes, indicating that biofilm is the right target for managing the bioburden barrier of chronic wounds⁽¹¹⁾. Additionally, the activity of the coagulase and lipase enzymes -both related to host tissue damage- was abrogated by propolis in strains of *Staphylococcus aureus* and significantly reduced in strains of *Staphylococcus* spp., respectively⁽²⁰⁾.

The expression of the highly conserved ancestral human skin AMPs (cathelicidin LL-37, beta Defensins), innate immune system molecules that collectively display broad antimicrobial/antibiofilm and high endotoxin neutralization activities, as well as keratinocytes proliferation and migration, is strongly reduced in the bed of chronic wounds⁽²³⁻²⁷⁾. The migration of keratinocytes to the more superficial and differentiated epidermal layers, driven by steadily increasing intracellular calcium concentrations resulting from the opening of BK_{Ca} channels⁽²⁸⁾, dramatically increases AMPs gene expression in the skin⁽²⁹⁾. As several propolis-derived polyphenols and CAPE are pharmacological openers of BK_{Ca} channels⁽³⁰⁻³²⁾ and display a strong antioxidant activity, they could increase AMPs production and protect BK_{Ca} channels functions challenged by the documented damage caused by sustained hyperglycemia⁽³³⁾ and by the high levels of ROS⁽³⁴⁾ and RNS⁽³⁵⁾ present in chronic wounds, thereby preserving AMPs production and facilitating re-epithelialization. Additionally, AMPs participate in the wound repair process^(24,36). Thus, a functional link between propolis and AMPs concerning their antibacterial and wound healing properties cannot be ruled out.

In vitro cellular studies showed that the levels and/or gene expression of skin inflammatory mediators were significantly reduced by propolis and/or some of its components. The synthesis of IL-1 β was reduced by 65% with 30 μ g/mL of propolis⁽³⁷⁾, whereas that of PGE₂, LTB₄ and LTC₄ were almost completely inhibited by 20-50 μ g/mL⁽³⁸⁾. The synthesis of ILs-1 β , -6, -8, TNF- α , MCP-1, PGE₂, LTB₄, LTC₄ and endothelial adhesion molecules was reduced by the flavonoids apigenin⁽³⁹⁻⁴³⁾, quercetin^(37,42-44), chrysin^(37,42,43,45), galangin⁽⁴³⁾ and kaempferol^(37,40,42,43) and by caffeic acid⁽⁴⁶⁾ and CAPE^(38,47,48). Also, the enzymes related to the synthesis of lipid mediators derived from arachidonic acid of cells membranes were inhibited by some propolis polyphenols: PLA₂ by quercetin⁽⁴⁹⁾, kaempferol⁽⁵⁰⁾ and caffeic acid⁽⁴⁶⁾; COX-2 by CAPE⁽⁴⁷⁾, apigenin⁽⁵¹⁾, kaempferol^(51,52) and quercetin⁽⁵²⁾; LOX-5 by CAPE and caffeic acid⁽⁴⁸⁾.

NO is a cellular mediator of physiological or pathological events in function of the amount produced and the enzyme involved in its synthesis. High levels of NO produced by the inducible isoform of nitric oxide synthase (iNOS) at micromolar range for hours or days are clearly proinflammatory and detrimental for healing due to the in vivo formation of peroxynitrite and hydroxyl radicals, after reacting with concomitantly produced superoxide anions. iNOS activity and gene expression induced by IL-1 β , TNF- α , bacterial molecules and hypoxia are significantly increased in chronic ulcers^(53 - 55). In vitro, 12.5 μ g/mL of propolis inhibited NO production by 65% by decreasing iNOS gene expression and directly inhibiting its catalytic activity⁽⁵⁶⁾. Another study showed a 65% reduction of iNOS expression with 30 μ g/mL propolis, whereas 30 μ M of chrysin, galangin, kaempferol or quercetin displayed stronger reductions⁽³⁷⁾. iNOS expression was also dose dependently-down regulated by apigenin, kaempferol and quercetin^(42,51,52). Additionally, in concentrations ranging from 5 to 15 μ g/mL⁽⁵⁷⁾ propolis showed a high ability to scavenge peroxynitrite, a powerful oxidizing and nitrating molecule.

Endothelial cells, senescent fibroblasts, macrophages, and particularly neutrophils are the sources of the overproduction of ROS, which are released within the chronic wound microenvironment, amplifying the unrestrained damage to cell membranes and structural proteins of the ECM. Thus, the disruption of this deleterious cycle is a valid therapeutic strategy to protect the regenerative tissue from damage⁽⁵⁸⁾.

ROS are generated mainly by the enzymatic activity of XO, MPO and NADPH oxidase. The activity of XO was almost completely inhibited with 50 µg/mL of propolis, and CAPE accounted for 33% of this inhibition; its scavenging capacity of superoxide anion was close to 100% with 3 µg/mL⁽⁵⁹⁾. In a cellular assay with human endothelial cells XO activity was inhibited by 50% with 2 µg/mL of propolis⁽⁶⁰⁾. In vivo, the increased MPO activity (fourfold) in the wounds of diabetic rats was prevented by the topical application of a single drop with 8 mg of propolis; concomitantly, wound healing rate and re-epithelialization improved⁽⁶¹⁾. NADPH oxidase is the major source of ROS in endothelial cells activated by cytokines and/or ischemia/hypoxia, but the expression of its Nox 4 isoform in epithelial cells leads to constitutive ROS generation⁽⁶²⁾. The antioxidant effect of Uruguayan EEPs with high content of polyphenols was investigated at cellular level using rabbit endothelial cells. EEP with a concentration of 5.3 mg of total polyphenols/mL significantly reduced the NADPH oxidase activity (≈20%) in association with a reduced expression of the Nox 4 isoform (≈30%). In vitro, its ROS scavenging activity measured by the oxygen radical absorption capacity was extremely high (8µmol Trolox equivalents/mg propolis); additionally, this propolis was an effective inhibitor of lipid and protein oxidation⁽⁶³⁾. Unchecked proteolytic activity, a cardinal feature of chronic wounds, is responsible, in synergy with ROS, for the increased degradation of ECM proteins, the impaired angiogenesis, and for the suppression of cell proliferation^(9,12,13,58). Among other proteases, high levels of elastase and MMP-9 released from infiltrating neutrophils were documented in the fluids of human chronic wounds⁽⁶⁴⁻⁶⁶⁾ and in experimental diabetic wounds⁽⁶⁷⁾. Moreover, increased levels of MMP-9 in diabetic foot ulcers were highly predictive of poor healing rate⁽⁶⁶⁾, and inhibition of the high levels of MMP-2 and -9 in chronic wounds exudates significantly increased in vitro angiogenesis⁽⁶⁸⁾. Thus, controlling this proteolytic activity is another complementary approach for the treatment of chronic wounds. Propolis showed a strong inhibitory effect on human neutrophil elastase activity (50% inhibition with 2µg/mL)⁽⁶⁰⁾ as well as on MMP-9 activity. CAPE was found responsible for the anti-MMP -9 activity (50% inhibition with 1.0-2.0 nmol/mL)⁽⁶⁹⁾ and for the reduction of induced MMP-9 expression by inhibiting the function of NF-κB⁽⁷⁰⁾. One single application of propolis to experimental diabetic wounds suppressed the increased level and activity of MMP-9 and reversed the significant decrease in epithelial closure rate⁽⁶⁷⁾.

Elevated concentrations of inflammatory mediators, proteases and ROS into the chronic wound cause pain⁽¹⁰⁾, and high levels of PGE₂ and LTB₄ reduce the stimulation threshold of pain receptors, thereby amplifying pain mechanisms⁽⁷¹⁾. Thus, the local analgesic effect displayed by propolis could be ascribed to the inhibition of the aforementioned events.

The low levels of NO produced by the constitutive endothelial nitric oxide synthase (eNOS) at nanomolar range and released for short periods of time shows the physiological side of NO. eNOS contributes to the proangiogenic program of capillary endothelium by triggering VEGF-induced endothelial cell proliferation and differentiation and, in turn, VEGF increases NO synthesis and activity by increasing eNOS expression^(53,55,72). So, NO from eNOS appears to play a central role in angiogenesis, a pivotal component of wound repair. Indeed, angiogenesis and

wound healing were markedly impaired in mice with eNOS gene disruption^(73,74), and eNOS expression was strongly reduced at the wound site in diabetes-impaired skin repair⁽⁷⁵⁾. However, in vivo gene therapy with eNOS promoted wound healing in diabetic mice⁽⁷⁶⁾. On the other hand, several pathophysiological conditions (ischemia, chronic hypoxia) and inflammatory mediators (TNF- α , NO, bacterial lipopolysaccharides) present in human chronic ulcers reduce eNOS expression^(77,78). In such a context, propolis can protect eNOS expression and activity due to its aforementioned abilities but, most importantly, due to the finding that at cellular level Uruguayan propolis, at concentrations ranging from 3.2 to 5.3 mg/mL, were effective for increasing eNOS expression and inhibiting Nox activity that, together, point to an increase in endothelial NO bioavailability⁽⁶³⁾. In conclusion, the improvement produced in the broad range of molecular targets involved in healing makes propolis an effective therapeutic tool for the treatment of chronic ulcers.

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