# Saliva and Wound Healing

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*Wounds in the oral cavity heal faster and with less scarring than wounds in other parts of the body. One of the factors implicated in this phenomenon is the presence of saliva, which promotes the healing of oral wounds in several ways. Saliva creates a humid environment, which improves the survival and functioning of inflammatory cells that are crucial for wound healing. Furthermore, saliva contains a variety of proteins that play a role in the various stages of the intraoral wound healing. Tissue factor, present in salivary exosomes, accelerates the clotting of blood dramatically. The subsequent proliferation of epithelial cells is promoted by growth factors in saliva, especially epidermal growth factor. The importance of secretory leucocyte protease inhibitor is demonstrated by the observation that in the absence of this salivary protein, oral wound healing is considerably delayed. Members of the salivary histatin family promote wound closure in vitro by enhancing cell spreading and cell migration. Cell proliferation is not enhanced by histatin. Cyclization of histatin increased its biological activity approximately 1,000-fold compared to linear histatin. These studies suggest that histatins could potentially be used for the development of new wound healing medications.*

**Key words:** *growth factor, histatin, peptides, saliva, wound healing*

Daily activities, such as eating, drinking, biting, chewing and speaking expose the oral mucosa frequently to mechanical forces and chemical stress. The layer of epithelium that covers the oral mucosa provides only limited protection against these forces. Salivary mucins are of paramount importance for an optimal protection of the oral mucosa. They are the major component of the hydrophilic mucus layers that cover the oral tissues. The mucus layers lubricate teeth and mucosa, offering protection against frictional forces. In addition, they protect the underlying tissues against desiccation, colonization and invasion by bacteria, and action of chemical substances. However, the protective factors are not able to prevent all damage to the oral mucosa.

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Until now, the role of saliva in the healing of intraoral wounds has received relatively little attention. This is remarkable, as licking of wounds is instinctive for animals and man. There have even been reports that Fijian fishermen allow dogs to lick their wounds to promote wound healing[1]. Recent studies support this wound healing activity of saliva.

## Healing of wounds in the skin and the oral mucosa

The healing of skin wounds is characterized by several, partly overlapping, stages:

- Immediately after the wound is created, blood vessels constrict and blood clotting begins. Both processes are aimed to limit further blood loss.
- This is followed by the inflammatory phase, during which macrophages and other inflammatory cells remove bacteria and necrotic cell debris. At the same time, the inflammatory cells secrete factors that stimulate cell division and migration of cells, such as epithelial cells and fibroblasts.
- The proliferative phase. In this phase many regenerative processes take place, including angiogenesis, deposition of a new collagen matrix and formation

of granulation tissue. The wound is gradually covered with epithelial cells and wound contraction may occur.
- In the final phase, collagen is remodeled and cells that are no longer needed are removed by apoptosis. The total wound healing process may take between one month and more than two years.

Despite the fact that the wound healing of oral mucosal follows the same phases of the healing of the skin, there is a clear difference. Intraoral wounds heal faster and with less scar formation than wounds of the skin. Not only is this the clinical observation of dentists, who observe that extraction sockets usually heal rapidly without complications. A study with pigs, whose skin closely resembles the human skin, also showed that intraoral wound healing occurs much faster than wound healing of the skin. Comparable surgical wounds were made in the palatal mucosa and in the skin. After 14 days, the palatal wounds were clinically closed and after 28 days the original location of the wound could hardly be recognized from the surrounding unaffected tissue. In contrast, skin wounds were still covered with a crust after 14 days, and after 28 days the original wound could still easily be recognized[2]. The size of standard circular wounds in the hard palatal mucosa of human volunteers decreased very rapidly, especially in male and younger individuals[3].

Several factors play a role in the more rapid wound healing of the oral cavity. First, the baseline turnover of cells in the oral mucosa is higher than in the skin, enabling a more rapid repair of the oral mucosa. Second, the oral mucosa is highly vascularized. This is beneficial for the recruitment of inflammatory cells, growth factors and nutrients to the wound and, at a later stage, for the removal of phagocytized bacteria and necrotic cells.

The humid environment of the oral cavity also promotes wound healing. The mechanism behind this has not entirely been clarified yet, but several factors play a role. The humid atmosphere in the oral cavity prevents dehydration of cells and associated death of cells. Many cells are involved in wound healing, such as neutrophils, macrophages, epithelial cells and fibroblasts. The survival of these cells is improved by the humid environment of the oral cavity. Re-epithelization also progresses faster in a humid environment, as epithelial cells migrate faster on the humid surface of a wound than under a dry crust. In addition, the supply of nutrients and oxygen will be better in a humid environment.

The presence of saliva promotes the wound healing of the oral mucosa. Saliva not only creates the humid conditions in the oral cavity, but saliva also contains several proteins and peptides that enhance wound healing directly and indirectly.

*Analgesia*

Several years ago, it was shown that human saliva contains opiorphins. Opiorphins are peptides with an analgesic effect[4] that prolongs the effect of enkephalins – natural analgesics secreted by the brains when pain is perceived. However, the duration of action of enkephalins is limited because they are rapidly enzymatically degraded. Opiorphins inhibit the activity of these enkephalin-degrading enzymes and thereby prolong the analgesic effect of enkephalins. In rats, 1 mg of opiorphin had a comparable effect as 3 mg of morphine.

*Hemostasis*

Wound healing begins with haemostasis, the essential primary step before tissue repair can occur. Immediately after a blood vessel has been damaged, platelets will adhere to the exposed underlying connective tissue. The platelets form an aggregate that provides a primary closure of the wound to limit further blood loss. An insoluble network of fibrillar fibrin is deposited on the aggregated platelets, as the final product of the coagulation cascade. The coagulation cascade encompasses a series of inactive pro-enzymes, the coagulation factors. In vivo, coagulation activation is initiated through tissue factor, a protein expressed by subendothelial cells and smooth muscle as well as other cells. As soon as blood leaks from a vessel, it is exposed to tissue factor.

Already in the 1920s and 1930s, studies were performed on the potential capacity of saliva to clot blood. Addition of small amounts of saliva accelerated the clotting time of blood to a large extend[5,6]. Recently, it was shown that saliva is a rich source of tissue factor. Tissue factor in saliva is bound to the surface of exosomes, small membrane encapsulated particles with a diameter of 30 to 90 nanometer. The exosomes in saliva are derived from epithelial cells, and are released when the membranes of multivascular bodies fuse with the plasma membrane[7].

*Antimicrobial activity*

Not all the conditions in the oral cavity are beneficial for wound healing. The intraoral conditions are optimal for the development of very complex microbiota that comprises more than 1,000 different species of bacteria, fungi and viruses[8]. The total number of bacteria in the oral cavity is estimated to be $10^8$–$10^9$. This implies that

**Table 1** Concentrations of growth factors in saliva and plasma (in ng/ml) (modified from Oudhoff[28])

| Growth factor | Human saliva | Human plasma | Murine saliva |
|---|---|---|---|
| EGF | 0.9 | 0.2 | 20,000 |
| NGF | 0.9 | 0.1 | 40,000 |
| VEGF | 1.4 | 0.5 | |
| FGF | < 0.001 | 0,2 | |
| IGF | 0.4 | 170 | 75 |
| TGF-α | 5.6 | 0.03 | 560 |
| TGF-β | 0.024 | 2.0 | |
| TNF-α | 0.003 | 0.008 | |
| Insulin | 0.2 | 925 | |

wounds of the oral mucosa can easily become infected and, after penetration of this infection barrier, microorganisms can enter the bloodstream and disseminate to other parts of the body. As the presence of bacteria initiates an inflammatory response, this will delay wound healing. Inflammatory cells that have been migrated to the damaged area will secrete interleukins and proteolytic enzymes in response to bacteria and bacterial toxins. The secreted interleukins and proteolytic enzymes may considerably delay wound healing[9].

Saliva contains several proteins and peptides that protect the oral cavity against microbial infections. Secretory-IgA, mucin 7 (MUC7) and salivary agglutinin prevent the adhesion of microorganisms to oral tissues. Another line of defense against microorganisms is provided by bactericidal proteins and peptides, including lysozyme, defensins, cathelicidins and histatins[10]. It has been suggested that nitric oxide, derived from salivary nitrite, also contributes to the antimicrobial effects of wound licking[11].

Some salivary proteins bind bacterial toxins, thereby neutralising the proinflammatory effect of these toxins[10]. In addition, saliva contains several proteins and peptides that are able to stimulate directly the activity of oral epithelial cells and fibroblasts.

*Growth factors*

Growth factors were originally discovered and characterised in saliva and salivary glands of rodents[12,13]. Later, it has been shown that human saliva contains similar growth factors. These growth factors are signal molecules that start a wide variety of intracellular processes, including cell division and cell migration.

During the past few decades, a substantial number of growth factors have been identified in human saliva. Some of these growth factors are present in concentrations that have biological activity *in vitro*, and therefore potentially could play a physiological role in the oral wound healing. These growth factors include, among others, epidermal growth factor (EGF), transforming growth factor-α and vascular endothelial growth factor[14-16]. In addition, saliva contains minute amounts of other growth factors and insulin, probably due to leakage of serum into the oral cavity. The first discovered growth factor, epidermal growth factor, has been investigated most extensively. It is a relatively small protein of 53 amino acids, mainly secreted by the parotid gland in man. It binds, like the other growth factors, to a specific receptor on the membrane of the target cell, initiating a cascade of processes. This results in migration, proliferation and differentiation of cells. Migration and proliferation of epithelial cells are activities in the initial stages of wound healing. In rodents, salivary epidermal growth factor has effects on wound healing of the skin and the stomach, in addition to local effects in the oral cavity[17-19].

When it was demonstrated in the 1980s that human saliva also contained EGF, it was assumed that this growth factor protects the human oral epithelium as it does in rodents. However, one has to consider that the concentration of this growth factor in human saliva in considerably lower than in saliva of rodents (see Table 1). In addition, a considerable part of EGF is present in human saliva in the form of an inactive precursor. Despite this, it has been shown that even in the low concentrations in which EGF is present in human saliva it has *in vitro* activity, suggesting that this growth factor can play a similar role in man as it does in mice and rats.

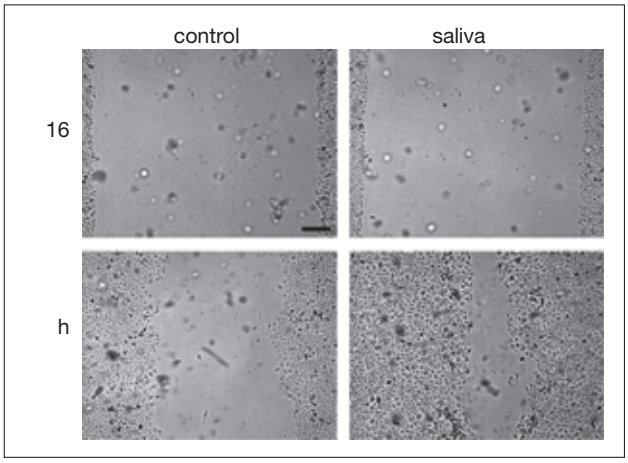


**Fig 1** Stimulating effect of parotid saliva on *in vitro* wound closure. Epithelial cells were grown in 12-well plates until confluence. Cells were removed with a sterile tip to create a standardized "wound". The width of the scratch was determined microscopically immediately after creation and 16 h after culture in medium in the absence (left panels) or presence of human parotid saliva (30% v/v right panels).

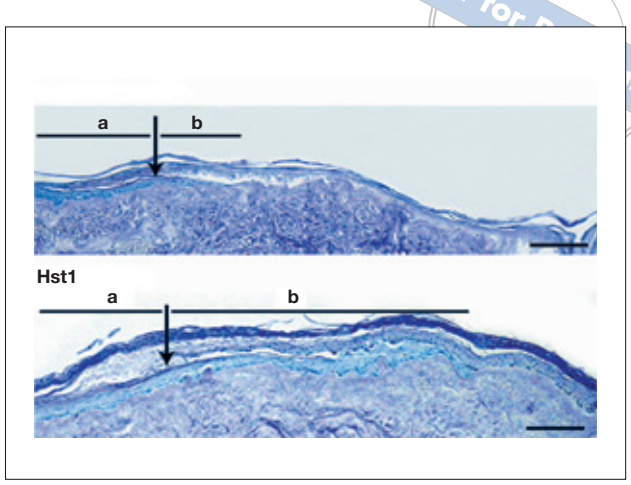


**Fig 2** Enhancement of wound healing by synthetic histatin, in a human epidermal skin equivalent that closely resembles healthy skin. Wounds were created with a glass rod cooled to –196 °C. This resulted in cell death of the entire treated area of the epidermis. Immediately after the creation of the wound, the epidermal equivalents were cultured for 6 days in medium in the absence (upper panel) or presence of histatin (lower panel). The arrow indicates the original border of the wound area. a = healthy epidermis. b = the length of the wound closure after 6 days.

Transforming growth factor-α is also present in human saliva. Its chemical structure shows a marked resemblance to the structure of epidermal growth factor. Both growth factors bind to the same receptor and show an almost identical range in biological activities.
A possible role of nerve growth factor in the maintenance of the oral health seems doubtful. In mice, no wound healing effects could be identified[20]. There are indications that this growth factor plays a role in the development and regeneration of nerve fibers[21].

Finally, saliva contains relatively large amounts of vascular endothelial growth factor. This growth factor is derived from the submandibular gland. It is a multifunctional protein, which, among others, stimulates angiogenesis. The concentration of this growth factor is increased in saliva of patients with periodontitis, which suggests a possible role in the healing of periodontal tissues[22].

### *Secretory leukocyte protease inhibitor*

Saliva also contains several proteins that enhance wound healing by inhibiting the inflammatory response. An example of these is secretory leukocyte protease inhibitor (SLPI), a physiological enzyme inhibitor originally isolated from human parotid saliva. SLPI is present in most mucosal secretions, including bronchial, nasal and cervical mucus, saliva and seminal plasma. It is a multifunctional protein that inhibits a large number of protein-degrading enzymes, including elastase, trypsin and cathepsin. In addition, it also has anti-HIV-activity, anti-inflammatory activity and antimicrobial activity.

The potential role of SLPI in wound healing was explored in animal studies. In SLPI knock-out mice, missing the gene of this enzyme-inhibitor, oral wound healing was delayed considerably and oral infections occurred more frequently. In SLPI-deficient animals, the degradation of connective tissue was increased due to an increased activity of elastase, a protein that degrades proteins in connective tissue. In mice where SLPI was topically applied, wound healing normalized indicating that this enzyme-inhibitor is crucial for wound healing[23].

### *Trefoil peptides*

Another protein important for oral wound healing is Trefoil Factor 3 (TFF3), a member of the Trefoil peptide family. This family of peptides is characterized by a 40 amino acid domain containing three conserved disulfide bridges resulting in three loops. Trefoil peptides are abundantly present on almost all mucosal surfaces of the human body, including the oral cavity, gastro-intestinal tract, gall bladder, pancreas, lungs and cervix. They improve the mechanical and chemical resistance of the mucus layer and are involved in the homeostasis and regeneration of the mucosa. The compact form of trefoil peptides makes them remarkably resistant against proteolytic degradation.

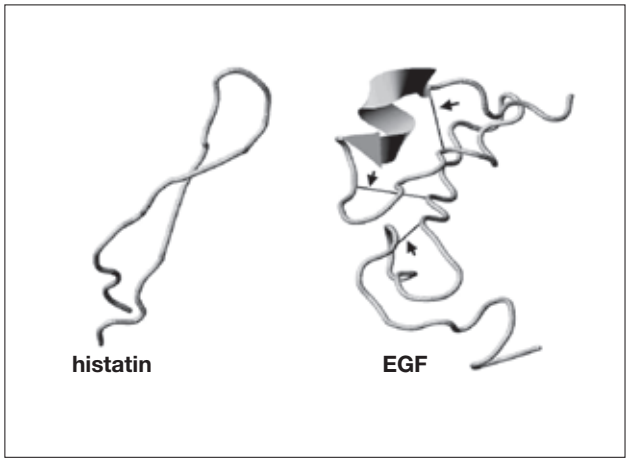


**Fig 3** Schematic structures of histatin and epidermal growth factor (EGF). Compared to histatin, chemical synthesis of EGF is more complicated because of its larger size (53 versus 38 amino acids) and the presence of disulphide bridges (indicated with arrows). These disulphide bridges are necessary for the three-dimensional structure of EGF, which is essential for its biological activity. Histatin is smaller than EGF, and does not need a fixed three-dimensional structure for activity.

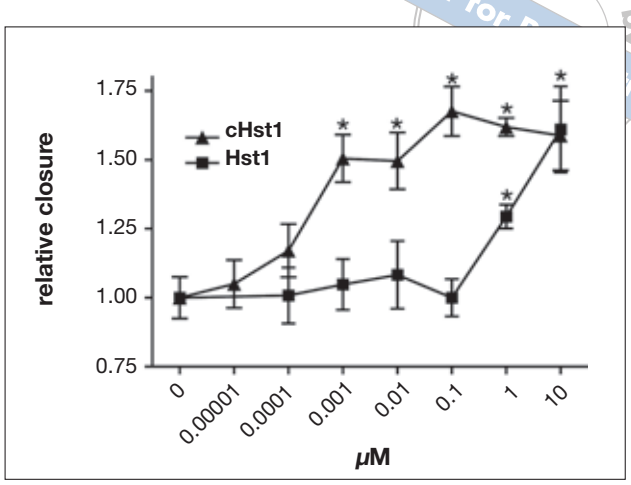


**Fig 4** Comparison of the wound closing effects of natural linear histatin and synthetic cyclic histatin, in concentrations ranging from 0.01 μM up to 10 μM. Cyclisation resulted in a 1,000-fold stimulation of the molar activity.

Trefoil factor 3 is the only member of the trefoil peptide family present in saliva. TFF3 is secreted by the submandibular and sublingual glad, and increases wound closure in a dose-dependent manner. Using *in vitro* culture system it was shown that TFF3 increases the migration of oral keratinocytes. TFF3 had no significant effect on the cell division of these cells[24].

### *Histatins*

Histatins are a family of histidine-rich peptides that are only present in the saliva of higher primates. Until now, they have not been identified in other tissues. Based on their structure, many functions have been attributed to histatins, including antimicrobial and anti-inflammatory activity, detoxification and remineralization of teeth.

Recently, it has been shown that some members of the histatin family are able to stimulate the migration of epithelial cells and fibroblasts, thereby enhancing wound closure[25]. First it was shown that addition of human parotid saliva improved wound closure in a relatively simple *in vitro* model (Fig 1). Several high performance liquid chromatography purification steps were used to isolate individual proteins from parotid saliva, and each purified fraction was tested for its wound closing capacity. From this analysis, one single active component emerged: histatin-1. Histatin-1 also enhanced wound closure in a more complex *in vitro* model for wound healing, using human epidermal skin equivalents (Fig 2)[26].

The discovery of the wound closing effects of histatins opens the way for the development of new wound healing medications. Histatins, as compared to EGF, are relatively simple chemical compounds that can easily be produced in large quantities (Fig 3). The low production costs of histatins will probably make them commercially more interesting than recombinant produced EGF and TGF-α, currently entering the market in small amounts. Another advantage of histatins over the classical growth factors is that chemical variants can easily be produced. Chemical cyclization of histatin increased its biological activity approximately 1,000-fold compared to linear histatin (Fig 4)[26]. Using sortase A for cyclisation, the efficiency of histatin cyclisation was optimised to approximately 90%[27].

Histatin-analogues seem to have very interesting clinical potential. For example, a histatin-containing gel could be used in the oral cavity to enhance the healing of mucositis or to treat aphthous ulcers. Hopefully such clinical trials will be conducted in due course.

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