effective solution to periodontitis and peri-implantitis

Introduction by Dr. Peter Blijdorp

Infection control is one of the most challenging and complex problems in dentistry today. The mouth is a sea of bacteria normally living in balance with the rest of the human body. Under certain conditions, disease-causing bacteria can become dominant, creating infection. These bacteria live together in the biofilm, which supports a mixture of bacteria, viruses, fungi and even parasites. The complication is that each of these require a different drug to eliminate its dominance.

Bluem active oxygen technology for oral wound healing, tissue regeneration and oral disease management is based on my experience over the last 25 years as a Maxillofacial Surgeon and Implantologist.

wound healing

Wound healing requires a variety of cells to increase their metabolic activity, resulting in a high oxygen demand. Oxygen at the wound site has been shown to promote wound healing by stimulating several processes including the following:

- Anti-bacterial activities.
- Neo-vascularisation.
- Collagen production.
- Epithelialisation.
- Phagocytosis (engulfing of micro-organisms, cells or debris by macrophages or neutrophils).
- Degradation of necrotic wound tissue.

Severe and destructive processes can also be caused by deficient oxygen efficiency. An optimal uptake of oxygen is necessary to maintain the bio reactions of the body and for a healthy life.

Interestingly, oxygen saturation of the blood and oxygen partial pressure (pO2) have been found to reduce with age, thus reducing the optimal healing environment.

Bluem can provide the oxygen necessary to allow these processes to proceed effectively.

oral wounds

Peri-Implantitis and periodontitis are bacterial infections with chronic inflammation characteristics. It has been shown that the pO2 value in areas of peri-implantitis is significantly lower than in healthy tissue. Moreover, the pO2 value correlated with pocket depth; the deeper the pocket, the lower the pO2 value. These lower oxygen levels reduce the body’s resistance to bacteria and healing potential.

However, changing this chronic wound to an acute wound through curettage, together with a local application of Bluem oral gel on the wound bed, will greatly aid in the repairing process and accelerate healing.

why bluem?

Often, products like chlorhexidine digluconate, hydrogen peroxide, triclosan and other local or systemic antimicrobials are used to support periodontal treatment. These products are known to have beneficial effects. They also have their limitations, disadvantages and side effects. The topical application of oxygen as a support for tissue regeneration and wound healing is a much safer alternative which benefits the human body.

Bluem increases the local active oxygen available in the wound area. It contains low concentrations of sodium perborate, and the enzyme glucose oxidase (GOX). On application, and in the presence of moisture, the sodium perborate converts to sodium borate and H2O2 (hydrogen peroxide), while the normally inactive GOX causes a gradual conversion of glucose into gluconic acid and H2O2. These low levels of hydrogen peroxide (0.003 – 0.015 %) act as a disinfectant and is present along with the ROS (Reactive Oxygen Species) during the respiratory burst of neutrophils found in normal body fluid. This has a chemotactic effect on leucocytes.

The concentration of oxygen is not comparable to the higher concentrations (1.5 – 3.0%) of H2O2 commonly used in other oral disinfectants. It has been proven that tissue damage can occur at these higher levels of oxygen due to the production of free radicals in the wound. Research has shown that a continuous presence of a low concentration of hydrogen peroxide, such as that found with Bluem, kills pathogenic bacteria much more effectively than a single high concentration without damage to fibroblasts.

tel: 01535 656 312 | www.swallowdental.co.uk/bluem
case study

A 39 year-old man signed up as new patient in our practice. Intra-oral examination showed peri-implantitis at 36 with bone loss and some mobility (Fig. 1). After oral hygiene advice we curetted the pyorrhoea around 36, which was then treated with Bluem gel. The oral gel was injected deeply around the implant with a syringe, at first weekly, then bi-weekly. The gel was rinsed away with NaCl after one minute and a further application made and left in situ. The patient was instructed to brush twice daily with Bluem toothpaste, to rinse twice daily with Bluem mouthwash and to rub Bluem oral gel around the implant once a day (in the evening after rinsing). After three months, we reduced the professional application of gel to once every three months, while the patient continued with the procedure described above. After a year, we took another X-ray (Fig. 2). This clearly shows new vertical bone growth. Pocket depth fell from nine to four mm. Tissue around the implant was no longer red and swollen. The gum again fit tightly around the implant. There was no bleeding during examination and the implant showed no mobility.

Dr. R. Muts, Apeldoorn, The Netherlands *References supplied upon request.

bluem contains:

- Sodium Perborate – a reaction occurs after contact with fluid (saliva/blood) which releases active oxygen that kills anaerobic bacteria.
- Honey – Anti-bacterial & Anti-Fungal – also releases oxygen/peroxide
- Xylitol – Natural sweetener that eliminates the need for fluoride in the product.
- Lactoferrin – Thickens the mucosa and accelerates bone growth

benefits for you and your patients

Bluem accelerates wound healing, implant integration and bone regeneration in a safe, effective and non-invasive way.

- Oxygen is released within 30 seconds and penetrates approx. 0.5 cm into tissues.
- Effective on all micro-organisms.
- Unlike anti-biotics, no immune resistance is developed.
- No side effects known.
- No toxic ingredients.
- Unlike chlorhexidine, Bluem only kills bad bacteria (anaerobic) not the good bacteria.
- Quick and easy to apply.
- Simple concept.
- Low cost treatment.

In addition, Bluem is fluoride free. Fluoride has been shown to attack the passive layer of titanium implants.

available in:

- Mouthwash - 50 ml & 500 ml
- Toothpaste - 15 ml & 75 ml
- Oral Gel - 15 ml

Oral Gel can be used post-surgery to enhance tissue healing as well as in periodontitis and peri-implantitis cases.

† Active Oxygen - Molecular oxygen only contributes to cell metabolism but does not function as a bio-signal for the growth of cells. By contrast active oxygen, derived from Reactive Oxygen Species (in this case H₂O₂), does function as a bio-signal for cell growth. Low dose H₂O₂ (below 1.5%) thus has three major functions in the wound healing process:

- Playing an important role in the antimicrobial host defense.
- Has a chemotactic effect on neutrophils as a first response to injury.
- Plays an important role in the redox signaling and as a gene regulator (e.g. VEGF and IL-8)
Why Oxygen?


Oxygen is a prerequisite for successful wound healing due to the increased demand for reparative processes such as cell proliferation, bacterial defence, angiogenesis and collagen synthesis. Even though the role of oxygen in wound healing is not yet completely understood, many experimental and clinical observations have shown wound healing to be impaired under hypoxia. Restricted oxygenation, common in chronic wounds, impairs the healing process. Several studies have demonstrated that enhancing wound tissue oxygenation improves wound healing and reduces bacterial colonization.

Adjunctive topical Reactive Oxygen Species (ROS) in periodontitis and periimplantitis – a pilot study Berendsen JLM1, el Allati I1, Sylva LH1, Blijdorp PA2, Meijer GJ3 1 Mondzorgkunde Hogeschool Utrecht, Oral Hygiene Practice C M M Berendsen-Wolters, Venlo, The Netherlands 2 Department of Oral & Maxillofacial Surgery, Rijnstate Hospital Arnhem, The Netherlands 3 Departments of Periodontology & Biomaterials and Oral & Maxillofacial Surgery, Radboud University Nijmegen, The Netherlands

The aim of the study was to gain insight into the healing effects of ROS in periodontitis and periimplantitis. Healing is induced through the release of active oxygen in the peri-dental and peri-implant area. As reported elsewhere, in addition to mechanical/instrumental treatment, bluem’s effect derives from oxygen’s effect on tissue and bacteria. The chlorhexidine gluconate rinse had the lowest MICs compared with honey mouthrinse effectively inhibited the six tested microorganisms. The chlorhexidine gluconate rinse had the lowest MICs compared with honey mouthrinse effectively inhibited the six tested microorganisms. The chlorhexidine gluconate rinse had the lowest MICs compared with honey mouthrinse effectively inhibited the six tested microorganisms.

Why Honey?

A Comparative Evaluation of the Antibacterial Efficacy of Honey In-Vitro and Antiplaque Efficacy in a 4-Day Plaque Regrowth Model In Vivo: Preliminary Results S. Aparna,* S. Srirangarajan,* Veena Malgi,* Krishnanand P. Setlur,† R. Shashidhar,‡ S. Srirangarajan,* Veena Malgi,* Krishnanand P. Setlur,† R. Shashidhar,‡ Swati Setty,§ and Srinath Thakur§ J Periodontol, September 2012, Volume 83, Number 9, pp1116-1121

The aims of this study were: 1) to evaluate the antibacterial efficacy of honey against oral bacteria and compare this with 0.2% chlorhexidine; and 2) to compare ant plaque efficacy in vivo with chlorhexidine. The study was conducted in two parts. 1) in vitro; the inhibitory effects of three test agents, 0.2% chlorhexidine gluconate, honey mouthwash, and saline, against six oral bacteria at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 mg/ml, tested in duplicate. 2) the in vivo part consisted of a double-masked parallel clinical trial based on a 4-day plaque regrowth model. Sixty-six volunteers, 20 to 24 years of age, participated in the study. The plaque scores were compared at baseline and at the end of 4 days. The mean plaque scores were 1.77 – 0.86, 1.64 – 0.90, and 3.27 – 0.83 for groups 1, 2, and 3, respectively. Results: The honey mouthrinse effectively inhibited the six tested microorganisms. The chlorhexidine gluconate rinse had the lowest MICs compared with honey and saline rinses for all test species examined. The in vivo results revealed that plaque formation was inhibited/reduced by chlorhexidine and honey rinses. Conclusion: Honey has a potent broad-spectrum antibacterial action that may make it suitable for “anti-infective” treatment of periodontal disease. Honey has antibacterial action against tested oral microorganisms and also has antiplaque action.

Why Xylitol ?


Xylitol is used as a diabetic sweetener which is roughly as sweet as sucrose with 33% fewer calories. Unlike other natural or synthetic sweeteners, xylitol is actively beneficial for dental health by reducing caries to a third in regular use and helpful to remineralisation. 3 bluem

Bluem has been developed by a team of implantologists, oral surgeons and dentists led by oral surgeon Dr. Peter Blijdorp in the Netherlands. Initially, bluem oxygen gel was used in larger maxillary reconstructions using iliac crest bone. Bluem oral gel was used in attempts to counteract that dehiscences and necrosis that often occur in such cases. It proved to work quite well. It is striking that there were fewer complications and faster healing.

bluem’s effect derives from oxygen’s effect on tissue and bacteria. Research on the use of the hyperbaric oxygen tank and the effects of oxygen on anaerobic bacteria support the working of bluem.

bluem releases oxygen as soon as it comes in contact with body fluids like saliva. This oxygen penetrates deeply into tissues and reaches the places it is really needed. This keeps the pockets of teeth and implants free of harmful anaerobic bacteria.
Berendsen JLM¹, el Allati I¹, Sylva LH¹, Blijdorp PA², Meijer GJ³

¹ Mondzorgkunde Hogeschool Utrecht, Oral Hygiene Practice C.M.M. Berendsen-Wolters, Venlo, The Netherlands
² Department of Oral & Maxillofacial Surgery, Rijnstate Hospital Arnhem, The Netherlands
³ Departments of Periodontology & Biomaterials and Oral & Maxillofacial Surgery, Radboud University Nijmegen, The Netherlands

Adjunctive topical Reactive Oxygen Species (ROS) in periodontitis and peri-implantitis – a pilot study

Abstract

Aims: The aim of the study was to gain insight into the healing effects of ROS in periodontitis and peri-implantitis. Healing is induced through the release of active oxygen in the peri-dental and peri-implant area. The results were to be compared with the generally accepted – ‘gold standard’ – treatment strategies for these disease entities. As reported in the literature, in addition to mechanical/instrumental treatment, antimicrobials such as chlorhexidine digluconate (CHX) and hydrogen peroxide (H₂O₂) are often prescribed and used in these situations, as well as in many other different oral and dental disorders. These medications are known to have advantageous effects, but they also have their limitations, disadvantages and adverse effects. This pilot study is meant to suggest that ROS might be a better alternative.

Material and methods: A case control study, in which 33 patients were included, has been carried out to examine the effects of adjunctive treatment with ROS in periodontitis situations. Full mouth dental pocket depth recordings have been made before and within 3 months after treatment with ROS. In the peri-implantitis study 34 patients were included, with a total of 40 dental implants. They were all treated according to a standardized ROS peri-implantitis protocol and were both clinically and radiographically re-examined after 3, 6 weeks and after 3, 6 months, respectively.

Results: In the periodontitis study, after 3 months treatment with ROS, the average total pocket depth decrease was 56%. Different values were scored for male and female patients, 66 and 49%, respectively. Improvement was perceptible in all age categories. The age category of 40-44 years showed the greatest improvement (71%) and the category of 65-69 years the least (36%). There were no remarkable differences in relation to cigarette smoking habits: the average pocket depth decrease in smokers was 56%, in patients who had smoked in the past 55%, and in non-smokers 56%.

In the peri-implantitis study, the affected tissue had clinically noticeably recovered after 3 and 6 weeks in all cases. After 3 months, 75% of the peri-implantitis situations had been cured (with radiographically definite re-osseointegration in 15% of the implants), 9 peri-implantitis cases had not been cured yet and 1 implant was lost. After 6 months, radiographical examination showed re-osseointegration of 3 mm in 15% of cases, of 2 mm in 60%, and no signs of re-osseointegration in 4 cases.

Conclusions: From the case control periodontitis study results could be concluded that adjunctive ROS yielded better average total pocket depth reduction percentages than generally reported in the literature for other treatment strategies. From the ROS protocol peri-implantitis study results could be concluded that the clinical situation around implants improved markedly within 3 to 6 weeks in all cases. After 3 months, 75% of cases were clinically cured. Radiographically evidenced re-osseointegration of 2 mm could be noted in 60 % and of 3 mm in 15 % of cases after 6 months. These figures are indicative for faster and better pocket and peri-implantitis healing than reported in the literature for the generally accepted – ‘gold standard’ – adjunctive treatment regimens. A prospective double masked placebo controlled split mouth model adjunctive periodontitis treatment study with ROS is in its final preparation phase.

Key words: periodontal disease, implantology, treatment, re-osseointegration, clinical, radiographic
Introduction

Infections around teeth (periodontitis) and around endosseous dental implants (peri-implantitis) frequently occur in the elderly adult population. The estimated prevalence is approximately 50% (19.2 – 62.7%) for periodontitis and 1.0% - 19.0% for peri-implantitis, respectively (Bourgeois et al. 2007, Kaptein et al. 1999, Leeuwangh et al. 2006, Roos-Jansáker 2007). Untreated they cause symptoms of disease and ultimately loss of teeth and/or dental implants. Healthy life style, balanced, i.e. varied fresh food intake and routine oral hygiene measurements (tooth brushing, interdental cleaning [dental floss, brushes], mouth rinsing, et cetera) are meant to prevent these diseases to occur. In the healthy situation there is a so-called gingival sulcus or crevice, on probing not deeper than 3 mm around natural teeth (Fig. 1), with underneath the gingival soft tissues a firm fibrous tooth-bone attachment – the periodontal ligament.

Fig. 1. Healthy gingival sulcus or crevice, on probing not deeper than 3 mm around the tooth.

In the healthy dental implant situation, underneath the soft tissue barrier there is direct implant-bone contact without any interface, so-called osseointegration (Fig. 2). Probing depths till 5 mm are interpreted as physiological.

Fig. 2. Tooth and implant comparison, with direct implant-bone contact without any interface.

The chronic presence of dental plaque biofilm, dental calculus, with inherent oral microflora are the causative mechanisms in the etiology and pathogenesis of these two parallel presentations of the same disease entity, pockets and intrabony defects (Fig. 3).
An aerobic gram negative micro-organisms induce a chronic inflammation and all inflammatory reactions are present (redness [rubor], bleeding, pain [dolor], swelling [tumor], loosening and hypermobility of teeth, suppuration, bad breath [foetor ex ore or halitosis]). Smokers and patients with a history of periodontal disease are at higher risk to develop peri-implantitis (Roos-Jansäker 2007). The indicated mechanical treatment of periodontitis and peri-implantitis lesions is rather invasive, painful (need for local anaesthesia and analgesics), intensive, time consuming, expensive and difficult (Greenstein 2005, Hanes & Purvis 2003). Additionally and alternatively chlorhexidine digluconate (CHX), hydrogen peroxide (H₂O₂)-solutions, triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), povidone-iodine, other locally delivered antimicrobials (LDAs) and (systemic) antibiotics are often prescribed and used in different dental disciplines (De Araújo Nobre 2006, Hanes & Purvis 2003). These remedies are known to have advantageous effects, but they have limitations, disadvantages and negative side effects as well (Ribeiro et al. 2004). CHX has been reported to be highly cytotoxic in vitro and to exert toxic effects on periodontal tissues (Giannelli et al. 2007). Hydrogen peroxide has been associated with DNA-damage and carcinogenesis (Naik et al. 2006, Ribeiro et al. 2007). The administration of local and systemic antibiotics has been criticized for reasons of creating (multi)resistant micro-organisms, allergic reactions, hypersensitivity, and other adverse reactions in patients (Bidault et al. 2007).

The ROS gel is capable of releasing oxygen, without freeing oxygen radicals as in hydrogen peroxide (H₂O₂)-solutions, and without inducing damage to the DNA of oral mucosal cells, or carcinogenesis (European Commission Health & Consumer Protection Directorate-General 2005, Li et al. 1998 a,b, Li & Ramaekers 2003, Ribeiro et al. 2004). The ROS gel was originally discovered by a Dutch dentist and has been patented in 1996 (Van den Bosch 1997. In-vitro studies have demonstrated that ROS gel obstructed the growth and development of harmful bacteria and fungi. These micro-organisms were not resistant to the high oxygen concentrations, as existing in the application of the oxygen complex in special care products (Kreis et al. 2004, Li et al. 1998 a). There is a choice of 10 concentrations of ROS solutions, of which the highest concentration (10 eq) is used for the successful treatment of chronic nail infections (onychomycosis) and the lowest concentration (1 eq) is used for the treatment of gingivitis. The effects of ROS tooth gel on bacteria involved in periodontitis have been studied by Camp (2002). There is limited literature data about other indications and applications of ROS, but it has been successfully used in halitosis, onychomycosis, tinea pedis, psoriasis, eczema, burn lesions and chronic wounds as in diabetes patients (Enoch & Harding 2003, Kreis et al. 2004, Meinardi et al. 2004).
This study aimed at investigating the healing effects of ROS on the periodontal ligament and peri-implant mucosa, thereby introducing ROS as an alternative for the above mentioned remedies in periodontitis and peri-implantitis. The results will be compared with the generally accepted – ‘gold standard’ – treatment strategies for these diseases, as described in the literature (Greenstein 2005).

Materials and Methods

Periodontitis study
A case control study was performed to analyze the effects of treatment with ROS in 33 (20 female and 13 male) patients with periodontitis (Table 1). The inclusion criteria were: clinical diagnosis of manifest periodontitis, pocket depth measuring with the Goldman–Fox/- Williams probe (Fig. 4) and recording before the start of the treatment with ROS according to the protocol (concentration 5eq and 2eq for gel and mouth wash, respectively) and after maximally 3 months therapy.

Table 1. Periodontitis patients

<table>
<thead>
<tr>
<th>Periodontitis patients</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>20</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

n = number of patients

Protocol ROS

The periodontitis / peri-implantitis treatment protocol in short:
- Curettage of the pocket
- Syringe the pocket with ROS gel (concentration 5eq), remaining in situ for 5 minutes
- Washing the gel away with saline
- Syringe the pocket again with ROS gel (concentration 5eq), application remains
- Instruct the patient to rinse and/or brush three times a day with ROS oral washing (concentration 2eq)

The protocol included that the pockets were curetted mechanically and ROS gel (concentration 5eq) was applied by a syringe. This was kept in situ for 5 minutes, was rinsed off with physiologic saline solution, and the same concentration 5eq gel was applied again and then remained. The patients were instructed to rinse and/or brush three
times a day with ROS oral washing (concentration 2eq). After maximally three months, the patients returned for a final clinical check-up. Based on the full mouth pocket depth probing values a so-called pocket status form was completed for each patient. The forms were numbered and the patients’ pocket depths were added up per pocket status. The differences between these sums before and after treatment with ROS were calculated per patient and transformed into difference percentages with the help of SPSS computer programs. Mutual comparisons have been made, between men and women, smokers, and patients who had smoked in the past and non-smokers, and between the different age groups, respectively.

**Peri-implantitis study**
A case control study to analyse the effects of treatment with ROS in 34 patients (25 female, 9 male) with a total of 40 dental implants, with peri-implantitis (Table 2).

<table>
<thead>
<tr>
<th>Peri-implantitis patients</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>25</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
</tr>
<tr>
<td>Total patients</td>
<td>34</td>
</tr>
<tr>
<td>Total implants</td>
<td>40</td>
</tr>
</tbody>
</table>

*n = number*

The inclusion criteria were the following: clinical diagnosis of manifest peri-implantitis, a minimal pocket depth of 5 mm (pocket depth measuring and recording with the Goldman- Fox/Williams probe) (Figure 4), with swollen soft tissues around the implant and bleeding occurring rapidly on inspection and initial cleaning (Fig. 5). A general characterization of inflammation/infection was required. The suprastructure had to be constructed according to the state of art techniques, and the patient’s general condition had to correspond to the present valid standards required for performing justified implantology.

*Fig. 5. Manifest peri-implantitis, with swollen soft tissues around the implant*
First of all, the pockets were curetted mechanically and ROS gel (concentration 5eq) was applied by a syringe. This was kept in situ for 5 minutes, was rinsed off with physiologic saline solution, and the same concentration 5eq gel was applied again and then remained. The patients were instructed to rinse and/or brush three times a day with ROS oral washing (concentration 2eq). After three weeks, the patients returned for a clinical check-up and after six weeks the patients returned for a second treatment, which was identical to the first treatment. After three months, again there was a clinical check-up. If there were still signs of infection, the patient went back to the six weeks’ schedule. When there were no further complications, the dental hygienist would see the patient for a clinical check-up every 2 years. The patients continued rinsing/brushing with the ROS oral washing (concentration 2eq).

The radiographs taken postoperatively and at 3 and 6 months after therapy respectively, were evaluated with respect to potential re-osseointegration around the implants and diminishing of the intrabony pocket radiolucencies (Fig. 6).

![Fig. 6. Details of panoramic radiographs showing intrabony pocket radiolucencies around the implants; left side pre-treatment, right side 3 months post-treatment with ROS](image)

### Results

#### Periodontitis study

The average decrease of the pocket depth after treatment with ROS was 56%, meaning that after maximally 3 months treatment the total pocket depth decreased by more than half (Table 3).

<table>
<thead>
<tr>
<th>Differences in pocket depth</th>
<th>n</th>
<th>average in %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>56</td>
<td>± 23</td>
</tr>
</tbody>
</table>

**Average improvement of pocket depth in %**

n = number of patients

SD = standard deviation
In the results a distinction could be made between men and women with regard to the average decrease of the pocket depth. The male patients had an average improvement of 66%, whereas the female patients had an average improvement of 49% (Table 4).

**Table 4.**

<table>
<thead>
<tr>
<th>Sex differences</th>
<th>n</th>
<th>average in %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>20</td>
<td>49</td>
<td>± 24</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>66</td>
<td>± 17</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>56</td>
<td>± 23</td>
</tr>
</tbody>
</table>

Average improvement of pocket depth in % with regard to sex  

- **n** = number of patients  
- **SD** = standard deviation

The improvement was perceptible in all age categories, but the age category of 40-44 years showed the greatest improvement (71%). The age category of 65-69 showed the slightest improvement (36%) (Table 5).

**Table 5.**

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>average in %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 - 39 years</td>
<td>3</td>
<td>66</td>
<td>± 7.5</td>
</tr>
<tr>
<td>40 - 44 years</td>
<td>5</td>
<td>71</td>
<td>± 31</td>
</tr>
<tr>
<td>45 - 49 years</td>
<td>3</td>
<td>54</td>
<td>± 16</td>
</tr>
<tr>
<td>50 - 54 years</td>
<td>2</td>
<td>53</td>
<td>± 35</td>
</tr>
<tr>
<td>55 - 59 years</td>
<td>7</td>
<td>66</td>
<td>± 18</td>
</tr>
<tr>
<td>60 - 64 years</td>
<td>6</td>
<td>51</td>
<td>± 21</td>
</tr>
<tr>
<td>65 - 69 years</td>
<td>7</td>
<td>36</td>
<td>± 19</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>56</td>
<td>± 23</td>
</tr>
</tbody>
</table>

Average improvement of pocket depth in % with regard to age  

- **n** = number of patients  
- **SD** = standard deviation

In addition, the results of the ROS treatment had been related to cigarette smoking habits. For smokers, patients who had smoked in the past and non-smokers, the respective percentages were not much different (Table 6).

**Table 6.**

<table>
<thead>
<tr>
<th>Smoking habits</th>
<th>Average improvement in %</th>
<th>n</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smoking</td>
<td>19</td>
<td>56</td>
<td>± 20</td>
</tr>
<tr>
<td>Smoked in the past</td>
<td>3</td>
<td>55</td>
<td>± 23</td>
</tr>
<tr>
<td>Smoking</td>
<td>11</td>
<td>56</td>
<td>± 30</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>56</td>
<td>± 23</td>
</tr>
</tbody>
</table>

Average improvement of pocket depth in % with regard to smoking habits  

- **n** = number of patients  
- **SD** = standard deviation
**Peri-implantitis study**

In general, already after three weeks of ROS protocol therapy, the peri-implant situation appeared clinically improved or even healthy in all cases (Fig. 7). After six weeks a decrease of the pocket depth was noticeable in all patients. In 30 cases, the soft tissue had settled more firmly and tight around the implants, which made it more difficult to probe. Additional curetting around these implants could and did not take place.

![Fig. 7. Soft tissue recovery after peri-implantitis (Fig. 5), healthier situation after three months ROS protocol therapy](image)

In order to avoid tissue damage, no mechanical cleaning was conducted, only the ROS gel (concentration 5eq) was syringed. In 4 cases repetition of the first treatment was indicated: curetting, syringing ROS gel (concentration 5eq) according to the protocol, washing the gel away and syringing the pocket again with ROS gel (concentration 5eq). Within three months, 30 implants (75%) could be cured clinically, 6 of which showed re-osseointegration on the radiographs all around the implants’ deep pockets (15%). Nine other implants had not been cured yet and 1 implant was lost. After six months, 6 implants showed a re-osseointegration of 3 mm on the radiographs (15%). The other 24 cured implants showed a re-osseointegration of 2 mm (60%)(Table 7). In 4 cases there were no signs of re-osseointegration.
### Table 7.

<table>
<thead>
<tr>
<th>Peri-implantitis patients</th>
<th>n = 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implants</td>
<td>n = 40</td>
</tr>
<tr>
<td>After 3 weeks, in patients</td>
<td>- In 34 patients clinical improvement – healthy look</td>
</tr>
<tr>
<td>After 6 weeks, in patients - probing</td>
<td>- In 34 patients pocket depth reduction</td>
</tr>
<tr>
<td></td>
<td>- In 30 patients soft tissue firmer/tighter around the implant(s)</td>
</tr>
<tr>
<td></td>
<td>- In 4 patients indication for repeating treatment: irrigation pockets with ROS gel concentration 5eq, et cetera (see protocol)</td>
</tr>
<tr>
<td>After 3 months, in implants (clinically and radiographically)</td>
<td>- 1 implant lost</td>
</tr>
<tr>
<td></td>
<td>- 9 implants not yet cured</td>
</tr>
<tr>
<td></td>
<td>- 30 implants free of pathology (75%)</td>
</tr>
<tr>
<td></td>
<td>- 6 implants re-osseointegration noticeable on the radiographs (15%)</td>
</tr>
<tr>
<td>After 6 months, in implants (radiographically)</td>
<td>- 6 implants re-osseointegration 3 mm (15%)</td>
</tr>
<tr>
<td></td>
<td>- 24 implants re-osseointegration 2 mm (60%)</td>
</tr>
<tr>
<td></td>
<td>- 4 implants no signs of re-osseointegration</td>
</tr>
</tbody>
</table>

n = number

### Discussion

In the literature series of generally accepted – ‘gold standard’ - treatment strategies for periodontitis have been described.

The effect of dental plaque control and surgical pocket elimination on the establishment and maintenance of periodontal health was already described by Lindhe & Nyman in 1975. Their results demonstrated that it was possible to treat periodontal disease successfully, even in advanced stages, in patients willing to maintain a plaque-free dentition. In this way an improvement of the pocket depth with more than 47% was perceptible.

The therapy defined by the American Academy of Periodontology (2000, Greenstein 2005) is called initial therapy and consists of supra- and subgingival scaling and rootplaning, and potentially supplementary periodontal surgery, such as flap operations. At the same time dental hygienic guidance and correct instruction are essential. From the publication of the American Academy of Periodontology (2000) on the parameter of chronic periodontitis and the loss of supporting tissue, it could be inferred that the initial therapy will not be successful with all patients or in all cases. In specific places or with certain patients extra therapy may be necessary.

Locally delivered antimicrobials (LDAs) may be used in support of the initial therapy as adjuncts to mechanical therapy in treatment of recalcitrant deep (> or = 5mm), active, non-responding sites, providing the patient's oral hygiene is adequate (Hussein et al. 2007).

Recent interest in the local application of antimicrobial and anti-inflammatory agents has stimulated interest in the efficacy of various treatment regimens. The clinical and microbiological effects of subgingival and gingival marginal irrigation with chlorhexidine digluconate (CHX) have been studied (Jolkovsky et al. 1990, Giannelli et al. 2007). The findings suggested that it was possible to achieve beneficial effects from adjunctive single professional 0.12% CHX irrigation and home 0.04% CHX...
subgingival irrigations in periodontal maintenance patients receiving supportive periodontal treatment. The average reductions in probing depth between the baseline and the three months’ visit were 4.2%.

Along with good subgingival cleaning and an optimal dental hygiene, the microbiological compounding of the subgingival plaque, is one of the modifying factors. The use of a systemic periodontal antimicrobial therapy should always be based on detailed clinical examination and microbiological analysis. The micro-organisms involved are mainly: Actinobacillus actinomycetemcomitans (A.a.), Porphyromonas gingivalis (P.g.), Tannerella forsythensis (T.f.) (formerly Bacteroides forsythus), Treponema denticola (T.d.), Prevotella intermedia (P.i.), Peptostreptococcus micros (P.m.), Fusobacterium nucleatum (F.n.), Campylobacter rectus (C.r.), Eubacterium nodatum (E.n.), Eikenella corrodens (E.c.), Capnocytophaga species (C.s.) (Camp 2002, Leeuwangh et al. 2006).

In a study of Abbas & Van Winkelhoff (2004) the impact of antibiotics on the indication for periodontal surgery was described. Based on scientific literature about the clinical effects of antibiotics on the treatment of periodontitis with regard to plaque related disorders, it was concluded that application of antibiotics could be a valuable addition to conventional periodontal treatment. However, not all patients benefited from an antimicrobial therapy. Provided that it was used in the right way, particular systemic antibiotics (amoxicillin, metronidazole, or a combination of both) could reduce the indication for periodontal surgery significantly, especially around teeth with one root and in aesthetically sensitive areas. In mouths in which the infection was under control, the indication for successful regenerative periodontal surgery could increase. This also counted for plastic periodontal surgery, such as the covering of gingival recessions.

According to Renvert et al. (2006) the adjunctive use of minocycline microspheres resulted in improvements of probing depths and bleeding scores, whereas the adjunctive use of chlorhexidine only resulted in limited reduction of bleeding scores. For the deepest sites of the treated implants in the minocycline group, the mean probing depth was reduced from 5.0 to 4.4 mm (i.e. 12% reduction) at 12 months (Renvert et al. 2006).

The abovementioned treatment strategy of periodontitis has less effect on smokers than on non-smokers (American Academy of Periodontology 2000). In view of this information it could be stated that the adjunctive treatment of periodontitis with ROS seems to be a positive turn for smokers.

In respect of the effect of age on the treatment results it is worthwhile to note that in general the patient numbers in the higher age category are rising. Since the amounts in the different age categories in this pilot study are rather low, a realistic outcome as to the effects of ROS with regard to age cannot be determined. For this matter a more extended study would be needed, with a larger population and sufficient patient amounts in each age category.

A logical continuation of this case control pilot study into the effects of adjunctive ROS in periodontitis patients would be a prospective study in a preferably larger patient population, with defined protocols and explicit guidelines. Considering the group to be examined, a university periodontal practice would be a sophisticated choice for the place of action. In this way it should be possible to obtain a more realistic - evidence based -
image of the effects of the adjunctive treatment of periodontitis with ROS on probing depth, bleeding scores, gingival recession, and clinical attachment level (CAL).

Notwithstanding the fact that most implant systems have a 90% or higher success rate (O’Neal et al. 1992), one of the major causes of implant loss is peri-implantitis. More or less the same groups of micro-organisms seem to be involved in peri-implantitis as in periodontitis (Botero et al. 2005, Laine et al. 2005, Leeuwangh et al. 2006).

Generally accepted – ‘gold standard’ - treatment strategies for peri-implantitis have been described in the literature as well (De Araújo Nobre 2006). Peri-implant tissues are treated in more or less the same way as the periodontium of natural dental elements in case of periodontitis: an initial treatment with conservative measures as (ultrasonic) curetting, scaling, planing, and 0.1% chlorhexidine digluconate (CHX) irrigation, based on the principles of mechanical and chemical cleaning and disinfection (Wetzel et al. 1999). The titanium surface of the implants easily gets scratched and damaged by metal instruments. Because of that the surface-qualities of the implant are deteriorated and plaque-retention increases. In order to prevent this, it is deemed better to use specially designed implant instruments of a synthetic material, spray instruments or ultrasonic instruments, in combination with hydrogen peroxide-solution (3%) (Speelman & Collaert 1990, Strooker et al. 1998). If this treatment strategy is not successful it is advised that the patient will be placed on a 10 day systemic antibiotic combination course (e.g. amoxicillin and metronidazole). In case of lack of improvement, a flap operation, with or without gingivectomy and polishing of the implant surface can be performed and that open site could also be treated with an antimicrobial solution. If there is pain afterwards, analgesic medication in combination with a systemic antibiotic regimen is advised.

According to Wetzel, it appears that if peri-implantitis is to be ‘cured’ and re-osseointegration is to be achieved, an effective antibacterial therapy has to be applied (Wetzel et al. 1999). Even then, it remains difficult to achieve true re-osseointegration. A published possible treatment modality of peri-implantitis to achieve bone formation around and re-osseointegration of dental implants is based on application of recombinant human bone morphogenetic protein type-2 (rhBMP-2) in peri-implantitis defects (Hanisch et al. 1997).

If all these measures have no noticeable effect and the patient remains in pain and discomfort, then removal of the implant would be the next step (Wiskott et al. 2004). Ultimately, approximately 10% of the implants fail.

Peri-implant problems could be looked upon as poorly healing chronic wounds (Blijdorp 2006). Generally, the vascularity, blood flow and oxygenation in the area surrounding chronic wounds are compromised, which hampers initiation and continuation of the healing process. Mainly due to the facts that tissue with a poor blood flow has a reduced resistance to infections and the process of self-healing is compromised, the role of adjunctive active oxygen is paramount (Blijdorp 2006).

With regard to the topical application of ROS active oxygen in peri-implantitis, in three weeks the clinical situation already looked healthy or at least healthier from the outside. Similarly, parallel studies of ROS topical application in onychomycosis, burn lesions and chronic wounds, as in diabetes mellitus patients, showed overwhelming results and the
researchers noticed a recovery after only a few weeks, which generally could not have been obtained in several months with other methods (Kreis et al. 2004, Enoch & Harding 2003).

On the whole, dental implantology is successful, however, the longer the speciality exists (actually, more than 20 years now), the more patients there will be who can only retain their implants with difficulties, for general or local reasons. One of the reasons is the ongoing increase of higher age group patient numbers and the inherent progressive diminishing of motor and mental abilities. The patients’ immune status also becomes weaker, and consequently dental implants, which are *corpora aliena* after all, may become more difficult to clean and to retain. Usually, after 8-10 years of presence, the lowest figures of implant loss are reached. Thereafter peri-implant problems tend to occur more frequently leading to higher implant losses. Up till now, the patients with peri-implantitis were treated by means of flap operations. The implants were partly uncovered, the surface of the implant would be polished and treated with disinfectants, etching gel and other (bio)materials (Strooker et al. 1998). The results of these treatment strategies were not always very encouraging. Chlorhexidine digluconate (CHX) was most frequently used in an attempt to eliminate the bacteria in the pocket. When intrabony problems underlie the soft tissue peri-implantitis, a surgical intervention with curetting might still be the right option. However, a surgical intervention alone is unlikely to reactivate the titanium surface or achieve wound recovery at both the soft tissue level and the bony structure level.

The Adjunctive topical Reactive Oxygen Species (ROS) application is claimed to be a valuable contribution to ‘diseased implant’ salvage and implant survival (Blijdorp 2006). The ROS gel not only has proven antibacterial and antifungal effects in-vitro, but it also has cleansing and purifying effects. The experience in general medicine with regard to chronic wound treatment is that ROS gel (concentration 5eq) cleans the wounds. One of the other in-vitro findings is that ROS gel is not mutagenic or cytotoxic, it does not cause cell death, neither of erythrocytes and leucocytes, nor oral mucosal cells and osteoblasts. It is thought to activate the release of wound-healing enzymes. There also are indications that it has pain and inflammation reducing effects. In comparison with hydrogen peroxide, which causes necrosis of erythrocytes and, in concentrations higher than 1%, stimulates radicals and therefore assumed to be carcinogenic, the ROS gel (concentration 5eq) stands out rather positively.

In fact, the exact working mechanism of ROS gel is not yet fully understood or known, i.e. why it activates wound healing so quickly and even brings about bone formation around infected implants. The clinical findings are promising. As mentioned above, more extensive clinical follow-up studies of ROS gel within the dental and medical field are indispensable (Blijdorp 2006). Some of the methods used in the actual pilot study have been proven to be adequate and detailed, specifically the technique of full mouth pocket probing with the Goldman–Fox/-Williams probe (Grisi et al. 1998, Araujo et al. 2003, Buduneli et al. 2004). In order to avoid traumatizing the healing tissue, the peri-implantitis patients should not be examined so early as after three weeks with a pocket probe. Other limiting factors are the radiographic representations of the buccal and lingual respectively palatal sides of the implants which are not perceptible. For that reason, in the future, 3-D cone beam CT-scanning would be an option.
Conclusion

This paper hypothesizes that the adjunctive treatment of periodontitis and peri-implantitis, according to the ROS gel protocol shows a larger pocket reduction and faster wound healing compared to the generally accepted treatment strategies as described in the literature. Further and additional studies on (non-)prescription antimicrobial oral care products may lead to new regimens for decreasing the burden of periodontal and peri-implant diseases in the population. A prospective double masked placebo controlled split mouth model adjunctive periodontitis treatment study with ROS is in its final preparation phase to prove above mentioned hypothesis.
References


26. Li, Y., Zhang, W., Xu, Y., Davis, J. & Klaunig, J. (1998a) Peroxide content and free radical generation by OT7051 tooth whitening gel, Ref: 98-004. Loma Linda University, Loma Linda, Ca, USA and Indiana University, Bloomington, In, USA.


Application of toothpaste and mouthwash “BLUEM” in complex hygienic oral care for patients with coronary heart disease

I.M. Makeyeva, N.V. Tambovtseva

I.M. Sechenov First Moscow State Medical University

Summary. For patients with coronary heart disease oral hygiene and treatment of inflammatory changes of oral mucosa and periodontal diseases is very important. Assessment of dental status of 110 patients routinely admitted to the cardiology department showed that they all require treatment of dental and periodontal diseases and diseases of oral mucosa. In 100% of cases it is necessary to perform training on proper hygienic oral care. Inclusion of toothpaste and mouthwash “Bluem” in complex hygienic oral care reduced severity of inflammatory changes and improved the hygienic status of the oral cavity.

Keywords: coronary heart disease, oral hygiene, oral inflammatory diseases.

Importance of the problem. Coronary heart disease is an essential problem of today’s medicine and according to data of E.I. Chazov (2004) it is on the first place among mortality causes of world’s population and it is also one of the most prevalent causes of loss of earning capacity and disability [5]. Problem of influence of chronic infection foci in oral cavity on development of cardiovascular diseases is still important for all countries throughout the world: presence of odontogenic infection foci and their determined influence on cardiovascular status in this category of patients impose necessity for studying dental status and determination of oral inflammatory diseases [1, 2, 6, 7]. According to A. Spahr (2006) and M. Stelzel (2003) the etiological model of influence of odontogenic infection foci on development of cardiovascular diseases is associated with the fact that periodontal pathogenic agents are able to contaminate endothelial cells of coronary arteries promoting platelet aggregation [8, 9]. Despite development of more and more effective products for hygienic
oral care, the prevalence of periodontal diseases does not decrease even in somatically healthy patients (A.I. Grudyanov, 2004). It is well known, that patients taking antiarrhythmic and antihypertensive drugs may develop dryness of mouth, which promotes foul breath, quick formation of dental plaque, worsens prognosis for dental diseases and negatively influences cardiovascular status [3, 4, 7] and exacerbates unfavorable hygienic status of oral cavity.

Taking into account the prevalence of coronary heart disease, in these patients it is required to perform comprehensive assessment of dental status, to reveal odontogenic inflammation foci, inflammatory periodontal diseases and their treatment, development of recommendations on personal hygienic oral care, considering possible development of dryness of mouth mucosa. Traditional inclusion of chlorhexidine-based toothpastes and mouthwashes in complex treatment may cause such adverse effects as dysbiotic changes, discoloration of tongue and tooth, taste disorders. Therefore those studies are important which are designed to determine and assess efficacy of products for personal hygienic oral care for constant use with anti-inflammatory, antiseptic and moisturizing effect, but causing no dysbiotic changes in oral cavity.

**Purpose of the study** is to determine dental status of the patients routinely admitted to the cardiology department of University Clinical Hospital № 2 of I.M. Sechenov First Moscow State Medical University and assess efficacy of hygienic complex “Bluem”, containing lactoferrin and releasing active oxygen during use. Complex of dental hygiene products “Bluem” (toothpaste and mouthwash) is intended for hygienic oral care, including patients with dental implants. The toothpaste and mouthwash does not contain antibacterial agents, its efficacy is determined by the release of active oxygen and content of lactoferrin. The toothpaste and mouthwash can be recommended for constant use.
Methods and materials of the study. One hundred and ten (110) patients with coronary heart disease routinely admitted to the cardiology department participated in this study. Only those patients participated in the study that could perform appropriate self-care and were ready to correct oral hygiene skills. During the study we assessed compliance of patients’ dental status with dentist’s conclusion included in the list of documents necessary for hospital admission.

Patients were examined on admission day. All patients underwent a standard dental examination with determination of hygienic and periodontal indexes: OHI-S index [Green J.C., Vermillion J.R., 1964], periodontal index – PI [Russell A., 1956], gingival bleeding index [Muhlemann H.P., 1971], papillary marginal alveolar index – PMA [Parma C., 1960]. After examination the patients obtained recommendations on necessary treatment and hygienic oral care. Patients with suspected periapical odontogenic foci and chronic generalized severe or moderate periodontitis underwent orthopantomography.

Further the patients were divided in two groups: study group where hygienic care was performed with toothpaste and mouthwash “Bluem”. Patients that continued using their own regular hygienic care products after training were included in control group. If they have not previously used dental floss, they were recommended to use it every day and appropriate training was performed. Protocol of “Bluem” complex application included tooth brushing with the toothpaste twice a day and use of the mouthwash three times a day. Another determination of indexes was performed on Day 10.

Study results. As a result of performed dental examination it was determined that among 110 patients oral cavity was treated only in six of them (5.45%) (i.e. no unsealed dental decay cavities, abscess formation, fistulas, manifestations of oral mucosa diseases were revealed), although all the patients submitted the
medical note on treatment. In patients’ words, they obtained the medical note on treatment without examination of oral cavity.

At examination of patients periodontal diseases were revealed in 100 patients (90.91%), provided that chronic generalized moderate periodontitis was revealed in 34 patients (36.36 %), and chronic generalized severe periodontitis was revealed in 66 patients or in 60% of cases. Analysis of patients complaints revealed during the interview provided us with data presented in Table 1.

**Complaints revealed during the interview**  
Table 1

<table>
<thead>
<tr>
<th>Complaints</th>
<th>Number of patients (total/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gingival bleeding</td>
<td>102 (92.73)</td>
</tr>
<tr>
<td>Pain caused by different irritants</td>
<td>86 (78.18%)</td>
</tr>
<tr>
<td>Burning tongue</td>
<td>73 (66.36%)</td>
</tr>
<tr>
<td>Dryness of mouth</td>
<td>19 (17.27%)</td>
</tr>
<tr>
<td>Foul breath</td>
<td>91 (82.73%)</td>
</tr>
</tbody>
</table>

Chronic recurrent ulcerative stomatitis was revealed in 14 patients (12.73%), angular cheilitis – In 34 patients (30.91%), lichen ruber planus (typical form) in 3 patients (2.73%).

At performance of index assessment the following data were obtained: mean value of OHI-S index was 3.6±0.6; PMA – 69.5±3.1; PI – 4.6±0.4. Gingival bleeding of first and second degree was revealed almost in all patients.

It was found that normalization of hygienic oral care provided positive effect on oral cavity and periodontal status, which was represented by the dynamics of hygienic and periodontal indexes (Table 2). However, in study group where patients used “Bluem” complex positive dynamics is more
pronounced. Patients who used “Bluem” complex noted its pleasant organoleptic properties and abundant foaming during use.

**Table 2**

**Dynamics of hygienic and periodontal indexes values**

<table>
<thead>
<tr>
<th>Index</th>
<th>Study group “Bluem”</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>after 10 days</td>
</tr>
<tr>
<td>OHI-S</td>
<td>3.6±0.6</td>
<td>1.7±0.4</td>
</tr>
<tr>
<td>PMA</td>
<td>69.5±3.1</td>
<td>52.1±2.4</td>
</tr>
<tr>
<td>PI</td>
<td>4.6±0.4</td>
<td>4.3±0.2</td>
</tr>
</tbody>
</table>

Analysis of dynamics of hygienic and periodontal indexes showed that patients who used “Bluem” complex had more positive tendency, than patients from control group. Thus, mean value of OHI-S index in study group is on the line between satisfactory and poor value, while in control group mean value of OHI-S index corresponds with poor result. In study group PMA index value decreased by 24.7%, while in control group this value was only 15.9%. Significant dynamics of PI index was not seen in any group as bone tissue status could not improve as a result of normalization of hygienic oral care.

Performed study enabled us to make the following conclusions:

1. Among 110 examined patients of cardiology department oral cavity was treated only in six patients and that comprises 5.45% cases. It is necessary to pay close attention to dental status of patients with coronary heart disease.

2. Data obtained in this study indicate that 100% of patients of cardiology department require dental treatment and/or professional oral hygiene and tooth brushing training.

3. Inclusion of toothpaste and mouthwash “Bluem” with active oxygen and lactoferrin provides positive dynamics of hygienic oral status and
decreases severity of inflammatory changes of gingival tissue in patients with coronary heart disease.

Literature

Oral oxygen therapy in dentistry

RONALD MUTS shares case studies in which he uses oxygen treatment for peri-implantitis and periodontitis...

Oxygen has been used to promote the healing of wounds in medicine for more than one hundred years. Since the sixties, hyperbaric oxygen therapy has been used successfully in the Netherlands, after heart surgery, for example, and in other cases of life-threatening infection with anaerobic organisms. Lives or limbs can often be saved.

Experiments have also been conducted with topical oral oxygen therapy in dentistry, primarily in larger maxillary reconstructions using iliac crest bone (figure 2). In such cases, bone loss regularly occurs as a result of dehiscences and necrosis of any exposed bone. In an attempt to prevent bone loss, and with the good results from hyperbaric oxygen therapy in mind, active oxygen was applied locally by means of a stabilised, active oxygen producing gel (figure 3).

According to the dental surgeons conducting these experiments, it was a notable clinical experience that fewer complications occurred, and that the healing process progressed faster and better. Based on these findings, we wondered whether these beneficial experiences would also lead to similarly positive results in our dental practice. I will discuss three typical cases.

Case 1 (figure 4 – 6)
A 39-year-old man presented as a new patient in 2011. During the intraoral examination, peri-implantitis was noticed around the implant of tooth 36 with a circular bone defect and a pocket of 4mm (figure 4). After extensive oral hygiene instruction and in order to create a fresh wound bed, we curedtted the mucous membrane around the implant of tooth 36 in a closed situation under anaesthetic. We did not clean the implant surface in this context. Immediately after this, we applied the active oxygen based Oral Gel (BlueM) in accordance with our TOOTh guidelines – see below.

After one year, new horizontal and vertical bone growth around the implant is clearly visible (figure 5). The pocket depth had reduced from 9 to 4mm. The redness and the swelling of the peri-implant tissue had disappeared, the mucous membrane was tight around the implant again, and there was no longer any bleeding after probing. Another year later the bone ingrowth had been maintained (figure 6). There even appears to be a slight improvement, and the pocket depth had reduced further to 3mm.

Case 2 (figure 7 – 8)
In a 59-year-old man, local periodontal problems occurred at the site of tooth 36. Figure 7 shows a distal angular bone defect with a pocket depth of 8mm and an attachment loss of 9mm. Under anaesthetic, the pocket was first cleaned ultrasonically, then with manual instrumentation and the application of blueM Oral Gel in accordance with the TOOTh guidelines. The x-ray taken two years later showed new bone growth and the pocket depth had been reduced to 4mm (figure 7). The bone level distal had returned to the same height as the mesial bone.

Case 3 (figure 9 – 11)
In a 62-year-old female patient, we encountered peri-implantitis in 2011. This is clearly visible radiographically (figures 9, 10). The maximum pocket depth was measured at 12mm. In this case, we raised a flap and encountered cement residue around the implant. We cleaned the implant surface with curettes and 35 per cent phosphoric acid, blueM Oral Gel was then applied to the implant surface, and to the flap after suturing. We instructed the patient to apply the oxygen gel three times daily around the implant, and to rinse three times daily with the blueM mouth rinse. After two years, a check-up x-ray (figure 11) was taken. This x-ray agrees with the clinical finding: the pocket depth has been reduced to 4mm.

Discussion
In order to increase the action of active oxygen locally, both low concentrations of sodium perborate and of the enzyme glucose oxidase (GOx) can be applied. In contact with water, sodium perborate is converted into sodium borate and H2O2. The GOx ensures a gradual conversion of glucose into gluconic acid and H2O2. GOx, which is normally dormant, but becomes active under the influence of moisture, for example from a wound. Small quantities of gluconic acid and hydrogen peroxide are released very gradually.

In low concentrations of 0.003 per cent-0.015 per cent, hydrogen peroxide has a disinfectant action, and occurs, together with the antibacterial ROS (reactive oxygen species) during the respiratory burst of neutrophils in normal wound fluid. It has a chemoattractant effect on leucocytes. The concentration of hydrogen peroxide in the blum products used here is not comparable to the high concentrations (1.5 – 3 per cent) of hydrogen peroxide used in medicine as a disinfectant. It is known that the production of free radicals with high concentrations of hydrogen peroxide causes tissue damage. Research has however shown that the continuous presence of a low concentration of hydrogen peroxide kills pathogenic bacteria much more effectively than a one-off high concentration and that fibroblasts are not damaged by this.8,9

Conclusion
Although the local use of a low dose H2O2 has a proven positive effect on wound healing, the exact mechanism of stabilised oxygen preparations on wound healing in general and on tissue regeneration in dentistry in particular is not yet fully understood. The TOOTh advice for application of active oxygen gel is only a treatment guideline, but has shown great patient benefits in our practice. Reference is made emphatically to the fact that the active oxygen therapy is based on the use of physiologically low-dose hydrogen peroxide and should not be compared to the.1 Five to three per cent concentration usual for disinfectants used in dentistry since these can actually cause tissue damage.
Wound Healing Essentials: Let There Be Oxygen

Chandan K. Sen, PhD, FACSM, FACN
The Comprehensive Wound Center, Departments of Surgery and Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, OH 43210

Abstract

The state of wound oxygenation is a key determinant of healing outcomes. From a diagnostic standpoint, measurements of wound oxygenation are commonly used to guide treatment planning such as amputation decision. In preventive applications, optimizing wound perfusion and providing supplemental O2 in the peri-operative period reduces the incidence of post-operative infections. Correction of wound pO2 may, by itself, trigger some healing responses. Importantly, approaches to correct wound pO2 favorably influence outcomes of other therapies such as responsiveness to growth factors and acceptance of grafts. Chronic ischemic wounds are essentially hypoxic. Primarily based on the tumor literature, hypoxia is generally viewed as being angiogenic. This is true with the condition that hypoxia be acute and mild to modest in magnitude. Extreme near-anoxic hypoxia, as commonly noted in problem wounds, is not compatible with tissue repair. Adequate wound tissue oxygenation is required but may not be sufficient to favorably influence healing outcomes. Success in wound care may be improved by a personalized health care approach. The key lies in our ability to specifically identify the key limitations of a given wound and in developing a multifaceted strategy to specifically address those limitations. In considering approaches to oxygenate the wound tissue it is important to recognize that both too little as well as too much may impede the healing process. Oxygen dosing based on the specific need of a wound therefore seems prudent. Therapeutic approaches targeting the oxygen sensing and redox signaling pathways are promising.

Keywords

hyperbaric oxygen; topical oxygen; angiogenesis; patient; redox; hypoxia; therapy; clinical

The clinical application of O2 to wound healing occurs at many levels: diagnostic, preventive and therapeutic. From a diagnostic standpoint, measurements of wound oxygenation (transcutaneous О2 measurements or TCOM) are commonly used to guide treatment planning such as amputation decision. In preventive applications, optimizing wound perfusion and providing supplemental О2 in the peri-operative period reduces the incidence of post-operative infections. Correction of wound pO2 may, by itself, trigger some healing responses. More importantly, approaches to correct wound pO2 favorably influence outcomes of other therapies such as responsiveness to growth factors and acceptance of grafts. This leads to the concept of correction of wound hypoxia as adjunct to other therapeutic modalities. Although the case for therapeutic approaches aimed at correcting wound tissue hypoxia is compelling, outcomes in the wound clinics have been inconsistent. The objective of this review article is to concisely address some of the fundamental and emergent concepts in tissue O2 sensing and response with the goal to illuminate salient complexities and perform critical analysis of what should help improve clinical outcomes in response to О2-based therapeutics.

Correspondence: Prof. Chandan K. Sen 513 Davis Heart & Lung Research Institute The Ohio State University Medical Center 473 W. 12th Avenue, Columbus, OH 43210 Tel. 614 247 7658 E. mail: E-mail: chandan.sen@osumc.edu.
Wound Ischemia and Hypoxia

Vascular complications commonly associated with problematic wounds are primarily responsible for wound ischemia. Limitations in the ability of the vasculature to deliver O\textsubscript{2}-rich blood to the wound tissue leads to, among other consequences, hypoxia. Hypoxia is a reduction in oxygen delivery below tissue demand, whereas ischemia is a lack of perfusion, characterized not only by hypoxia but also by insufficient nutrient supply. Hypoxia, by definition, is a relative term. It is defined by a lower tissue partial pressure of oxygen (pO\textsubscript{2}) compared to the pO\textsubscript{2} to which the specific tissue element in question is adjusted to under healthy conditions in vivo. Depending on the magnitude, cells confronting hypoxic challenge either induce an adaptive response that includes increasing the rates of glycolysis and conserve energy or undergo cell death. Generally, acute mild to moderate hypoxia supports adaptation and survival. In contrast, chronic extreme hypoxia leads to tissue loss. While the tumor tissue is metabolically designed to thrive under conditions of hypoxia, hypoxia of the wound primarily caused by vascular limitations is intensified by coincident conditions (e.g. infection, pain, anxiety and hyperthermia) and leads to poor healing outcomes.

Three major factors may contribute to wound tissue hypoxia: (i) peripheral vascular diseases, (ii) increased O\textsubscript{2} demand of the healing tissue, and (iii) generation of reactive oxygen species (ROS) by way of respiratory burst and for redox signaling (Fig. 1). Other related factors such as arterial hypoxia (e.g. pulmonary fibrosis or pneumonia, sympathetic response to pain, hypothermia, anemia caused by major blood loss, cyanotic heart disease, high altitude) may contribute to wound hypoxia as well. Depending on factors such as these, it is important to recognize that wound hypoxia may range anywhere from near-anoxia to mild-moderate hypoxia. In this context it is also important to appreciate that point measurements performed in the wound tissue may not provide a complete picture of the wound tissue biology because it is likely that the magnitude of wound hypoxia is not uniformly distributed throughout the affected tissue especially in large wounds. This is most likely the case in chronic wounds presented clinically as opposed to experimental wounds which are more controlled and homogenous in nature. In any single problem wound presented in the clinic, it is likely that there are pockets of near-anoxic as well as that of different grades of hypoxia (Fig. 2). As the weakest link in the chain, tissue at the near-anoxic pockets will be vulnerable to necrosis which in turn may propagate secondary tissue damage and infection. Pockets of extreme hypoxia may be flooded with hypoxia-inducible angiogenic factors but would fail to functionally vascularize because of insufficient O\textsubscript{2} that is necessary to fuel the repair process. Indeed, uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis. Whether cells in the pockets of extreme hypoxia are O\textsubscript{2}-responsive is another concern. Even if such cells may have passed the point of no return in the survival curve, correction of tissue oxygenation is likely to help clean-up the dead or dying tissue and replace the void with proliferating neighboring cells. Pockets of moderate or mild hypoxia are likely to be the point of origin of successful angiogenic response as long as other barriers such as infection and epigenetic alterations are kept to a minimum.

Wound Hypoxia: The Imbalance between Limited Supply and High Demand

Limited supply: peripheral vascular diseases

Peripheral vascular disease (PVD) can affect the arteries, the veins as well as the lymph vessels. The most common and important type of PVD is peripheral arterial disease, or PAD, which affects about eight million Americans. The ankle brachial pressure index (ABPI) represents a simple non-invasive method to detect arterial insufficiency within a limb. Arterial diseases, especially those associated with diabetes, represent a major complicating factor in wound healing. PAD is the only identifiable etiology in approximately 10% of leg ulcers. In an
ischemic limb, peripheral tissues are deprived of blood supply as PAD progresses causing tissue loss, ulcers and gangrene.

Venous insufficiency, on the other hand, is the root cause of most leg ulcers. Chronic venous insufficiency, characterized by the retrograde flow of blood in the lower extremity, is associated with changes in the venous wall and valves generally caused by inflammatory disorders induced by venous hypertension and associated fluid shear stress. Factors causing arterial hypoxemia may also limit $O_2$ supply to the wound tissue. Compromised pulmonary health, loss of hepatic function, hemodialysis, anemia, altitude hypoxemia, nitroglycerin therapy, nasal packing, critical illness, pain and hypothermia, are some examples of conditions associated with arterial hypoxemia. Vasoconstricting drugs may contribute to tissue hypoxia as well.

High demand: increased demand of the healing tissue

Mitochondrial respiration is responsible for more than 90% of $O_2$ consumption in humans. Cells utilize $O_2$ as the final electron acceptor in the aerobic metabolism of glucose to generate ATP which fuels most active cellular processes such as during wound healing. Increased energy demand of the healing tissue leads to a hypermetabolic state wherein additional energy is generated from oxidative metabolism increasing the $O_2$ demand of the healing tissue. ATP thus generated powers tissue repair. At the injury site, extracellular ATP may be contributed by platelets and other disintegrating cells. Extracellular ATP liberated during hypoxia or inflammation can either signal directly to purinergic receptors or, after phosphohydrolytic metabolism, can activate surface adenosine receptors. Purinergic signaling may influence numerous aspects of wound biology including immune response, inflammation, vascular as well as epithelial biology. ATP may be immunostimulatory or vice versa depending on extracellular concentrations as well as on expression patterns of purinergic receptors and ecto-enzymes. Extracellular ATP induces receptor activation in epithelial cells. ATP, released upon epithelial injury, acts as an early signal to trigger cell responses including an increase in heparin-binding EGF-like growth factor shedding, subsequent transactivation of the epidermal growth factor (EGF) receptor and its downstream signaling, resulting in wound healing.

Absolute requirements for $O_2$ arise in several points along the angiogenic sequence. For instance, all vessels require a net or sheath of extracellular matrix, mainly collagen and proteoglycans, to guide tube formation and resist the pressures of blood flow. Conditions for collagen deposition and polymerization can be created only if molecular $O_2$ is available to be incorporated into the structure of nascent collagen by prolyl- and lysyl hydroxylases. Without the obligatory extracellular, hydroxylated collagen, new capillary tubes assemble poorly and remain fragile. This has a convincing clinical correlate in scurvy, i.e. ascorbate deficiency. Scurvy may result from insufficient intake of ascorbate which is required for correct collagen synthesis in humans. Ascorbate is required for the post-translational hydroxylation of collagen that enables the matured collagen molecules to escape to the extracellular space and provide the necessary tensile strength. In scurvy, the collagenous sheath cannot form...
because, under ascorbate-deficient conditions, collagen cannot be hydroxylated. Consequently, new vessels fail to mature. Older vessels weaken and break, and wounds fail to heal. In this context it is important to recognize that the collagen hydroxylation process requires molecular oxygen. Thus, even under ascorbate-sufficient conditions collagen may fail to mature if there is insufficient supply of oxygen to the tissue. Collagen deposition proceeds in direct proportion to \( pO_2 \) across the entire physiologic range, from zero to hundreds of mm Hg. The \( K_m \) for \( O_2 \) for this reaction is approximately 25 and the \( V_{max} \) is approximately 250 mm Hg, suggesting that new vessels cannot even approach their greatest possible rate of growth unless the wound tissue \( pO_2 \) is high. Angiogenesis is directly proportional to \( pO_2 \) in injured tissues.

Hypoxic wounds deposit collagen poorly and become infected easily, both of which are problems of considerable clinical significance.

**High demand: increased production of reactive species**

**Phagocytic NADPH oxidases**—Sbarra and Karnovsky’s 1959 discovery of the leukocyte oxidase in phagocytes came into limelight when in the late 1970s, the pioneering works of Bernard Babior that linked the explosive production of superoxide ions (\( O_2^- \)) by leukocyte oxidase to bacterial killing. During phagocytosis of microbial intruders, professional phagocytes of our innate immune system increase their \( O_2 \) consumption through the inducible activity of NADPH-oxidase (NOX) that generates \( O_2^- \) and \( H_2O_2 \). These oxygen-derived metabolites give rise to yet other ROS that are potently anti-microbial but which may also cause damage by destroying surrounding tissue and cells. NADPH oxidase, catalyzing the deliberate production of ROS by cells, has been extensively investigated in phagocytes (neutrophilic and eosinophilic granulocytes, monocytes, and macrophages). Exposure of these cells to any of a large number of stimuli activates a “respiratory burst”, caused by an activation of the plasma membrane bound NADPH oxidase (NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+). The \( O_2^- \) then rapidly dismutates to \( H_2O_2 \). Approximately 98% of the \( O_2 \) consumed by wound neutrophils is utilized for respiratory burst. NADPH Oxidase supports macrophage survival and enables dead cell cleansing by phagocytosis. Appropriate infection management may therefore spare precious \( O_2 \) at the wound site which would otherwise be utilized via respiratory burst. Overt infection poses the risk of intensifying wound tissue hypoxia.

The NOX of ‘professional’ phagocytic cells transfers electrons across the wall of the phagocytic vacuole, forming \( O_2^- \) in the lumen. It is generally accepted that this system promotes microbial killing through the generation of ROS and through the activity of myeloperoxidase. In response to bacterial infection, the neutrophil NADPH oxidase assembles on phagolysosomes to catalyze the transfer of electrons from NADPH to \( O_2 \), forming \( O_2^- \) and derivative ROS. The active oxidase is composed of a membrane-bound cytochrome (e.g. gp91phox and p22phox) together with three cytosolic phox proteins, p40phox, p47phox, and p67phox, and the small GTPase Rac2, and is regulated through a process involving protein kinase C, MAPK, and phosphatidylinositol 3-kinase. In the resting cell, two of the subunits, p22phox and gp91phox, are located in the membrane, and the remaining components are present in the cytosol. The electron-carrying components of the oxidase are located in gp91phox. The NADPH binding site is generally regarded to be in gp91phox as well, but there is some evidence that it may be in p67phox. The catalytic subunit gp91phox, dormant in resting cells, becomes activated by assembly with cytosolic regulatory proteins. When the oxidase is activated, p47phox is phosphorylated at specific sites, and the cytosolic components together with Rac2 migrate to the membrane to assemble the active oxidase. Mutations in p47phox are a cause of chronic granulomatous disease, an immune-deficient condition characterized with impaired healing response. Rac2 mutation is another factor responsible for impaired human neutrophil NADPH oxidase function, low \( O_2^- \) generation and compromised wound healing. The concentration of \( O_2 \) necessary to achieve half maximal...
ROS production (the $K_m$) is in the range of 45-80 mmHg, with maximal ROS production at $pO_2$ at $>300$ mmHg $^{24}$. Thus, the maximal effects of respiratory burst dependent wound infection management can only be achieved through the administration of supplemental $O_2$ to attain wound $pO_2$ levels beyond those encountered when breathing room air $^{85}$. This also explains why the state of wound tissue oxygenation is a sensitive indicator for the risk of infection in surgical patients $^{8, 9, 86, 87}$.

**Oxygen free radicals and reactive derivatives: a paradigm shift and emergence of redox signaling**—In the 1980s, oxygen free radicals drew much attention in biomedical research. Limitations in methodological approaches to sensitively detect and monitor the extremely short-living reactive species clouded a true appreciation of the significance of oxygen-derived free radicals and reactive species in health and disease. The paradigm that emerged was too simple to be meaningful in its complete sense. The primary identity of free radicals was that they were destructive to biological tissues, and that approaches to antagonize free radicals i.e. antioxidants are helpful $^{88-96}$. Based on this crude preliminary concept, numerous clinical trials testing the efficacy of antioxidants were hastily started and the results were understandably disappointing $^{97-101}$. Lack of consideration of a very important aspect of free radical biology which started to crystallize only in the late 1990s proved to be very expensive in many ways. Work during the mid-late 1990s led to the recognition that at very low levels oxygen-derived free radicals and derivative species such as $H_2O_2$ may serve as signaling messengers $^{102-104}$. The field of redox signaling was thus born $^{102, 105-107}$ with a dedicated international peer-reviewed Journal (www.liebertpub.com/ars). Today, the concept that reactive derivatives of $O_2$ may serve as signaling messengers has revolutionized cell biology $^{108-123}$ and has led to the concept of redox-based clinical therapeutics $^{124-129}$.

**Non-phagocytic NADPH oxidases**—Given the traditional bad and ugly image of oxygen free radicals and its derivatives few would have imagined that even non-phagocytic cells of the human body have a dedicated apparatus to generate ROS. In 1999, the cloning of mox1 marked a major progress in categorically establishing the presence of distinct NADPH oxidases in non-phagocytic cells $^{123}$. Mox1 or p65Mox was described as encoding a homologue of the catalytic subunit of the $O_2^-$-generating NADPH oxidase of phagocytes, gp91phox. Mox1 messenger RNA is expressed in colon, prostate, uterus and vascular smooth muscle, but not in peripheral blood leukocytes. Later, Mox1 was renamed as NOX1 referring to NADPH oxidase $^{130}$. Over the last years, six homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Together with the phagocyte NADPH oxidase itself (NOX2/gp91(phox)), the homologs are now referred to as the NOX family of NADPH oxidases. Activation mechanisms of these enzymes and tissue distribution of the different members of the family are markedly different. The physiological functions of NOX family enzymes include host defense, post-translational processing of proteins, cellular signaling, regulation of gene expression, cell differentiation and renewal of precursor cells $^{131-135}$. NOX enzymes also contribute to a wide range of pathological processes. NOX deficiency may lead to immunosuppression, lack of otoconogenesis, or hypothyroidism. Increased NOX activity also contributes to a large number or pathologies, in particular cardiovascular diseases and neurodegeneration $^{136}$. Thus, optimal generation of $O_2^-$ is required to sustain healthy living.

Acute inflammation following injury is the site for abundant production of ROS by phagocytic NADPH oxidases. As inflammation resolves and phagocyte count at the wound site falls, several aspects of healing such as cell proliferation and migration are supported by redox signaling where low-level ROS produced by non-phagocytic oxidases serve as messenger molecules $^{57}$. The critical significance of the NADPH oxidases in wound healing is rapidly unfolding. As discussed previously, NADPH oxidase deficient mice and humans suffer from impaired healing. As an integral part of the healing response, wounding induces $H_2O_2$
production\textsuperscript{56}. This response is also conserved in plants\textsuperscript{137}. Wound fluid from healing tissues contains the highest concentration of H\textsubscript{2}O\textsubscript{2} compared to all other bodily fluids\textsuperscript{56, 138}. Of note, selective decomposition of H\textsubscript{2}O\textsubscript{2} at the wound site using catalase overexpression approaches impairs the healing process demonstrating the key significance of H\textsubscript{2}O\textsubscript{2} in wound healing\textsuperscript{56}. Importantly, catalase-dependent decomposition of H\textsubscript{2}O\textsubscript{2} generates O\textsubscript{2} as end-product. Thus, molecular O\textsubscript{2} is not sufficient if NADPH oxidase dependent O\textsubscript{2}-consumption and redox signaling is impaired. How redox signals may contribute to tissue repair has been recently reviewed elsewhere\textsuperscript{57, 139} and is beyond the scope of this article. In the context of this article it is important to appreciate that redox signals are generated at the cost of tissue O\textsubscript{2}. Thus, tissue hypoxia will limit redox signaling and disable the function of several growth factors (e.g. PDGF, VEGF, KGF, IGF, TGF\textsubscript{a}) and numerous molecular mechanisms (e.g. leukocyte recruitment, cell motility, integrin function) which rely on redox signaling\textsuperscript{57, 139, 140}.

Collagen deposition provides the matrix for angiogenesis and tissue remodeling. Maturation of collagen is O\textsubscript{2}-dependent. Of the O\textsubscript{2} dependent enzymatic processes, the rate of collagen synthesis is reflected by the rate at which prolyl hydroxylation occurs\textsuperscript{141}. Collagen synthesis is half-maximal (K\textsubscript{m} using Michaelis-Menton equation) at a pO\textsubscript{2} of 20-25mmHg\textsuperscript{66, 142}, with V\textsubscript{max} at levels approaching 250 mmHg. This represents levels of O\textsubscript{2} availability that exceeds the pO\textsubscript{2} normally present in the wound tissue and suggests that adequate wound tissue oxygenation is crucial to support collagen synthesis and maturation. Indeed, increasing wound oxygenation results in increased collagen deposition and tensile strength\textsuperscript{143-145}.

NO synthases—NO is widely recognized as a major signaling messenger that drive numerous aspects of (patho)physiology\textsuperscript{146-149}. O\textsubscript{2} consuming nitric-oxide synthases (NOS) catalyze NO formation from the amino acid L-arginine. The reaction of NOS with O\textsubscript{2} is fast and takes place within several steps\textsuperscript{150}. NOS are known to catalyze more than one reaction: the NO-producing reaction is considered to be the coupled reaction, and the uncoupled reactions are those that produce ROS, such as O\textsubscript{2}- and H\textsubscript{2}O\textsubscript{2}\textsuperscript{151}. The key significance of NO in wound healing has been reviewed elsewhere\textsuperscript{152, 153}. In the context of this article it is important to note that O\textsubscript{2} is often the overlooked substrate in NO synthesis. To date there has been little consideration of the role of O\textsubscript{2} tension in the regulation of nitric oxide production associated with wound healing. Tissue O\textsubscript{2} tension is known to significantly alter endogenous NO production in articular cartilage where the tissue pO\textsubscript{2} is comparable to that of ischemic wounds\textsuperscript{134}. The preliminary observation that hyperbaric oxygen (HBO) therapy may significantly increase local wound NO levels is therefore understandable\textsuperscript{155}. Once generated, the biological significance of NO also depends on the tissue oxygenation status\textsuperscript{156}. As NO gas based therapies are being considered for healing wounds clinically, it is important to recognize that NO can block mitochondrial function by interacting with the cytochrome c oxidase (complex IV) of the electron transport chain in a manner that is reversible and in competition with O\textsubscript{2}. Concentrations of NO too low to inhibit respiration can trigger cellular defense response mechanisms. Inhibition of mitochondrial respiration by NO at low O\textsubscript{2} concentrations can cause so-called “metabolic hypoxia” and divert O\textsubscript{2} towards other oxygen-dependent systems. Metabolic hypoxia refers to a state wherein although O\textsubscript{2} is available the cell is unable to utilize it for respiration\textsuperscript{157}. Such a diversion reactivates prolyl hydroxylases and thus accounts for the prevention by NO of the stabilization of the hypoxia inducible factor (HIF). When NO inhibits mitochondrial respiration under hypoxia, it prevents mitochondria from depleting local oxygen, enabling the continued hydroxylation and degradation of HIF-1\textalpha, thus leading to a situation in which the cell may fail to register hypoxia. Furthermore, in a wound setting where O\textsubscript{2}- production is highly active, NO is likely to generate peroxynitrite which can affect the action of key enzymes, such as mitochondrial complex I, by S-nitrosation\textsuperscript{157}. NO-based wound therapeutics should be designed in light of these complexities.
The stability of HIF, and therefore its ability to drive HIF-dependent gene transcription, is differentially regulated by NO under conditions of normoxia and hypoxia. While NO stabilizes HIF under normoxia, the effect is exactly opposite under conditions of hypoxia. Under conditions of normoxia, NO may attenuate the ubiquitination of HIF-1α and thus abrogate binding of pVHL to HIF-1α. Ubiquitination of HIF would not take place if HIF is not hydroxylated by PHDs. Indeed, NO inhibits PHD activity. Fe^{2+}-coordination by NO seems to be the explanation for how NO inhibits PHDs. The stabilization of HIF under normoxia is also explained by the induction of HIF-1α synthesis by NO. Although speculative, different redox-active products, derived from chemically distinct NO donors, use divergent transmission systems to stabilize/express HIF-1α. Under conditions of hypoxia, NO and its derivatives inhibit hypoxia-induced HIF-1α accumulation. In light of the observation that NO attenuates PHD activity under normoxia to stabilize HIF-1α, raises the question whether PHD activity is regained under conditions of hypoxia-NO co-existence. An affirmative answer to this question came from the observation that oxygen-dependent death domain of HIF-1α, which accounts for protein stability, is needed for NO and its derivatives to reverse hypoxic HIF-1α stabilization. Several mechanistic hypotheses have been proposed to explain how NO impairs accumulation of HIF-1α under hypoxia. The scenario gets even more complicated in a wound setting where both phagocytic as well as non-phagocytic NADPH oxidases generate copious amounts of superoxide anion radicals. Furthermore, hypoxic tissues are known to generate more ROS. The HIF system has revealed an unexpectedly direct connection between molecular oxygen, superoxide and NO in achieving or attenuating responses to hypoxia. The reaction between O_2^- and NO represents a primary biochemical path in vivo. Flux rates of NO and O_2^-, as well as the presence of antioxidant enzymes, can modulate HIF-1α stabilization. Understanding the multiple signals, that have the potential to deliver a flexible and controlled response to hypoxia, will be critical to develop therapeutic maneuvers. Thus, a clear appreciation of the specific wound tissue redox environment becomes critically important in the context of planning NO-based therapeutics.

The Normoxic Setpoint and Oxygen Sensing

Cellular O_2 homeostasis is tightly maintained within a narrow range (“normoxia”) due to the risk of oxidative damage from excess O_2 (hyperoxia), and of metabolic demise from insufficient O_2 (hypoxia). Vast majority of the current literature focuses on the sensing of hypoxia, and the work on hyperoxic sensing is limited. Both hypoxia and hyperoxia are relative terms. They refer to a state of oxygenation that departs from the normoxic setpoint, i.e. the pO_2 to which cells or tissues are adjusted to under basal conditions. For any given cell or tissue, normoxic setpoint represents that state of oxygenation where the cell or tissue does not report hypoxia, and hyperoxic setpoint represents that state of oxygenation where the cell or tissue does not report hyperoxia. It is likely that this set-point would represent a range of pO_2, the span of which might depend on the tissue in question. Any change of O_2 ambience exceeding that span would result in the switching on of a hypoxic or hyperoxic response. In the finest of scales, such response would be detected in the molecular scale such as HIF-stabilization or HRE-transactivation for hypoxia and say p21-induction for hyperoxia. In a relatively coarser scale, oxygen-sensitive changes in cellular phenotype may be noted. Of note, different organs of the body have different normoxic set-points. While the lung and arterial vasculature represent the high end, organs such as the liver have very low basal pO_2. pO_2 ranges from 90 to below 3 Torr in mammalian organs under normoxic conditions with arterial pO_2 of about 100 Torr or ∼14%O_2.

Hypoxia sensing

Hypoxia sensing and response is activated upon exposure to a state of oxygenation that is lower than the pO_2 to which the cells or tissue is adjusted to under basal conditions. This response
cascade is centrally important in coping with the challenge of O\textsubscript{2} deficiency. Hypoxia response has been mostly studied in transformed and tumor cells. It is important to recognize that findings from such cells may not be directly applicable to non-transformed primary cells which are involved in wound healing \cite{167}. Hypoxia is a hallmark of all ischemic diseases but is also noted under several physiological processes where exposure to a dynamic state of oxygenation is an integral component. During early pregnancy, trophoblast differentiation occurs in an environment of relative low O\textsubscript{2} tension which is essential for normal embryonic and placental development \cite{168}. O\textsubscript{2} supply to the human embryo in the first trimester is tightly controlled, suggesting that too much O\textsubscript{2} may interfere with development. Relative to maternal tissue \(p\text{O}_2\), embryo is normally in a state of partial hypoxia \cite{169,170}. Thus, hypoxia sensing and response is not only implicated in ischemic disease conditions but is also required for development where a changing state of oxygenation seems to serve as a cue for successful development. Whether this is nature’s approach to quality check each healthy birth for the ability of the new-born to cope with ischemic diseases later on in their lives may be viewed as a matter of interesting speculation.

Hypoxia sensing and response mechanisms may be broadly classified into two general categories: HIF-dependent and HIF-independent. Extensive discussion of these pathways is beyond the scope of this article and the readers are referred to excellent review articles \cite{171-173}.

**HIF-dependent pathways**—The basic helix-loop-helix (bHLH) proteins form a large superfamily of dimeric transcriptional regulators that are found in organisms from yeast to humans and function in critical developmental processes. One basis for the evolutionary classification of bHLH proteins is the presence or absence of additional domains, of which the most common are the PAS, orange and leucine-zipper domains. PAS domains, located carboxy-terminal to the bHLH region, are 260-310 residues long and function as dimerization motifs. They allow binding with other PAS proteins, non-PAS proteins, and small molecules such as dioxin. The PAS domain is named after three proteins containing it: Drosophila Period (Per), the human aryl hydrocarbon receptor nuclear translocator (Arnt) and Drosophila Single-minded (Sim). HIFs belong to the bHLH-PAS family of environmental sensors which bind to canonical DNA sequences call hypoxia response elements (HRE) in the promoters or enhancers of target genes \cite{174}. HIF is able to direct transcription from either of two transactivation domains, each of which is regulated by distinct mechanisms. The O\textsubscript{2}-dependent asparaginyl hydroxylase factor-inhibiting HIF-1alpha (FIH-1) is a key regulator of the HIF C-terminal transactivation domain, and provides a direct link between O\textsubscript{2} sensing and HIF-mediated transcription. Additionally, there are phosphorylation and nitrosylation events reported to modulate HIF transcriptional activity, as well as numerous transcriptional coactivators and other interacting proteins that together provide cell and tissue specificity of HIF target gene regulation \cite{175}.

HIF-1 consists of a constitutively expressed subunit HIF-1beta and an oxygen-regulated subunit HIF-1alpha (or its paralogs HIF-2alpha and HIF-3alpha). The transcriptional role of HIF is primarily dependent on the stabilization of HIF-1alpha or its paralogs under hypoxic conditions. Under O\textsubscript{2}-replete conditions HIF-1alpha is very labile \cite{176}. Molecular O\textsubscript{2} targets HIF for degradation by post-translational hydroxylation at specific prolyl residues within the alpha subunits. Hydroxylation at two prolyl residues within the central degradation domain of HIF1-alpha increases the affinity for the von Hippel-Lindau (pVHL) E3 ligase complex by at least three orders of magnitude, thus directing HIF-alpha polypeptides for proteolytic destruction by the ubiquitin/proteasome pathway. Because the HIF hydroxylases have an absolute requirement for molecular O\textsubscript{2} this process is suppressed in hypoxia allowing HIF-alpha to escape destruction and activate transcription.

*Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.*
The O$_2$ sensitive prolyl-hydroxylase domain enzymes (PHDs) and the asparagines hydroxylase (FIH, factor inhibiting HIF) regulate the transcriptional activity of HIFs. The unusual high $K_M$ of PHDs for oxygen allows small changes in the oxygen supply to affect enzyme activity, which makes this system an ideal oxygen sensor. In hypoxia, FIH-1 hydroxylation of Asn803 within the C-terminal transactivation domain (TAD) does not occur and HIF-1alpha fails to form a fully active transcriptional complex. Thus, HIF prolyl hydroxylation regulates proteolytic degradation of HIF whereas HIF asparaginyl hydroxylation modulates interaction with transcriptional co-activators. These hydroxylations are catalysed by a set of non-haem Fe(II)- and 2-oxoglutarate (2-OG)-dependent dioxygenases. During catalysis, the splitting of molecular O$_2$ is coupled to the hydroxylation of HIF and the oxidative decarboxylation of 2-OG to give succinate and CO$_2$. The von Hippel-Lindau tumor suppressor gene product, pVHL, functions as the substrate recognition component of an E3-ubiquitin ligase, which targets the O$_2$-sensitive alpha-subunit of hypoxia-inducible factor (HIF) for rapid proteasomal degradation under normoxic conditions and as such plays a central role in molecular O$_2$ sensing.

Stabilization of HIF under hypoxic conditions is followed by nuclear localization where HIF may bind to DNA sequences and other transcriptional regulators to influence gene expression (Table 1). The passage of transcription factors e.g. HIF-1alpha into the nucleus through the nuclear pore complex is regulated by nuclear transport receptors. Therefore nucleocytoplasmic shuttling can regulate transcriptional activity by facilitating the cellular traffic of transcription factors between both compartments.

Shortly after the cloning of HIF-1alpha, a closely related protein, HIF-2 alpha [also known as endothelial PAS protein, HIF-like factor (HLF), HIF-related factor (HRF), and member of the PAS superfamily 2 (MOP2)] was identified and cloned. HIF-2alpha regulates erythropoietin production in adults. HIF-1alpha functions as an up-stream player in the p21-mediated growth arrest of keratinocytes. Thus, HIF may antagonize certain aspects of skin repair. Negative pressure wound therapy, known to be effective in healing wounds clinically, is known to antagonize the stabilization of HIF-1alpha. HIF-dependent pathways for survival and vascularization can function under conditions where hypoxia is moderate and not extreme. As long as there is a threshold level of oxygenation sufficient to sustain life, HIF-dependent survival responses may benefit wound healing.

HIF-independent pathways—Conservation of ATP under conditions of limited O$_2$ supply is a HIF-independent survival response that is not compatible with the energy-demanding healing process. For example, HIF-independent hypoxic inhibition of protein synthesis and cell growth is mediated by: i) hypoxia-induced cellular energy depletion; ii) mTOR inhibition via the AMPK/TSC2/Rheb pathway; iii) eEF2 inhibition mediated by AMP-activated protein kinase (AMPK); and iv) induction of ER stress that leads to eIF2α inhibition. mTOR is a Ser/Thr kinase that integrates signals from growth factors and nutrients to increase ribosome biogenesis. Upon hypoxic energy starvation, AMPK phosphorylates eEF2 kinase (eEF2K) on Ser398 and activates its kinase activity. eEF2K then phosphorylates elongation factor eEF2 at Thr56, resulting in the inhibition of peptide elongation. mRNA translation is a critical component of cell growth and proliferation that is critically supported by eIF2α. Hypoxia causes ER stress which in turn inhibits eIF2α. Wound healing requires protein synthesis. Hypoxia causes global downregulation of protein synthesis. Hypoxia-induced translational attenuation may be linked to ER stress and the unfolded-protein response. The translational efficiency of individual genes is dynamic and changes with alterations in the cellular environment. Whereas changes in transcription can take hours to achieve, translational regulation is rapid and reversible. Preferential translation of select mRNA is another hallmark of response to hypoxia. Roughly 2.5% of total cellular transcripts are
preferentially translated, despite arrest of global protein synthesis, in response to sustained extreme hypoxia \(^{194}\). Taken together, while all these hypoxia-responses represent important HIF-independent mechanisms of energy conservation that promote survival under low O\(_2\) conditions, they are not compatible with the formation of new tissue as required during wound healing.

**Intermittent hypoxia**

O\(_2\) sensing is no longer a unique property limited to chemoreceptors but is a common property of tissues \(^{195}\). The classic concept of intermittent hypoxia has been markedly revised in light of our current understanding of O\(_2\) sensing. Intermittent hypoxia (IH), or periodic exposure to hypoxia interrupted by return to normoxia or less hypoxic conditions, occurs in many circumstances. Chronic intermittent hypoxia (CIH) is a common life-threatening condition that occurs in many different diseases, including sleep-disordered breathing manifested as recurrent apneas. Excessive ROS have been identified as one of the causative factors in a variety of morbidities \(^{196}\). In experimental models, CIH activates ROS dependent responses that include (a) altered carotid body function, the primary chemoreceptor for sensing changes in arterial blood O\(_2\); (b) elevated blood pressure; (c) enhanced release of transmitters and neurotrophic factors; (d) altered sleep and cognitive behaviors; and (e) activation of second-messenger pathways and transcriptional factors. Considerable evidence indicates elevated ROS levels in patients experiencing CIH as a consequence of recurrent apneas \(^{196}\). Recently we evaluated the prevalence of obstructive sleep apnea (OSA) in the patient population of the OSU Wound Center. Between August 15th and September 30th of 2007, 105 consecutive unscreened patients of the wound center completed a sleep screening questionnaire. In this representative sample of patients of the wound center, fifty-one percent either were diagnosed with, or were at very high risk for OSA. Forty-three percent of patients with chronic non-healing wound were deemed at high risk for OSA \(^{197}\). Whether intermittent hypoxia associated with OSA complicates wound healing warrants further investigation. Results of our survey may be explained by the association that many with chronic wounds are overweight due to metabolic complications (e.g. PAD and type II diabetes), and sleep apnea is more prevalent in overweight individuals. Merit of the hypothesis that sleep disorder may complicate wound healing is supported by the extensive literature identifying OSA as a causative factor underlying vascular disorders \(^{198}, 199\).

**Hyperoxia sensing**

O\(_2\) got its name from “Principe Oxygene”, which means the acidifying principle. “Oxy” is from Greek, and means sharp or acid; “gen” is also from Greek, and means the origin of. Taken together, oxygen means “the origin of acid”. Joseph Priestly’s (1774) “dephlogisticated air” \(^{200}\) and Carl Scheele’s (1771) “fire air” were soon characterized by Antoine Lavoisier as pure respirable air \(^{201}\). Within decades of the first realization that oxygen is the element of life, Brizé-Fradin \(^{202}\) noted in 1808 that “vital air” or pure oxygen would soon wear life out instead of maintaining it. That oxygen may be harmful to human health was first postulated in the late 19th century with Paul Bert’s work (1878) on oxygen sickness. Paul Bert’s work is regarded as one of the cornerstones of HBO medicine \(^{203}\). He concluded that to avoid harmful effects, oxygen should not be inhaled at a concentration above 60% at 1ATA. Bert’s observation was extended through Michaeli’s theoretical considerations, Gerschman’s experimental verification and finally caught the interests of biomedical scientists when in 1969 McCord & Fridovich demonstrated that a metalloenzyme produced H\(_2\)O\(_2\) by combining O\(_2\) with hydrogen \(^{204}, 205\). Today, H\(_2\)O\(_2\) is widely known to function as a cellular messenger \(^{108-123}\). Hyperoxia-inducible molecular biomarkers have been characterized \(^{164}, 165\) enabling us to detect hyperoxic insult long before overt signs of oxygen toxicity and adverse clinical symptoms are manifested \(^{206}\).
Although marginal hyperoxic challenge may induce favorable responses\(^207\), a state of tissue oxygenation that far exceeds the normoxic setpoint of a given tissue is a clear risk factor that deserves appropriate attention\(^208\). In a wound with pockets of hypoxia ranging in magnitude from extreme to marginal (Fig. 2), the goal should be to re-establish normoxia in the worst affected hypoxic pockets without exposing other parts of the wound tissue to such high levels of \(pO_2\) that would antagonize healing by hyperxia-induced growth arrest or simply overt oxygen toxicity. One needs to be cautious about too much of a good thing\(^209\). Endothelial progenitor cells (EPCs) are essential in vasculogenesis and wound healing, but their circulating and wound level numbers are decreased in diabetes. Hyperoxia reverses the diabetic defect in EPC mobilization\(^210\). Moderate hyperoxia increases the appearance of new blood vessels in wounds\(^11\). In addition to inducing VEGF gene expression, moderate hyperoxia enhances the expression of VEGF\(_{121/165}\) proteins and facilitates the release of VEGF\(_{165}\) from cell-associated stores\(^211\). Among the factors that may oppose wound healing, extreme hyperoxia causes growth arrest\(^212-215\) and cell death by a mitochondria-dependent apoptosis pathway\(^171, 216, 217\). In addition, extreme hyperoxia does pose the threat of oxidative stress\(^218, 219\).

### Tuning the normoxic setpoint

When cells grown under standard culture conditions of 20% O\(_2\) are moved to 5% O\(_2\) ambience, hypoxia is reported by way of HIF-response elements. When the same cells are maintained at 5% O\(_2\) over long periods of time the O\(_2\)-sensitive molecular machinery undergoes adjustment such that the same cells no longer report hypoxia. Interestingly, if these cells are maintained under mild hyperoxic conditions, e.g. 30% O\(_2\), and then brought down to 20% O\(_2\) culture conditions they report hypoxia\(^163\). These simple observations establish two important points: (i) that it is not the actual \(pO_2\) but the \(\Delta pO_2\) that seems to matter; and (ii) that the normoxic setpoint in a cell can be reset by the adjustment of O\(_2\)-sensing machinery that is capable of responding to changes in the O\(_2\) ambience. In this simplified example, the machinery is represented by the PHD family of proteins the expression of which is upregulated under conditions of hypoxia and down-regulated under conditions of hyperoxia. This is noted not only in vitro but also in vivo. Here, although the example is limited to PHDs to keep the discussion simple it is important to recognize that there are numerous other O\(_2\)-sensitive functions in a cell that would contribute to its overall response to any \(pO_2\) outside the normoxic setpoint. Thus, the normoxic setpoint in a biological cell is tunable. For example, under conditions of no change in ambient O\(_2\) condition a cell may be made to report hypoxia, as measured by HIF-transactivation, simply by knock-down of the PHDs\(^163\). In response to down-regulated PHD1, cells not only report HRE-dependent gene expression but causes metabolic adaptations lowering tissue O\(_2\) consumption\(^220\). Conditional inactivation of PHD2 in mice is sufficient to activate a subset of HIF target genes, including erythropoietin, leading to striking increases in red blood cell production\(^221\). Tuning of the normoxic-setpoint when the cells are exposed to modest changes in O\(_2\) ambience seems to happen physiologically perhaps as an adaptive response. Comprehension of the pathways involved in such process should help us employ pharmacological and/or genetic approaches to therapeutically adjust the normoxic setpoint on an as needed basis. For example, moderate hypoxia is known to be a robust cue to initiate the angiogenic response. One can reap the angiogenic benefits of that knowledge by adopting therapeutic approaches that would lead to suppression of PHD function resulting in HIF stabilization and HRE-dependent transactivation. Indeed, this approach is being explored for wound therapies.

### Tissue Oxygenation and Wound Therapy

#### HIF PHD-directed Wound Therapeutics

The PHD inhibitor FG-4497 readily stabilizes HIF-1alpha and subsequently drives the expression downstream of HIF target genes. FG-4497 is helpful in colitis perhaps by benefiting...
wound healing at the site of inflammation. Extracellular matrix (ECM) is predominantly collagen, and the imino acids (Pro and HyPro) comprise 25% of collagen residues. The final step in collagen degradation is catalyzed by prolidase, the obligate peptidase for imidodipeptides with Pro and HyPro in the carboxyl terminus. Defective wound healing in patients with inherited prolidase deficiency is associated with histologic features of angiopathy suggesting that prolidase may play a role in angiogenesis. Recently it has been demonstrated that prolidase inhibits PHD activity to induce HRE-dependent transactivation and facilitate angiogenic signaling. HIF-specific PHD inhibitors are being tried out for their efficacy in treating wounds. It is likely that such approaches to pharmacologically stabilize HIF will facilitate responses such as generation of angiogenic factors. Whether that response translates to functionally successful angiogenesis and improvements in wound closure will depend on other fundamental pre-requisites such as a threshold level of tissue oxygenation is present to fuel the healing process. This is of particular concern for ischemic wounds that suffer from extreme chronic hypoxia. If hypoxia alone would have been sufficient to heal, all ischemic wounds would have undergone rapid healing. Clinical observation is exactly the opposite. The key here is to couple hypoxia-response signaling with conditions such as appropriate tissue oxygenation that could sustain the healing process. PHD inhibitors alone are not likely to yield favorable outcomes in extremely hypoxic wounds. Furthermore, it is important to note in this context that PHD inhibition may stabilize HIF but does not guarantee transcriptional function. Co-substrate and co-factor requirements for Fe(II), ascorbate, and the Krebs cycle intermediate 2-OG, and inducible changes in the cellular abundance of three closely related HIF prolyl hydroxylases (PHD1-3) provide additional interfaces with cellular O₂ status that may be important in regulating the oxygen-sensitive signal. Although under conditions of acute hypoxia PHD inactivation supports tissue survival, recently it has been demonstrated that under conditions of chronic hypoxia PHD overactivation is necessary as a survival response. Chronic ischemic tissue overactivates all three isoforms of PHD to survive. The merit of PHD inhibition for the treatment of ischemic wounds involving chronic hypoxia warrants reconsideration in this new light.

First and foremost it needs to be borne in mind that the overarching goal of oxygen therapy should be to correct wound hypoxia. While to some extent hyperoxia may be well tolerated by tissues, it would be prudent to avoid extreme hyperoxia. Although oxygen toxicity may not be imminently overt, an overdose of O₂ is likely to trigger molecular responses such as cell cycle arrest and epigenetic modifications which would oppose healing. Second, approaches to keep a wound oxygenated over a longer period of time, as opposed to a few hours usually targeted in HBO therapy, should prove to be beneficial. In response to HBO, there is no sustained change in tissue O₂ tension beyond the period of treatment.

The most fundamental factors in wound care are fluid management, temperature management, pain control, increased arterial O₂ tension, the use of appropriate sterile techniques, and administration of prophylactic antibiotics. In addition, numerous cellular and molecular players are required to act in concert to successfully execute wound healing. While examining the efficacy of O₂ therapy in wound healing, it is critically important to recognize that O₂ cannot act in isolation. Oxygen therapy may be only expected to benefit in those cases where the remaining essential players are functional and hypoxia is the only rate limiting factor. Thus, oxygen therapy is generally recommended as an adjunct to other forms of wound care.

Hyperbaric oxygen therapy represents an effective approach to bolster tissue O₂ levels and has been found to benefit wound healing under specific conditions. Importantly, HBO may potentially work synergistically with growth factors such as PDGF to improve the
outcomes of ischemic wounds. Because PDGF requires O$_2$-derived H$_2$O$_2$ for successful function, this finding is not surprising. HBO causes sharp elevation in tissue pO$_2$. The administration of two atmospheres of 100% O$_2$ for 2h may raise tissue pO$_2$ by 10-20 folds over the values under basal room air conditions. This systemic approach to oxygenate tissues seems to offer some unique potential advantages. HBO may increase bone marrow NO in vivo thereby increasing the release of EPC into circulation. EPC mobilization into circulation is triggered by hyperoxia through induction of bone marrow NO with resulting enhancement in ischemic limb perfusion and wound healing. HBO may also increase nitric oxide levels in perivascular tissues via stimulation of NOS. Exposures to 2.0 and 2.8 ATA O$_2$ stimulated neuronal (type I) NOS (nNOS) and significantly increased steady-state NO concentration, but the mechanism for enzyme activation differed at each partial pressure. Enzyme activation at 2.0 ATA O$_2$ appeared to be due to an altered cellular redox state. Exposure to 2.8 ATA O$_2$, but not 2ATA O$_2$, increased nNOS activity by enhancing nNOS association with calmodulin. Thus, dosing does seem to matter in HBO therapy. Yet, in the clinics HBO is applied in a standard format to all patients regardless of their individual needs. Could this be an important factor in explaining the less than satisfactory results that HBO is generally thought to have produced in clinical settings? When a flat dose of oxygen is provided to all wound patients, it is possible that the specific dose applied is successful in oxygenating the pockets of extreme hypoxia in some wounds. In these cases, beneficial outcomes should be expected to follow. In the same vein it may be hypothesized that for some other cases, the dose applied is excessive compared to the need of the wound. In these wound with pockets of more moderate hypoxia, the same dose of HBO may be excessive negating the beneficial effects of hypoxia. This is of outstanding interest because excessive oxygen is known to cause growth arrest and accelerate cellular senescence. Because the ability to handle oxygen toxicity is dependent on the expression of genes encoding antioxidant proteins, it is possible that in some patients pre-disposed to oxidative stress the massive increase in tissue pO$_2$ following HBO results in molecular responses such as growth arrest which may not manifest overt signs of oxygen toxicity but does resist wound healing. Another consideration in this regard would be the observation that a large fraction of chronic wound patients suffer from malnutrition. Such individuals are also known to be pre-disposed to oxidative stress and are limited in their ability to fend against oxygen toxicity. It is therefore reasonable to propose that chronic wound patients suffering from malnutrition are pre-disposed to HBO-induced oxidative stress. Taken together, such hypotheses would explain the inconsistent outcomes reported following HBO treatment and call for HBO dosing regimens where physicians would prescribe the target wound pO$_2$. This approach would be consistent with the emerging concept of personalized healthcare and would require the design of new HBO devices fitted with the capability of real-time mapping of wound O$_2$ tension as can be made possible via technologies such as electron paramagnetic resonance spectroscopy.

Topical oxygen

Studies reported during the last five years renew interest in examining the significance of topical approaches to oxygenate cutaneous wounds as adjunctive therapy. Topically applied O$_2$ gas is able to modestly raise the pO$_2$ of the superficial wound tissue. In cases where hypoxia of the superficial wound tissue is a key limitation, topical oxygenation should prove to be helpful. Encouraging results obtained from the use of topical O$_2$ gas in both clinical and pre-clinical settings warrant serious consideration of this approach. Recently, perfluorocarbon droplets encapsulated in aqueous continuous phase has been used as topical O$_2$ emulsion to treat experimental wounds. Results from this double-blind in vivo study demonstrate that topical approaches to oxygenate the wound significantly enhance the rate of epithelialization of partial-thickness excisional wounds and second-degree burns. Whether the emulsion was able to increase wound tissue pO$_2$ was not examined.
Epithelial wound healing is improved by transdermal sustained-delivery treatment with 100% O\textsubscript{2} \textsuperscript{14}. A recent clinical study testing the effects of topical O\textsubscript{2} gas application on chronic wound presented clinically reports significant improvement in wound size. Interestingly, topical oxygen treatment was associated with higher VEGF expression in the wound edge tissue \textsuperscript{18}. Pure O\textsubscript{2} is known to induce VEGF \textsuperscript{15, 63, 219}. Findings of the study testing the effects of topical oxygen gas on chronic wounds are consistent with previous findings suggesting that topical treatment may induce wound angiogenesis \textsuperscript{278}. Randomized clinical trials testing the effects of topical oxygenation on wound outcomes are warranted.

HBO and topical oxygen approaches have several contrasting features. The systemic effects of HBO, both favorable as well as unfavorable, may not be expected with topical oxygen. Topical oxygenation can only modestly raise tissue $pO_2$ \textsuperscript{277} and cannot match the large increases in tissue $pO_2$ typically noted in response to HBO \textsuperscript{242, 243}. If the goal is to correct hypoxia of the superficial tissue, topical approaches should be helpful. However, if the goal is to achieve larger supraphysiological levels of tissue $pO_2$, HBO would represent the approach of choice. An advantage of topical approaches is that they are portable and therefore applicable in a field or home setting. The cost advantage of topical oxygenation over HBO is another practical consideration \textsuperscript{276, 279, 280}.

**Summary**—The etiology of chronic ischemic wounds is generally multi-factorial of which hypoxia is a common factor in most cases. Primarily based on the tumor literature, hypoxia is generally viewed as being angiogenic. This is true with the condition that hypoxia be acute and mild-modest in magnitude. Extreme hypoxia, as commonly noted in problem wounds, is not compatible with life or tissue repair. Adequate wound tissue oxygenation is required but may not be sufficient to favorably influence healing outcomes. Success in wound care depends on a personalized health care approach. The key lies in our ability to specifically identify the key limitations of a given wound and in developing a multifaceted strategy to address those limitations. In considering approaches to oxygenate the wound tissue it is important to recognize that both too little as well as too much may impede the healing process. Oxygen dosing based on the specific need of a wound therefore seems prudent. Therapeutic approaches targeting the oxygen sensing and redox signaling pathways are promising as well. Investment in bringing such capabilities to clinical practice should yield lucrative returns.

**Acknowledgment**
Supported by NIH awards RO1 HL073087, GM 077185 and GM 069589 to CKS.

**Literature Cited**


Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.


Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.


Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.
of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat Genet. 2008

Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.

Wound Repair Regen. Author manuscript; available in PMC 2010 January 1.
Figure 1. Significance of molecular oxygen and its derivatives in wound healing
In its molecular form, oxygen is required for oxidative metabolism derived energy synthesis, protein synthesis and the maturation (hydroxylation) of extracellular matrices such as collagen. Molecular oxygen is also required for NO synthesis which in turn plays a key role in the regulation of vascular tone as well as in angiogenesis. In a wound setting, large amounts of molecular oxygen are partially reduced to form reactive oxygen species (ROS). ROS includes oxygen free radicals such as superoxide anion as well its non-radical derivative hydrogen peroxide ($\text{H}_2\text{O}_2$). Superoxide anion radical is the one-electron reduction product of oxygen. NADPH oxidases represent one major source of superoxide anion radicals at the wound-site. NADPH oxidases in phagocytic cells help fight infection. Superoxide anion also drives endothelial cell signaling such as required during angiogenesis. In biological tissues, superoxide anion radical rapidly dismutates to hydrogen peroxide-either spontaneously or facilitated by enzymes called superoxide dismutases. Endogenous hydrogen peroxide drives redox signaling, a molecular network of signal propagation that supports key aspects of wound healing such as cell migration, proliferation and angiogenesis. Neutrophil-derived hydrogen peroxide may be utilized by myeloperoxidase to mediate peroxidation of chloride ions resulting in the formation of hypochlorous acid (HOCl), a potent disinfectant.
Figure 2. Heterogeneous distribution of oxygen in the wound tissue: hypothetical pockets of graded levels of hypoxia
Structures outside the illustrated magnifying glass represent the macro tissue structures. Objects under the glass represent a higher resolution. Shade of black (anoxia) or blue represents graded hypoxia. Shade of red or pink represents oxygenated tissue. Tissue around each blood vessel is dark pink in shade representing regions that are well oxygenated (oxygen-rich pockets). Bacteria and bacterial infection are presented by shades of green on the surface of the open wound.
### Table 1

HIF-1 target genes

<table>
<thead>
<tr>
<th>Erythropoiesis/ Iron Metabolism</th>
<th>Cell Survival/ Proliferation</th>
<th>Angiogenesis</th>
<th>Vascular tone</th>
<th>Glucose Metabolism</th>
<th>Matrix Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO</td>
<td>IGF2</td>
<td>VEGF</td>
<td>NOS2</td>
<td>HK1,2</td>
<td>MMPs</td>
</tr>
<tr>
<td>Tf</td>
<td>TGF-α</td>
<td>Leptin</td>
<td>HO1</td>
<td>LDHA</td>
<td>PAR/PAI</td>
</tr>
<tr>
<td>Tfr</td>
<td>ADM</td>
<td>TGF-β3</td>
<td>ETβ1</td>
<td>PKM</td>
<td>Coll PHD</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>BNip3</td>
<td>EG-VEGF</td>
<td>ADM</td>
<td>PFKL</td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>α1b</td>
<td></td>
<td></td>
<td>PGK1</td>
<td></td>
</tr>
<tr>
<td>NDRG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIX</td>
<td>PGK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α1b, α1b-adrenergic receptor; ADM, adrenomedulin; AK, adenylate kinase; ALD, alldolase; BNip3, Bcl-2/adenovirus EIB 19kD-interacting protein 3; CA, carbonic anhydrase; Coll PHD, collagen prolylhydroxylases; EG-VEGF, endocrine gland-derived VEGF; ENO, enolase; EPO, erythropoietin; ET, endothelin; GAPDH, glyceraldehyde phosphate dehydrogenase; GLUT, glucose transporters; HK1,2, hexokinase 1,2; HO, heme oxygenase; IGF, insulin-like growth factor; LDH-A, lactate dehydrogenase-A; MMP, matrix metalloproteinases; NDRG, N-Myc downstream-regulated genes; NIX, Nip 3-like protein X; NOS, nitric oxide synthase; PAR/PAI, plasminogen activator receptors and inhibitors; PGK1, phosphoglycerate kinase 1; PFKL, phosphofructokinase L; PKM, pyruvate kinase M; TGF, transforming growth factor; TF, transferrin; Tfr, Tf receptor.
How Oxygen Works in Wound Healing

The following is a summary overview of the current scientific literature regarding the efficacy of oxygen in wound healing. The primary modes of action listed below are detailed in the following pages. Literature references are included at the end.

1. Increases Cell Metabolism and Energy Production
   Oxygen is required for intracellular processes like biosynthesis, movement, and transport need energy to be functional, as well as for cell survival
   * Oxygen dependent enzymes include:
     - Adenosine triphosphate (ATP) for chemical energy, which fuels most active cellular processes such as during wound healing.
     - Increased energy demand of the healing tissue leads to a hypermetabolic state wherein additional energy is generated from oxidative metabolism increasing the oxygen demand of the healing tissue.
     - ATP thus generated powers tissue repair
     - NADPH oxygenase for respiratory burst (reactive oxygen species release), the activity of which is critically required to produce the redox signals required for wound healing
       - Aerobic glycolysis, β-oxidation of fatty acids, and the citric acid cycle are tightly attached to the energy acquisition by oxidative phosphorylation and are therefore oxygen dependent
     - If oxygen levels are too low (<20 mmHg pO2), cells convert to anaerobic metabolism and go into survival mode in which wound healing activities such as mitosis (cell division, and therefore reepithelialization) and collagen production are impaired
     - Prolonged exposure to extremely low oxygen levels, if not alleviated by oxygen, can result in cell death and tissue necrosis due to the inability of the cells to repair the spontaneous decay of cell components (DNA, RNA, proteins) and inability to maintain calcium pumps which require ATP to function

2. Increases Rate of Cell Proliferation and Reepithelialization
   Epithelial cells “march in” from the sides to close the wound and form a barrier between the wound and the environment – this is the foundation for forming new skin
   * The addition of pure oxygen over a diabetic wound has been shown to increase the rate of wound closure, as measured by endothelial gap closure, by as much as 69%, indicating more rapid reepithelialization
   * Fibroblast proliferation and protein production have been reported to be optimal at 160 mmHg, i.e. at pO2 levels 2-fold to 3-fold higher than those found in healthy tissues, indicating that supplemental oxygen increases the rate of wound repair
   * Endothelial progenitor cells (EPCs) are essential in wound healing, but their circulating and wound level numbers are decreased in diabetes. Elevated oxygen levels (hyperoxia) reverse the diabetic defect in EPC mobilization
   * EPC mobilization into circulation is triggered by hyperoxia through induction of nitric oxide (NO) with resulting enhancement in ischemic limb perfusion and wound healing

3. Increases Collagen Synthesis and Tensile Strength
   Oxygen is essential to make and properly organize collagen, which is the primary component of skin, accounting for 70-80% (dry weight – without water) and acts as the structural scaffold of skin. Organized collagen is bundled into fibers (like strands in rope), which are interwoven and can be stretched in multiple directions without tearing (the collagen fibers are woven similar to fabric)
Oxygen plays a crucial role in various physiological processes. It promotes growth factor signaling transduction, increases anti-bacterial activities, and enhances angiogenesis and revascularization. Oxygen levels directly affect the rate and quality of collagen deposition and tensile strength. At the wound site, ROS are generated by almost all wound-related cells, including neutrophils and macrophages. Optimal ROS production is seen at oxygen levels of greater than 300 mmHg, which can only be achieved with supplemental oxygen. The efficacy of supplemental oxygen has been shown to be similar to antibiotic administration and has additive effects when used together.

### 4. Increases Anti-bacterial Activities
Oxygen is essential for respiratory burst, the production of reactive oxygen species (ROS), and the bactericidal activity of phagocytes. It is required for respiratory burst, the production of ROS, and the bactericidal activity of phagocytes such as neutrophils and macrophages in bactericidal activity and the removal of necrotic cellular debris. Oxygen is necessary for respiratory burst, the production of ROS, and the bactericidal activity of phagocytes.

### 5. Increases Angiogenesis and Promotes Revascularization
The creation of new blood vessels, angiogenesis, is essential for the growth and survival of repair tissue. Oxygen levels directly affect the rate and quality of new blood vessel growth. Sufficient oxygen levels are required for correct collagen synthesis (posttranslational hydroxylation), without which the new capillary tubes assemble poorly and remain fragile. Supplemental oxygen accelerates blood vessel growth, and moderate hyperoxia increases the appearance of new blood vessels in wounds. The rate of angiogenesis is directly proportional to oxygen levels in injured tissues, and rates of collagen deposition increase proportionally with oxygen levels to more than 250 mmHg.

### 6. Promotes Growth Factor Signaling Transduction
Reactive oxygen species (ROS) are essential for the signaling processes of growth factors and processes such as leukocyte recruitment, cell motility, angiogenesis, and extracellular matrix formation. Signal transduction of growth factors happens through ROS.

### Notes:
- Some sections of the above text with citations are directly extracted from the referenced literature without quotations.
- Recommended summary articles on oxygen in wound care include articles by Sen, Tandara and Mustoe, and Gordillo and Sen.

### References
Oxygen in acute and chronic wound healing


Departments of Dermatology and *Plastic Surgery, University Hospital Regensburg, 93042 Regensburg, Germany

Correspondence
Philipp Babilas.
E-mail: philipp.babilas@klinik.uni-regensburg.de

Accepted for publication
31 March 2010

Key words
hyperbaric oxygen therapy, luminescence imaging, oxygenation, reactive oxygen species, wound oximetry

Conflicts of interest
The work was supported by grants of the German Research Foundation (Deutsch Forschungsgemeinschaft DFG, BA 341013-1 and W0669/9-1) and the Novartis Foundation (S. S., Novartis Graduate Scholarship).

DOI 10.1111/j.1365-2133.2010.09804.x

Summary

Oxygen is a prerequisite for successful wound healing due to the increased demand for reparative processes such as cell proliferation, bacterial defence, angiogenesis and collagen synthesis. Even though the role of oxygen in wound healing is not yet completely understood, many experimental and clinical observations have shown wound healing to be impaired under hypoxia. This article provides an overview on the role of oxygen in wound healing and chronic wound pathogenesis, a brief insight into systemic and topical oxygen treatment, and a discussion of the role of wound tissue oximetry. Thus, the aim is to improve the understanding of the role of oxygen in wound healing and to advance our management of wound patients.

An injury to the skin may disturb the integrity of the epidermis, the dermis, the connective tissue and the microcirculation, and thus inevitably results in a wound. The disturbed equilibrium of the local environment induces wound healing.1 Physiological wound healing is a well-regulated stepwise process that ends with wound closure within days or weeks, depending on diameter and depth of the wound.2,3 One critical parameter for wound healing is oxygen that is required for almost every step of the healing process.4–7 The oxygenation of wound tissue is dependent on both the oxygen supply to the wound tissue – that is determined by the pulmonic gas exchange, the blood haemoglobin level, the cardiac output, the peripheral perfusion rate, and by the capillary density in the wound tissue and its periphery – and on the oxygen consumption rate of parenchymal, stromal and inflammatory cells of which the wound tissue is composed. Interestingly, in literature dealing with wound tissue oxygenation, the unit mmHg for oxygen partial pressure (pO2) is still widely used even though it is not the standard SI unit, which is pascal (1 Pa = 7-5066 × 10-3 mmHg). Therefore, we use mmHg throughout this review to make it easier for the reader to compare data from the different publications cited herein.

In wound healing, biochemical energy supply is a basic requirement. Oxygen is essential for the production of biological energy equivalents (e.g. adenosine triphosphate, ATP) in aerobic glycolysis, the citric acid cycle, and the oxidation of fatty acids.4,5 Therefore, sufficient oxygenation of tissue is a prerequisite for adequate energy levels, which are essential for proper cellular function.

In healing tissue, sufficient oxygenation is particularly relevant because of the increased energy demand for reparative processes such as cell proliferation, bacterial defence and collagen synthesis. The strictly oxygen-dependent NADPH-linked oxidase represents a further highly important enzyme in wound healing; it catalyses the production of reactive oxygen species (ROS) such as peroxide anion (HO2−), hydroxyl ion (HO·) and superoxide anion (O2−).8 ROS play a prominent role in oxidative bacterial killing9,10 and coregulate prevalent processes in wound healing such as cytokine release, cell proliferation and angiogenesis.8,11

Against this background, the crucial role of reduced oxygen supply in chronic wound pathogenesis becomes obvious. Chronic wounds are characterized by an insufficient repair process that precludes the establishment of a sustained anatomical and functional result in an appropriate length of time.1,2 Chronic wounds represent a frequent interdisciplinary disease affecting about 1% of the European population. According to the United Nations (see http://www.un.org/), the population of Europe was approximately 830 million in 2009, using a definition including the whole of the transcontinental countries of Russia and Turkey. Based on these figures, about 8 million people in Europe suffered from chronic wounds in 2009. Besides the tremendous impact on the quality of life of the affected patients, chronic wounds are
of fundamental economic relevance: nearly 2% of European health budgets are spent on the impaired healing of chronic wounds. In Germany, over 2.8 million sick days per year are caused by chronic wounds. In the U.S.A., approximately one-third of the dermatological health budget is spent on the treatment of chronic wounds.

This review summarizes the role of oxygen in the sequential steps of physiological wound healing. The pathogenesis of chronic wounds is explained against the background of impaired wound tissue oxygenation. Moreover, we question the benefits of treatment strategies for improving wound tissue oxygenation and discuss the role of wound tissue oxygen measurement either to classify chronic wounds or to monitor different treatment approaches in clinical routine.

**Physiological wound healing**

Physiological (syn. acute) wound healing is a dynamic stepwise process consisting of partially overlapping phases that are determined by interacting events on a molecular, cellular and extracellular matrix (ECM) level. This process, which is not yet fully understood, starts with a disturbance of tissue integrity and ends with a restitution ad integrum or a scar formation within an appropriate length of time. Nearly every step of wound healing requires oxygen. For didactic reasons, the course of physiological wound healing is schematically divided into three overlapping phases: the inflammatory phase, the proliferative phase (neoangiogenesis, tissue formation, re-epithelialization) and the tissue remodelling phase (Fig. 1).

**Inflammatory phase**

Physiologically, the inflammatory phase lasts between 4 and 6 days and starts immediately after wounding. Blood vessels constrict after traumatization, and platelets aggregate along the activated endothelium. Vascular disruption and vasocstriction cause a hypoxic microenvironment that is intensified by increased oxygen consumption due to metabolically active cells contributing to wound healing. Hypoxia actuates the initial steps of wound healing by boosting ROS activity, by activating platelets and endothelium, and by inducing cytokines released from platelets, monocytes and parenchymal cells [e.g. vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, tumour necrosis factor (TNF)].

However, even if acute hypoxia initiates wound healing, the recovery of wound tissue oxygenation is of major importance for physiological healing as chronic hypoxia impairs all processes necessary for healing. The aggregated platelets initiate the coagulation cascade leading to a blood clot, which prevents the leakage of blood and forms a provisional ECM. The provisional ECM, which is composed of fibronectin, fibrinogen, fibrin, thrombospinoid and vitronectin, fills the tissue defect and enables migration of the different cytokines and cells required for the healing process. Besides these structural contributions, the activated platelets direct the healing process through the secretion of several mediators of wound healing such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-β1 and TGF-β2. ROS stimulate cytokine and chemokine release as well as their functions. The primary effect of these mediators is the recruitment and activation of neutrophils and macrophages to the wound site and the activation of fibroblasts. However, these platelet-derived processes are not the only ones that initiate healing. The injury activates epithelial and nonepithelial cells in the wound area. Consecutively, cytokines and chemokines are secreted, which initiate stress pathways and activate the complement cascade, both oxygen-dependent processes. In consequence, a set of secreted factors [TGF-α, TGF-β1, keratinocyte growth factor (KGF), EGF, PDGF and insulin-like growth factor (IGF)] is released, which attracts and stimulates relevant players of wound healing such as inflammatory leukocytes and fibroblasts. Just recently, it has been shown that hydrogen peroxide (H₂O₂) is an important mediator in wound–leucocyte interaction. A tail-fin model of a zebrafish with a genetically encoded H₂O₂ sensor showed that H₂O₂ at the wound margins peaks as early as a few minutes after injury, and that leukocytes are recruited to the wound site by a tissue-scale H₂O₂ gradient. Whether H₂O₂ is produced by damaged cells or by their neighbours, and how neutrophils sense H₂O₂ gradients yet remains to be answered.

Even very superficial traumas without the destruction of blood vessels and activation of platelets initiate wound healing. Injured parenchymal cells secrete prostaglandins, histamine, bradykinin and serotonin, which induce vasodilatation and increase capillary permeability. Subsequently, diapedesis of cells is accelerated and oxygen supply to the wound site is
increased. Clinical correlates of these processes are erythema and oedema, which become apparent at the wound edges during the inflammatory phase. The infiltration of leukocytes, monocytes, and – 24–48 h later – macrophages is the key event in initial wound healing; their functions, such as degradation of cell detritus, counteraction of tissue infection and phagocytosis of microorganisms, are indispensable for wound healing. The amount and proportion of these inflammatory cells as well as the duration of the inflammatory phase depend on the wound extent, the degree of tissue infection, and the extent of debris that needs to be removed. Metabolically, inflammatory cells are extremely active and therefore depend on high amounts of oxygen. The high consumption of oxygen may lead to areas of hypoxia, even in well-oxygenated wounds. During the inflammatory phase, one central product of neutrophils, macrophages, monocytes, endothelial cells and fibroblasts is ROS. Thrombin, PDGF and TNF stimulate the release of ROS from endothelial cells, whereas intercellular (IL)-1 and platelet-activating factor stimulate ROS release from fibroblasts. ROS are the main force against microorganisms and thus wound infection. As mentioned above, NADPH-linked oxygenase is responsible for the production of ROS, a highly oxygen-dependent process: the $k_m$ (half maximal velocity) for NADPH-linked oxygenase with oxygen as a substrate is a $pO_2$ value of 40–80 mmHg. Neutrophils were shown in vitro to lose their bacterial killing capacity at a $pO_2$ level below 40 mmHg. This loss may explain the significant bacterial colonization apparent in hypoxic chronic wounds. Besides their role in the oxidative killing of bacteria, ROS are able to augment neutrophil chemotaxis. Activated inflammatory cells themselves produce cytokines and growth factors such as IGF, leucocyte growth factor, IL-1, IL-2, TNF, TGF-α, TGF-β, VEGF, PDGF and lactate. VEGF and PDGF are both potent chemoattractants and mitogens for fibroblasts and angiogenic growth factors; their release initiates the formation of granulation tissue and thus the proliferative phase at day 4–5 after wounding.

Proliferative phase

The proliferative phase lasts – depending on the extent of the wound – for a few weeks and comprises elementary processes such as neovascularization, formation of granulation tissue and ECM, and re-epithelialization. Endothelial cells and fibroblasts simultaneously invade the initially built haemostatic clot. Macrophages lead the way by degrading the clot and by releasing cytokines and chemokines that attract fibroblasts and stimulate angiogenesis. Particularly macrophages and their metabolites play a pivotal role in granulation tissue formation as depletion of macrophages was shown to lead to impaired wound healing in an in vivo porcine model. Fibroblasts and keratinocytes also secrete growth factors. Hereby, the cytokines of the TGF-β superfamily seem to play the most prominent role in granulation tissue formation. Interestingly, ECM molecules such as fibrinogen, fibronectin, fibrin and vitronectin are interactive with cytokines and also regulate the proliferation, differentiation and migration of fibroblasts. Important stimulators of angiogenesis are hypoxia and ROS. Both stimulate macrophages, fibroblasts, endothelial cells and keratinocytes to synthesize VEGF. Again, acute hypoxia is the initiator of this process, whereas chronic hypoxia impairs neovascularization. Hypoxia activates the transcription factor hypoxia-inducible factor (HIF)-1α. HIF-1α binds to the hypoxia response element in the gene promoter region of the VEGF gene, which in turn upregulates VEGF. As the major angiogenic growth factor, stimulates endothelial cells to migrate, proliferate and form countless new capillaries. Rossiter et al. showed in a murine model system that keratino- cyte-specific deletion of VEGF resulted in delayed wound healing due to impaired neoangiogenesis. Complementarily, Hong et al. showed enhanced wound healing in transgenic mice with overexpression of VEGF in the skin. The new capillaries branch out and invade the provisional wound matrix, which is replaced piecemeal by a new ECM produced and deposited by fibroblasts. The emerging ECM, in which fibroblasts, myofibroblasts, leucocytes and macrophages are embedded, consists of immature collagen (type III), proteoglycans, glycosaminoglycans, fibrin, fibronectin and hyaluronic acid. In this context, the production and deposition of collagen represents a fundamental process as it reconstitutes skin alignment and integrity. The production and deposition of collagen is proportional to oxygen tension: fibroblasts need a $pO_2$ of 30–40 mmHg for collagen synthesis. A central oxygen-dependent step in the synthesis of collagen is the hydroxylation of proline and lysine residues. In addition, hydroxylase activity is critically dependent on cofactors such as iron and vitamin C. Lysyl hydroxylase and lysyl oxidase, both oxygen-dependent enzymes, catalyse collagen cross-linking, a step that aims at wound stability. Again, in hypoxia, acute hypoxic conditions must be distinguished from chronic hypoxia. Acute hypoxia may stimulate fibroblast proliferation, collagen synthesis and expression of TGF-β1, whereas chronic hypoxia decreases these processes as shown in vitro by Siddiqui et al. in human dermal fibroblasts. Angiogenesis and ECM synthesis are interdependent processes as new blood vessels need new ECM as a three-dimensional scaffold for their ingrowth while the cell metabolism of, for example, fibroblasts needs new blood vessels that deliver oxygen and other nutrients. The fact that the same cytokines stimulate each process interconnects these steps of wound healing. Parallel to the formation of granulation tissue, re-epithelialization is initiated. Re-epithelialization aims at covering the wound surface by a layer of epithelium and is based on the differentiation, proliferation and migration of epidermal keratinocytes. The stress pathways activated by injury lead to the oxygen-dependent release of certain cytokines and chemokines (TNF, TGF-α, TGF-β1, KGF, IGF, PDGF and IGF) by parenchymal cells such as keratinocytes. These cytokines, foremost TNF, seem to stimulate epidermal cells at wound edges and hair follicles in an autocrine manner to restructure their cytoskeleton, a process that is oxygen dependent and starts within a few hours after injury. The cells retract their intracellular tonofilaments,
dissolve the desmosomal or hemidesmosomal connections, but establish adhesion structures for gripping to the ECM and develop cytoplasmic actin filaments for cell migration.\(^{51-53}\) Stimulated by EGF, TGF-\(\alpha\), KGF, TGF-\(\beta 1\), hepatocyte growth factor (HGF) and IGF-1, cell migration toward the wound’s central point, called shuffling, takes place.\(^{44}\) Hereby, TGF-\(\beta 1\) is a key cytokine as it controls the expression of integrins in keratinocytes. Integrons are cell surface receptors that interact with ECM, particularly with fibrin and fibronectin.\(^{44}\) Migration through the wound matrix, which is composed of necrotic material, bacteria, a haemostatic clot of platelets and fibrin, and later on of granulation tissue, is further supported by the activation of plasmin. This process is caused by a plasminogen activator produced by both epidermal cells\(^{46}\) and matrix metalloproteinases (MMPs). MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13) are released mainly by macrophages, keratinocytes, endothelial cells and fibroblasts\(^{67}\) and degrade certain constituents of provisional wound tissue such as collagen I, III, IV and VII. It is of outstanding importance that MMP inhibitors such as \(\alpha \_2\)-antiplasminase, secretory leucocyte protease inhibitor (SLPI), \(\alpha _2\)-macroglobulin and tissue inhibitors of MMPs (TIMPs) are sufficiently present, once provisional wound tissue has been removed. To achieve complete closure of larger wound areas, cell migration has to be accompanied by oxygen-dependent cell proliferation. For this, cytokines and chemokines (EGF, TGF-\(\alpha\), KGF, HGF, nerve growth factor, IGF-1, IL-1 and IL-6), most possibly released from keratinocyte stem cells, stimulate the proliferation of keratinocytes. Integrins are cell surface receptors that interact with ECM, particularly with fibrin and fibronectin.\(^{45}\) Migration gains increased wound tensile strength. The collagen fibres are deposited mainly by macrophages, keratinocytes, endothelial cells and fibroblasts. Of great importance are the TIMPs, which contribute to a concerted maturation process that leads to a restitution ad integrum or a scar formation depending on the MMP/TIMP ratio and activity.

**Tissue remodelling phase**

The tissue remodelling phase starts as early as a few days after injury and lasts up to 2 years thereafter. In the beginning, wound contraction contributes to wound closure. This process is enabled due to the differentiation of a subgroup of fibroblasts to contractile myofibroblasts triggered by oxygen\(^{53}\) and mediated by TGF-\(\beta 1\), TGF-\(\beta 2\) and PDGF.\(^{54-56}\) After the main steps of the proliferative phase are fulfilled, unknown stop signals induce a redifferentiation of fibroblasts, keratinocytes and endothelial cells so that the accelerated proliferation and migration normalizes. Gradually, the provisional collagen (type III) is replaced by the more stable collagen type I that is produced strictly oxygen dependently by fibroblasts and is deposited in a physiological alignment. Thus, the healing wound gains increased wound tensile strength. The collagen fibres contract so that the wound tissue shrinks.\(^{55,56}\) Prominent mediators of collagen anabolism and catabolism are MMPs, which are released oxygen dependently by macrophages, keratinocytes, endothelial cells and fibroblasts. Of great importance are the TIMPs, which contribute to a concerted maturation process that leads to a restitution ad integrum or a scar formation depending on the MMP/TIMP ratio and activity.

**Chronic wound healing**

Chronic wounds are defined as wounds that do not follow the well-defined stepwise process of physiological healing but are trapped in an uncoordinated and self-sustaining phase of inflammation (Fig. 1).\(^5\) This impairs the constitution of anatomic and functional integrity in a physiologically appropriate length of time. The aetiology of chronic wounds is diverse, but more than 80% are associated with venous insufficiency, high blood pressure or diabetes mellitus.\(^3,57\) Despite the different underlying aetiology, most chronic wounds show a similar behaviour and progress. This uniformity is due to consistent components of the multifactorial pathogenesis of most chronic wounds: local tissue hypoxia, bacterial colonization, repeated ischaemia-reperfusion injury and cellular as well as systemic changes of ageing (Fig. 2).\(^1,4,5,8,59\)

Common causes of local wound tissue hypoxia are pathological alterations of the vascular bed (arteriosclerosis, micro- or macroangiopathy, venous hypertension), periwound fibrosis and a subsequent local reduction of tissue perfusion, or oedema, which increases the distance between capillaries (Fig. 2). Local tissue hypoxia has been widely accepted to impair wound healing profoundly. Mathematical models showing the importance of oxygen for physiological wound healing and ischaemic wounds have recently been published.\(^{60,61}\) Sheffield measured a \(pO_2\) of 5–20 mmHg in chronic wound tissue as compared with 30–50 mmHg in control tissue.\(^62\) Ahn and Mustoe\(^63\) showed a wound healing deceleration of 80% in an ischaemic rabbit ear model evoked by a \(pO_2\) decrease from 40–45 mmHg to 28–30 mmHg. The initial implication of tissue hypoxia on the molecular level is the impairment of mitochondrial oxidative phosphorylation with a subsequently reduced ATP production. As a consequence, ATP-dependent membrane transport proteins such as Na\(^+\)/K\(^+\)-ATPase or Ca\(^+\+\)-ATPase drop out, which leads to a loss of the transmembrane potential with subsequent cell swelling. Particularly intracellular accumulation of calcium ions activates a signal transduction pathway that ends up in cell membrane disruption,\(^64\) which results in a promotion of inflammatory cascades via various signal pathways. Proinflammatory cytokines and chemokines such as TNF and IL-1 are
released, which attracts and activates neutrophils and macrophages. In addition, hypoxia induces a pronounced expression of endothelial adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and the corresponding ligands leucocyte function-associated antigen-1 and very late antigen-4 that enhance the extravasation and invasion of neutrophils and macrophages into the wound site with a subsequent autocrine synthesis of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF. Growth factors and cytokines are released in a self-perpetuating manner, macrophages are attracted, and tissue degenerating enzymes, e.g. serine proteases (neutrophil-derived elastases, cathepsin) and MMPs, particularly MMP-8, are generated. Wound fluid of chronic wounds has been demonstrated to show elevated levels of MMPs released from neutrophils (MMP-8) and fibroblasts (MMP-1, MMP-2, MMP-3, MMP-9, MMP-13). In physiological wound healing, processes are inhibited by α1-antiproteinase, SLPI, α2-macroglobulin and TIMPs, once all necrotic tissue and debris have been removed. In chronic wound healing, certain proteases (e.g. MMP-8 and MMP-9) exceed their inhibitors, leading to an excessive degradation of growth factors and ECM components such as collagen, fibronectin and vitronectin. The breakdown products further promote inflammatory reaction, changing the inflammation phase from a self-limiting to a self-sustaining process. Accordingly, the activity of neutrophils is long lasting in chronic wounds but is limited to the first 72 h in acute wound healing.

Neutrophils and macrophages produce ROS like HO2, HO and O2-. As mentioned above, ROS in low concentrations provide signalling and defence against microorganisms and thus play an important role in acute wound healing. A prerequisite for this process is a delicate balance between the amount of oxidants and antioxidants, as high amounts of ROS impair wound healing due to oxidative damage. High amounts of ROS not only damage extracellular structure proteins, lipids and DNA, but also stimulate complex signal transduction pathways, leading to an enhanced expression of MMPs, serine proteases and inflammatory cytokines. The toxic effects of high amounts of ROS were shown by the severe endothelial damage in wounds of mice which lack the ROS-detoxifying enzyme peroxiredoxin-6. The most effective antioxidant is nitric oxide (NO) that is produced by NO synthase in a strictly oxygen-dependent manner. NO not only detoxifies ROS but also switches off nuclear factor-kB, an important transcriptional activator of inflammatory proteins. Lymphocytes that invade wound tissue also activate the oxygen-dependent oxidoreductase thioredoxin as a protective mechanism against oxidative stress. Under oxidative stress, macrophages express the oxygen-dependent haem oxygenase and cysteine transporter to protect themselves against ROS. Thus, nearly all detoxification mechanisms are strictly oxygen dependent. Under hypoxic conditions, as prevalent in chronic wounds, the detoxification process is hindered, leading to a persistent and uncontrolled production of ROS and to a further potentiation of the inflammatory state. The resulting perpetual degradation of wound tissue impairs the maturation of wounds. However, hypoxia promotes wound healing not only by means of enhancing the inflammatory state but also through the impairment of countless other metabolic processes on the molecular, cellular and supracellular level. Siddiqui et al. demonstrated in vitro that the collagen synthesis rate of human fibroblasts is decelerated under chronic hypoxia. α1-procollagen was significantly downregulated at the mRNA and at the protein level. Accordingly, Hunt et al. showed in vivo that fibroblasts were actively proliferating only in wound tissue with a pO2 level of at least 15 mmHg. Jonsson et al. demonstrated this causality in a clinical investigation, in which the amount of deposited collagen was proportional to the pO2 value present in the respective wound. In another ischaemic rabbit ear model, Wu et al. demonstrated significantly...
impaired epithelial ingrowth and granulation tissue deposition under ischaemia.

Bacterial colonization, obligatory in all chronic wounds, attracts leucocytes, which results in high levels of proinflammatory cytokines and proteases and therefore directly initiates and maintains the inflammatory cascade. Different authors investigated wound fluids from acute and chronic wounds and showed an increased level of proinflammatory cytokines and proteases as well as a decreased level of growth factors in chronic wounds. Correspondingly, in healing wounds, a reduction in bacterial counts and markers of inflammation was reported. A direct correlation between bacterial colonization and the hypoxic state of the wound was shown in numerous studies. Knighton et al. performed a prospective study on 500 patients with colorectal resection. They compared the wound infection rates in two patient groups who had received 80% vs. 30% oxygen peri-operatively and 2 h postoperatively. Wound infection rates were 5.2% (80% oxygen) and 11.2% (30% oxygen), respectively. Other studies showed that even a moderate decrease of the tissue oxygen level significantly increases the risk of infection. Correspondingly, in an in vitro experiment, neutrophils were shown to lose their bacterial killing capacity at a pO2 level below 40 mmHg.

In the past years, different authors postulated ischaemia-reperfusion injury as an important aetiological factor of chronic wounds. Patients with impaired circulation due to venous insufficiency, arteriosclerosis, diabetes mellitus etc. suffer cyclic intervals of ischaemia and reperfusion in their lower legs when changing posture (leg elevation or leg dependency). As the leg position is changed repetitively, injury occurs in a self-potentiating cycle. Ischaemia in combination with subsequent hypoxia induces a proinflammatory state (see above). With reperfusion, oedema is increased. Moreover, additional neutrophils flood into the wound tissue and transmigrate to the activated endothelium, which further contributes to the inflammatory vicious circle leading to cell death and tissue damage. Besides, reperfusion accounts for partial reoxygenation with a subsequently enhanced production of ROS. In turn, ROS have a deleterious effect on vascular and cellular processes. The impact of repetitive ischaemia-reperfusion injury on wound healing was demonstrated in animal models in which the tissue damage correlated with the number of ischaemia-reperfusion cycles. Remarkably, repeated ischaemia-reperfusion cycles seem to be more deleterious for wound healing than prolonged phases of single ischaemia.

The fact that ageing cells show reduced cell viability and proliferative capacity, altered patterns of gene expression and decreased response to growth factors is of great importance for our understanding of abnormal healing, as most chronic wounds occur in the elderly (average age over 60 years). Senescent fibroblasts showed an increased generation of MMPs and a decreased release of MMP inhibitors, which could explain the well-documented fact that MMP inhibitor (TIMP-1) and serine protease inhibitor (α1-antitrypsin, antileucocyteprotease SLPI, α1-macroglobulin) activity is reduced in chronic wound fluids. The healing response in an aged organism is basically and essentially delayed and additional pathogenetic factors such as local tissue hypoxia soon overpower response. Here, tissue hypoxia as a mainstay of chronic wound pathogenesis plays a crucial role because aged cells are significantly more susceptible to hypoxia than young adult cells. In an ischaemic rabbit ear model, aged human fibroblasts showed decelerated migration under stimulation with TGF-β, depression of PDGF receptor β, and decreased TGF-β1 mRNA expression compared with young controls. Under hypoxic conditions, aged human keratinocytes showed a decelerated motility and a decreased proliferation rate in vitro compared with younger cells. Tandara et al. demonstrated increased cell death of aged human fibroblasts compared with young adult cells if exposed to oxidant stress plus ischaemia, conditions that are analogous to chronic wounds.

Interestingly, cells of chronic wounds show signs of senescence even independently of a patient’s age. Compared with fibroblasts taken from the healthy leg, fibroblasts harvested from the margin and bed of chronic wounds exhibited characteristics of premature senescence. An additional explanation might be that fibroblasts are driven through countless cell divisions to induce wound healing. Due to ongoing stimulation, cells seem to lose their proliferative capacity. This causality could explain the success of wound debridement as this procedure removes senescent cells from the ulcer surface. These data demonstrate that stress factors apparent in chronic wounds, specifically hypoxia, and the resulting premature senescence create a vicious circle that is difficult to break, particularly in elderly patients.

From the clinical point of view, the main surface area of a common nonhealing wound shows extensive fibrin deposition and necrosis due to the prevalent inflammatory state. However, a typical hallmark of chronic wounds is the persistent occurrence of islands of granulation tissue or epithelialization within the inflammatory battlefield. Hunt et al. showed that wound areas with actively proliferating fibroblasts were seen only at pO2 above 15 mmHg. Only rarely are these islands the origin of a structured healing process as, in most cases, they are overwhelmed by inflammation. The conditions in chronic wounds are changing repeatedly, not only temporally but also spatially. Thus, a chronic wound represents an extremely heterogeneous structure.
**Therapeutic wound oxygenation**

The ability of systemic oxygen therapy as well as topical oxygen therapy (TOT) to improve wound healing and prevent infection is documented in animal models and clinical trials. Hyperbaric oxygen therapy (HBOT) delivers 100% O2 at 2–3 atmospheres of pressure over 60–120 min 5 days a week in a specialized patient chamber. Usually, 10–30 treatments are performed. HBOT has turned out to be an effective tool to increase pO2 values in wound tissue, and the effects of HBOT on chronic wound healing have been described by a mathematical model. Sheikh et al. demonstrated increased VEGF expression in rats treated with HBOT, and Hopf et al. showed a stimulation of neovascularization in hypoxic tissue after HBOT. Moreover, oxygen administration has been shown to increase VEGF mRNA levels in endothelial cells and macrophages and VEGF protein expression in wound fluids in vivo. Knighton et al. reported accelerated vessel growth following supplemental oxygen administration. The transcutaneous pO2 in wound-surrounding tissue measured during HBOT correlated directly with the improvement in wound healing of chronic wounds. In a randomized controlled trial in diabetic patients (n = 68) with ulcers of the lower legs, Faglia et al. ascertained that treatment with HBOT and standard care vs. standard care alone resulted in a significant lower amputation rate in the HBOT group. Kranke et al. assessed the benefits and harms of HBOT for treating chronic ulcers of the lower legs and found that HBOT both significantly reduced the risk of major amputation and improved the chance of healing. Abidia et al. evaluated in a double-blind study the role of HBOT in the management of ischaemic lower-extremity ulcers. Patients were given 30 treatments of 100% oxygen vs. air. Healing was achieved in five of eight ulcers in the treatment group compared with one of eight ulcers in the control group. Despite the expense of HBOT, the authors stated reduced total treatment costs for every patient during treatment. Another controlled study demonstrated that HBOT did not reduce hospital days of wound patients but the amount of bacterial colonization. Boonomo et al. showed that HBOT stimulates the release and activity of growth factors and their receptors. Zhao et al. measured the amount of epithelial regrowth and granulation tissue production following HBOT alone and in combination with PDGF or TGF-β1 in an ischaemic rabbit ear ulcer model. They demonstrated that HBOT alone increased the production of new granulation tissue. However, the addition of growth factors to HBOT synergistically led to increased healing rates. This supports the clinical experience that, in the majority of cases, the different treatment strategies for chronic wounds are successful only if combined. In vitro experiments of Roy et al. demonstrated that oxygen triggers the differentiation of fibroblasts to myofibroblasts – a possible explanation for the accelerated healing. It has also been shown in vitro that HBOT may increase the susceptibility of certain bacteria to antimicrobial agents. For example, Kenward et al. reported that under HBOT the zones of inhibition were reduced in Gram-negative bacterial cultures, whereas for Gram-positive bacteria a mixture of effects was found. It seems that these effects are quite strain specific and may not easily be generalized to all aerobic or anaerobic bacteria. Grief et al. reported significantly fewer postoperative infections in patients who had received 80% oxygen compared with patients who had received 10% oxygen during surgery and 2 h afterwards. However, costs of therapy, the risk of systemic oxygen toxicity, and the lack of large evidence-based studies with standardized treatment protocols impede the general acceptance of oxygen therapy as a standard treatment option in wound care.

TOT is characterized by the administration of pure oxygen to the wound area using a portable inflatable device. A major advantage of TOT is its independence of the wound’s microcirculation. Other advantages are lower costs, the lower risk of oxygen toxicity, and the possibility of home treatment. Fries et al. studied the efficacy of TOT in excisional dermal wounds in pigs. They showed that exposure of open dermal wounds to TOT increases wound tissue pO2 and, if repetitively applied, accelerates wound closure. Kallainen et al. conducted a retrospective uncontrolled study on 58 wounds in 32 patients given TOT. They documented a complete healing of 38 wounds in 15 patients during TOT and concluded that TOT had no detrimental effects on wounds and showed beneficial indications in promoting wound healing. However, the data currently available have a restricted informative value because of small sample sizes, the inclusion of different wound types and patient ages, additionally applied wound care regimens, nonstandardized treatment protocols, or a poor evaluation of comorbidities. Therefore, additional evidence-based studies with standardized treatment protocols are required to evaluate the efficacy of oxygen therapy.

**Oxygen monitoring in wounds**

Different methods allow the evaluation of wound tissue oxygenation. Direct and indirect as well as invasive and noninvasive methods must be distinguished, whereas the noninvasive methods are preferable in routine clinical settings. Indirect methods estimate tissue oxygenation only by calculating the relation of oxygenated to nonoxygenated haemoglobin. This calculation is possible as haemoglobin changes its spectroscopic as well as its magnetic properties with its degree of oxygenation. Respective methods (for instance, near-infrared spectroscopy, tissue reflectance photometry, magnetic resonance chemistry, magnetic resonance saturation, blood oxygen-dependent magnetic resonance imaging) are rather imprecise: first, they do not allow the measurement of absolute values; second, the exact penetration depth of light is unknown; and third, they are falsified by certain disturbance variables such as other tissue chromophores or global perfusion (for further details see reviews). Direct methods allow direct measurements of oxygen or pO2 values. Tissue oxygen-dependent magnetic resonance imaging is based on the paramagnetic properties of oxygen in tissue. However, this technique is not applicable at the skin surface as the skin–air interface evokes a severe artifact. The polarographic electrode technique allows the measurement of pO2 and is still
the gold standard for assessing tissue oxygenation. This technique is usually applied using a planar electrode for surface measurements or a needle electrode for measurements within the tissue.\textsuperscript{124,125} An alternative application method for measurements in wound tissue is to place the polarographic electrode in a subcutaneously implanted tonometer or to implant the polarographic electrode directly into subcutaneous tissue.\textsuperscript{80,90,126} However, placing an electrode into tissue (i) is invasive and painful, (ii) causes tissue injury that alters microcirculation and thus PO\textsubscript{2} levels, and (iii) may lead to irritation at the electrode membrane. A further fundamental limitation of the polarographic technique is the oxygen consumption during measurement, which makes long-term measurements impossible and overestimates PO\textsubscript{2} values as the electrode "sucks" oxygen through the tissue.\textsuperscript{127} Besides, this technique provides only scattered single measurements, making multiple measurements necessary. The required calibration before and after each measurement and the lacking spatial resolution further limits the application of the polarographic electrode technique.\textsuperscript{124,128} Therefore, the available methods suffer from disadvantages when measuring PO\textsubscript{2} levels in tissue. Because of these disadvantages, only a few studies exist on tissue oxygenation in acute wounds and hardly any study on tissue oxygenation in chronic wounds.

The use of luminescence lifetime imaging (LLI) overcomes these limitations.\textsuperscript{127–130} This method for two-dimensional PO\textsubscript{2} measurements is based on the oxygen-dependent quenching of phosphorescence of the indicator platinum(ii)-octaethylporphyrin. Hereby, the indicator is immobilized in a polystyrene matrix as a transparent planar sensor. This method was validated in vitro and in vivo, as well as in clinical settings.\textsuperscript{127–129,131} This body of work has characterized this method as particularly suitable for surface measurements. First, sensors are transparent, allowing a simultaneous visualization of the underlying wound tissue. Second, sensor sensitivity remains stable during measurement because alterations due to ageing, moisture, toxic cell products, local enzymes or photo-bleaching as a result of light exposure could be excluded in calibration sequences over 8 days both in vivo and in vitro.\textsuperscript{128,130} Third, PO\textsubscript{2} levels can be visualized in two dimensions with a high resolution (approximately 25 \textmu m) over large areas,\textsuperscript{128,131} thus allowing the simultaneous visualization of PO\textsubscript{2} gradients in different skin conditions. This fact is of fundamental relevance in heterogeneously oxygenated tissues such as chronic wounds as the heterogeneity can be registered simultaneously in a single measurement. Single point measurements as provided by the Clark electrode are unable to provide oxygen gradients. Fourth, in vitro experiments documented a high sensitivity over a broad PO\textsubscript{2} range (±0.2 mmHg at 0 mmHg; ±1.5 mmHg at 160 mmHg PO\textsubscript{2}).\textsuperscript{128} The Clark electrode provides a sensitivity of at least ±10%. Fifth, in clinical investigations, accurate and reproducible PO\textsubscript{2} values were provided under changing microcirculatory conditions. The lack of oxygen consumption during measurement allowed both a more realistic estimation of PO\textsubscript{2} values compared with the gold standard and the permanent use in regions with critical oxygen supply.\textsuperscript{127} Sixth, this noninvasive and rapid technique is simple to perform and prevents patient discomfort. Zhang et al.\textsuperscript{132} recently published a dual-emissive material for luminescence imaging of PO\textsubscript{2} in tumours, which may also be modified for use in two-dimensional wound oximetry.

Because of the great interest in new technologies that advance research on the role of oxygen in wound healing, Hopf and Rollins listed attributes of an ideal wound oximeter: noninvasive; repeatable; simple to use; stable for at least 24 h in vivo; not disturbed by motion, pH and CO\textsubscript{2}; provides accurate, precise and easily interpretable results in the range from 0 to 2300 mmHg. Furthermore, such an oximeter should enable continuous long-term measurements, require just a single point calibration at room conditions in vivo, and simultaneously measure oxygen and temperature at the same site.\textsuperscript{5} All these requirements are fulfilled by LLI.

**Conclusions**

Oxygen is well known to be required for wound healing. The \( K_m \) for enzymes involved in bacterial killing, collagen synthesis, angiogenesis and epithelialization requires PO\textsubscript{2} levels in wound tissue ranging from 25 to 100 mmHg.\textsuperscript{5,21,32} Therefore, restricted oxygenation, as common in chronic wounds, impairs the healing process. Several studies have demonstrated that enhancing wound tissue oxygenation improves wound healing and reduces bacterial colonization. Further research should establish LLI as a new, promising tool for wound oximetry. This will probably enhance our understanding of the pathophysiology of chronic wound healing and of oxygen delivery and metabolism in the different healing zones of chronic wounds. Of special interest is monitoring the wound oxygenation under different therapeutic regimens to enable the well-founded selection of a suitable and efficient therapeutic modality for the individual patient. This will contribute to cost-effectiveness and hopefully will improve the quality of life of the affected patients.

---

**What’s already known about this topic?**

- Oxygen is a prerequisite for wound healing due to the increased demand for reparative processes such as cell proliferation, bacterial defence, angiogenesis and collagen synthesis.
- Wound healing is impaired under hypoxia and topical oxygen therapy is known to affect healing.

**What does this study add?**

- We present an up-to-date overview on the role of oxygen in wound healing and chronic wound pathogenesis, a brief insight into the impact of systemic and topical oxygen treatment, and a discussion of the role of wound tissue oximetry.
Acknowledgments

The excellent editorial assistance of Ms Monika Scholl is gratefully acknowledged. This work was supported by grants of the German Research Foundation (Deutsche Forschungsgemeinschaft, BA 3410/3-1) and the Novartis Foundation (S.S., Novartis Postgraduate Scholarship).

References

Oxygen in wound healing, S. Schreml et al.


Oxygen in wound healing, S. Schreml et al. 11

On site noninvasive assessment of peri-implant inflammation by optical spectroscopy


Background and Objective: Optical spectroscopy has been proposed to measure regional tissue hemodynamics in periodontal tissue. The objective of this study was to further evaluate the diagnostic potential of optical spectroscopy in peri-implant inflammation in vivo by assessing multiple inflammatory parameters (tissue oxygenation, total tissue hemoglobin, deoxyhemoglobin, oxygenated hemoglobin and tissue edema) simultaneously.

Material and Methods: A cross-sectional study was performed in a total of 64 individuals who presented with dental implants in different stages of inflammation. In brief, visible–near-infrared spectra were obtained, processed and evaluated from healthy (n = 151), mucositis (n = 70) and peri-implantitis sites (n = 75) using a portable spectrometer. A modified Beer–Lambert unmixing model that incorporates a nonparametric scattering loss function was employed to determine the relative contribution of each inflammatory component to the overall spectrum.

Results: Tissue oxygenation at peri-implantitis sites was significantly decreased (p < 0.05) when compared with that at healthy sites, which was largely due to an increase in deoxyhemoglobin and a decrease in oxyhemoglobin at the peri-implantitis sites compared with the mucositis and healthy sites. In addition, the tissue hydration index derived from the optical spectra in mucositis was significantly higher than that in other groups (p < 0.05).

Conclusion: In summary, the results of this study revealed that hemodynamic alterations can be detected around diseased peri-implant sites by optical spectroscopy, and this method may be considered an alternative and feasible approach for the monitoring and diagnosis of peri-implant diseases.

Peri-implantitis is a destructive infectious inflammatory disease, which presents with a prevalence ranging from 7 to 20% and affects soft and hard tissue around osseo-integrated dental implants under functional loading. Mucositis is a reversible inflammation confined to peri-implant soft tissues, which may progress to peri-implantitis and has a prevalence of about 65% (1–5). Peri-implant and periodontal diseases present some common clinical and microbiological characteristics, including pockets, bleeding on probing, bone loss, suppurition and a complex pathogenic microbiota (1,6,7). However, some histological and anatomical aspects differ substantially between peri-implant and periodontal tissue, potentially altering the course of the...
An inflammatory process around implants compared with natural teeth. The perimucosal seal around dental implants, for example, is comprised of collagen fibers in a simple nonattached and circular arrangement that differs from the attached and perpendicular arrangement of the fibers around natural teeth (8–11).

To date, the following parameters are considered the only available tools in the differential diagnosis of peri-implant diseases: (i) implant mobility, which is an indicator of lack of osseointegration; (ii) sulcular probing, which is used to monitor marginal bleeding (an indicator of inflammation), probing depth (the distance between the soft tissue margin and the bottom of the peri-implant pocket or sulcus) and suppuration; and (iii) radiography, which is widely employed to detect bone loss around dental implants (1,6,7,12).

However, these tools are not sensitive enough to accurately detect early lesions, progressive lesions and sometimes even late lesions, which are important in avoiding further loss of soft and hard peri-implant tissues and eventually implant failure. Therefore, there is an increased interest in finding alternative accurate methods for the monitoring and diagnosis of peri-implant diseases.

As peri-implant diseases are local infections initiated by accumulation of bacterial biofilm (6,7), microbial testing has been proposed for diagnosis and prognosis of peri-implant lesions. In a 2 year longitudinal study, presence of one or more pathogenic species (i.e., Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis and Treponema denticola) was reported to enhance the prognostic features of bleeding on probing in predicting disease activity (13). In addition, the presence of specific biomarkers, including cytokines, enzymes and proteases, in peri-implant crevicular fluid or saliva has been suggested as a useful tool in monitoring healthy or diseased peri-implant tissues (14–17).

However, considering that inflammatory biomarkers work more often in a network-like organization than individually, one or two specific markers are very unlikely to stand alone as valuable diagnostic tools for peri-implant diseases.

Based on the optical characteristics of chemical components related to tissue inflammation, it is possible to characterize the inflammatory process by optical infrared spectroscopy. It has been proposed that several absorption bands in the visible and near-infrared spectral region reflect key inflammatory events (18,19). For instance, tissue edema, a clinical sign commonly used as a marker of mucosal inflammation (20,21), can be measured using near-infrared spectroscopy (18,19,22–24).

In addition, optical spectroscopy can gauge the relative concentrations of oxygenated hemoglobin (HbO₂) and deoxygenated hemoglobin (Hb) by fitting optical attenuation spectra to the known optical properties (extinction coefficients) of oxygenated hemoglobin and deoxygenated hemoglobin (18,22–26). A previous study by our research group showed that optical spectroscopy could indeed determine various inflammatory indices simultaneously in the periodontal tissues, representing a potential means for diagnosis of inflammation at specific periodontal sites (22). In principle, this newly emerging technology can differentiate periodontitis from gingivitis and healthy periodontal sites based on these optical parameters, specifically, tissue perfusion, oxygenation and hydration (22).

Based on the limitations of the currently available methods for the diagnosis of peri-implant diseases, in this study we propose that optical spectroscopy might also be a feasible alternative for the detection of peri-implant inflammation and possibly for the differentiation of peri-implantitis and mucositis from healthy sites.

**Material and methods**

**Subject population**

A total of 64 systemically healthy subjects were recruited from the population referred to the Oral Implantology Clinic of Guarulhos University (Guarulhos, São Paulo, Brazil) from June 2009 to July 2009. Medical and dental histories were obtained, and full-mouth periodontal and peri-implant examinations were performed. Subjects who fulfilled the following inclusion/exclusion criteria were invited to participate in the study. The study protocol was explained to each subject, and signed informed consent was obtained. This study protocol was approved by Guarulhos University’s Ethics Committee in Clinical Research (CEP no. 113/2008) and National Research Council Research Ethics Board (REB).

**Inclusion/exclusion criteria**

Inclusion criteria were those subjects who were totally or partly edentulous, and had been treated with at least one screw-shaped machined-surface titanium implant that had been functional for at least 1 year (6). They were non-smokers, not pregnant or lactating, systemically and periodontally healthy or periodontally treated and engaged in supportive periodontal therapy (for partly edentulous subjects). Exclusion criteria were those subjects who had moderate to advanced chronic periodontitis (i.e. bleeding on probing in more than 30% of the subgingival sites or any site with probing depth > 5 mm), had been treated with antibiotics or anti-inflammatory drugs and local antimicrobial agents within the preceding 6 months, and had peri-implant therapy within 6 months. To avoid occlusal interference, those subjects who had an implant-supported prosthesis with mobile abutments and/or screws, as well as fractured prosthetic crowns of ceramic or resin, were also excluded.

**Clinical and radiographic examination**

Clinical examinations were performed by the same trained examiner after the spectral acquisitions (X.M.X.). The following parameters were assessed at six sites in each implant (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) using a periodontal probe: (i) visible plaque accumulation, assessed as the plaque index, the presence (score 1) or absence (score 0) of plaque along the gingival-mucosal margin (27);
mucosal marginal bleeding, assessed as the presence (score 1) or absence (score 0) of bleeding obtained by running a probe along the soft tissue margin without probe penetration into the sulcus or pocket (27); (iii) bleeding on probing, obtained 20 min after recording mucosal marginal bleeding, when the presence (score 1) of bleeding on probing was considered when bleeding occurred up to 15 s after gentle probe penetration into the sulcus or pocket; (iv) suppuration, assessed as the presence (score 1) or absence (score 0) of spontaneous suppuration or suppuration on probing; and (v) probing depth (in millimeters), assessed as the distance between the mucosal margin and the bottom of the sulcus/pocket. The probing depth measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

Standardized intra-oral periapical radiographs were obtained, using a dental X-ray machine equipped with a 35-cm-long cone. Exposure parameters were 70 kV (peak), 15 mA, and 0.25 s at a focus-to-sensor distance of 30 cm. The radiographs were captured with a dental X-ray machine equipped with a 35-cm-long cone. Exposure parameters were 70 kV (peak), 15 mA, and 0.25 s at a focus-to-sensor distance of 30 cm. The radiographs were displayed on a monitor, and linear measurements were taken using imaging software (Image Pro-Plus 4.5; Media Cybernetics Inc., Silver Spring, MD, USA). The radiographs were analyzed for peri-implant bone loss (i.e. the linear distance in millimeters analyzed for peri-implant bone loss) using software was used to store the digitized images. Subsequently, the images were collected using a 0.03 s integration time. The spectral range between 500 and 1100 nm at 5 nm resolution was used. A 99% Spectralon® reference standard (LabSphere, North Sutton, NH, USA) was used as a reference to convert raw data into reflectance spectra. The spectral range between 500 and 1100 nm at 5 nm resolution was used. A 99% Spectralon® reference standard (LabSphere, North Sutton, NH, USA) was used as a reference to convert raw data into reflectance spectra. Each reflectance spectrum was collected prior to clinical measurements from 75 peri-implantitis sites, 70 mucositis sites and 151 normal sites, as shown in Table 1.

**Definition of diagnostic peri-implant sites**

Based on the clinical and radiographic parameters, the peri-implant sites were assigned to one of the following groups: healthy, defined as absence of mucosal marginal bleeding, bleeding on probing, suppuration and radiographic bone loss; mucositis, defined as mucosal marginal bleeding and/or bleeding on probing and absence of radiographic bone loss and suppuration; or peri-implantitis, defined as probing depth ≧5 mm with bleeding on probing and/or suppuration and concomitant radiographic sacuer-shaped osseous defects ≧3 mm (6,7,17). All spectra were collected prior to clinical measurements from 75 peri-implantitis sites, 70 mucositis sites and 151 normal sites, as shown in Table 1.

### Acquisition of optical spectra

Spectra were collected using a portable PDA512-ISA spectrophotometer (Control Development Inc., South Bend, IN, USA) interfaced to a customized bifurcated fiberoptic probe designed for use in the oral cavity (Fiberguide Industries, Stirling, NJ, USA). The intra-oral probe was designed in detail previously (22). The outer fibers of the probe are coupled to the entrance slit of the spectrograph and collect light subsequently back-scattered from the tissue (Fig. 1). The inner fibers at the bifurcated end of the probe were coupled to a 5 W tungsten halogen light source (Spectral Products, Putnam, CT, USA) that provides a stable light output. Each reflectance spectrum consisted of 16 co-added scans collected using a 0.03 s integration time. The spectral range between 500 and 1100 nm at 5 nm resolution was used. A 99% Spectralon® reflectance standard (LabSphere, North Sutton, NH, USA) was used as a reference to convert raw data into reflectance spectra. The spectral range between 500 and 1100 nm at 5 nm resolution was used. A 99% Spectralon® reflectance standard (LabSphere, North Sutton, NH, USA) was used as a reference to convert raw data into reflectance spectra. During the collection of the spectra, the subjects were comfortably seated in a relaxed, standard semi-reclined position on a dental chair, and the regular overhead dental operating light was turned off to avoid any potential interference.

### Calculation of hemodynamic indices from optical spectra

The derivation of the relative contribution of Hb and HbO2 to the optical attenuation spectrum obtained from tissue was described in detail previously (28). Briefly, a modified Beer–Lambert unmixing model that incorporates a nonparametric scattering loss function was used to determine the relative contribution of Hb and HbO2 to the spectrum by using the known absorption coefficients of Hb and HbO2 to fit the spectrum. The visible region between 510 and 620 nm of the measured tissue attenuation spectrum, A, was modeled as a sum of two parametric terms, Hb and HbO2, that contribute to the spectrum and a nonparametric term m(λ) modeling a vector of covariates, primarily the Rayleigh and Mie scattering losses that contribute to the attenuation of measured light.

\[
A(\lambda) = \sum_{i=1}^{3} \xi_i(\lambda) c_i L + m(\lambda) + \text{error}
\]

The concentrations of Hb and HbO2 per unit photon path length were estimated by solving the equation (28) using a noniterative partially linear method based on kernel smoothing, as first described by Speckman (25). Tissue hemoglobin oxygen saturation, S \( \text{O2} \), and a measure of tissue perfusion, tHb, were derived from the predicted Hb and HbO2 relative concentrations as follows:

\[
S \text{O2} = \frac{[\text{HbO2}]}{[\text{HbO2}]+[\text{Hb}]}
\]

\[
tHb = [\text{HbO2}]+[\text{Hb}]
\]

### Statistical analysis

The hemodynamic indices, Hb, HbO2, S \( \text{O2} \) and tHb, derived from the optical spectra, were analyzed separately using a one-way analysis of variance to test the hypothesis that the indices from the three groups of sites, healthy, gingivitis and periodontitis, would differ significantly. The unequal Tukey HSD was

---

**Table 1. Spectral site distributions**

<table>
<thead>
<tr>
<th></th>
<th>Buccal</th>
<th>Lingual</th>
<th>Upper</th>
<th>Lower</th>
<th>MB/ML</th>
<th>IP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peri-implantitis</td>
<td>43</td>
<td>32</td>
<td>47</td>
<td>28</td>
<td>17</td>
<td>58</td>
<td>75</td>
</tr>
<tr>
<td>Mucositis</td>
<td>39</td>
<td>31</td>
<td>33</td>
<td>37</td>
<td>38</td>
<td>32</td>
<td>70</td>
</tr>
<tr>
<td>Healthy</td>
<td>94</td>
<td>57</td>
<td>72</td>
<td>79</td>
<td>72</td>
<td>79</td>
<td>151</td>
</tr>
</tbody>
</table>

Abbreviations: IP, interproximal; MB, mid-buccal; ML, mid-lingual.
used for the post hoc pairwise comparisons of mean differences between clinical groups. Pearson product-moment correlation coefficients were calculated between the hemodynamic indices to summarize the linear association between the variables. Statistical calculations were performed with Statistica 7.1 (Statsoft, Tulsa, OK, USA).

Results

Clinical parameters from the study group

The mean age of the recruited patients was 53.6 ± 11.2 years, with a range from 26 to 82 years, and 16 (25.4%) of the subjects were male. The mean pocket depth for the peri-implantitis group was 5.7 (±0.9) mm, while that in the mucositis group was 2.4 (±0.6) mm. Regarding the attachment loss, the mean was 6.2 (±1.5) mm for the peri-implantitis group, with the upper molars being the most affected region, and 3.1 (±1.5) mm for the mucositis group.

Representative pictures of dental implants diagnosed as healthy and peri-implantitis are displayed in Fig. 2. The probing depth of healthy peri-implant sites were <2 mm (Fig. 2A), and radiography revealed no bone loss, since bone tissue filled up the first thread of the implant (indicated by the arrows). In peri-implantitis, the peri-implant tissue exhibited severe inflammation, i.e. bleeding on probing, deep probing depth (7 mm) and suppuration (Fig. 2B). The radiographic examination of this implant showed peri-implant bone loss, extending beyond the fourth thread of the implant (indicated by the arrow).

Peri-implant hemodynamics extrapolated from optical spectra

Based on the information embedded in the spectra, several important local peri-implant hemodynamic indices were calculated for the three groups of sites. The most striking difference was the oxygen saturation of the tissue presented in Fig. 3. There was a significantly lower oxygen saturation in sites diagnosed as peri-implantitis when compared with healthy sites ($p < 0.05$). As mentioned in the material and methods section, the tissue hemoglobin oxygen saturation is derived from the predicted Hb and HbO$_2$ relative concentrations. Figure 4 presents the mean (±95% confidence interval) relative concentrations of Hb and HbO$_2$ parameters obtained from the optical attenuation spectra measured in vivo from healthy, mucositis and peri-implantitis sites. Although not statistically significant, there was a trend towards a higher relative concentration of Hb and a lower relative concentration of HbO$_2$ in peri-implantitis sites ($p > 0.05$). Moreover, the tissue hydration index was higher in diseased sites than in healthy sites, as shown in Fig. 5. In particular, the
increased tissue hydration in mucositis sites was significant when compared with both healthy \((p < 0.05)\) and peri-implantitis sites, whereas no significant difference was seen between healthy and peri-implantitis sites.

**Discussion**

The development of more accurate methods for diagnosis of peri-implant inflammation is crucial for the prevention of dental implant failures and monitoring of the outcomes of peri-implant therapies. Therefore, this study assessed, for the first time, multiple inflammatory parameters (tissue oxygenation, total tissue hemoglobin, deoxyhemoglobin, oxygenated hemoglobin and tissue edema) around healthy, mucositis and peri-implantitis sites by near-infrared spectroscopy, which has recently been proposed to measure tissue hemodynamics in periodontal tissues (22). In general, the results demonstrated that hemodynamic changes can be clearly detected around diseased peri-implant sites by this convenient noninvasive technique, especially in relation to tissue oxygenation and tissue hydration. The data provide new insights into the diagnosis of peri-implant diseases.

Peri-implant diseases are diagnosed mainly on their clinical and radiographic signs, including bone level, clinical attachment loss, probing depth, bleeding on probing, implant mobility and suppuration (6, 7, 17, 29–34). Although clinical and radiographic parameters are currently well accepted for the diagnosis of peri-implant lesions, these methods inherit some undesirable limitations that might lead to either an under- or overdiagnosis of the presence and severity of the disease, especially when taken alone. Therefore, considerable research has been done in the past on peri-implant inflammation with the intent of developing new alternative diagnostic tools to overcome these limitations. Optical spectroscopy provides a means to assess peri-implant inflammation rapidly, conveniently and without costly consumables or reagents.

As demonstrated in the present study, the spectral profile for the peri-implant sites resembled those observed for periodontal tissues (22), despite some anatomical and histological differences between teeth and dental implants, which include periodontal ligament space and fiber arrangements (9, 35, 36). For instance, tissue oxygen saturation was significantly lower in the peri-implantitis group than in the healthy and mucositis groups (Fig. 3). However, subtle differences were observed between the previous study (22) that used optical spectroscopy to characterize periodontal inflammation and the present study examining peri-implant inflammation. For example, there is a significant offset between the measured values of tissue oxygen saturation between the two studies. The fact that the two studies used different fiberoptic probes and spectrometers makes absolute comparison of the oxygenation values difficult. In addition, there was not as distinct a difference in oxygenation between the healthy and mucositis sites as observed between healthy and gingivitis sites measured in our previous study. Decreased oxygen saturation may be explained by tissue hypoxia resulting from the infectious inflammatory process in peri-implantitis (37, 38). In fact, it has been demonstrated that active inflammation is characterized by dramatic shifts in tissue metabolism, including low levels of oxygen (hypoxia), subsequent accumulation of lactate and metabolic acidosis (39, 40). In healthy tissues, the oxygen tension is usually between 20 and 70 mmHg, whereas in
diseased tissues, with transient or chronic areas of hypoxia, the oxygen tension is <10 mmHg. The microenvironmental metabolic changes (e.g. hypoxia) are related to profound recruitment of inflammatory cells, such as neutrophils and monocytes, that contribute to disease progression (37,38). It has previously been demonstrated that tissue oxygen saturation correlates with oxygen tension in periodontal pockets, because oxygen tension tends to decline as pocket depth increases (39,40). Another possible explanation for the low tissue oxygen saturation observed in the peri-implantitis sites may be the colonization of the pocket by specific pathogens. The peri-implantitis sites are predominately colonized by gram-negative facultative or obligate anaerobic microorganisms (6,7,41,42) that are related to a decrease in the oxygen tension in deep pockets (39,40). Furthermore, the low tissue oxygen saturation observed in the peri-implantitis sites may relate to the fact that loss of osseo-integration is related to decreased vascular proliferation in peri-implant diseases (43,44).

Another important profile detected in this study was the hydration index, which was significantly higher in mucositis sites than that in peri-implantitis and health control sites (Fig. 5). This result was somewhat expected, since mucositis is the first stage of peri-implant disease, characterized by signs of acute inflammation, including vessel dilatation, increased intercellular spacing and accumulations of interstitial fluid and inflammatory exudates (7,32). Our finding parallels the clinical signs of mucositis, which is characterized by swelling and redness due to local edema and inflammation. However, when the disease progresses to peri-implantitis, the primary pathological changes are the destruction of the supporting bone along with a certain degree of inflammatory infiltrate (41,45,46). At this stage, the microbial challenge might change; therefore, the peri-implantitis sites may or may not be inflamed (6,23,24), reflecting the lower level of tissue hydration in our peri-implantitis group.

As neither tissue oxygen saturation nor subtle local edema is readily detectable clinically, our data suggest that optical spectroscopy could be a useful supplementary tool for diagnosis and monitoring of inflammation around dental implants. In principle, optical spectroscopy is an entirely noninvasive technique that uses low-energy radiation; spectra can be captured instantly; no consumables need to be purchased or developed; and minimal training is required to obtain reliable and reproducible data (22–24). This leads us to believe that optical spectroscopy can be an important tool in the near future, to help determine healthy and diseased dental implants, as well as to help distinguish between progressing lesions and lesions that represent the consequences of a previous disease (23,24).

In conclusion, the results of this proof-of-concept study reveal that hemodynamic alterations can be detected around diseased peri-implant sites by optical spectroscopy, offering a feasible approach for the monitoring and diagnosis of peri-implant diseases. Prospective longitudinal clinical studies are warranted to demonstrate the role of this tool in differentiating progressive from stable lesions, and in monitoring the effect of peri-implant therapies.

### Acknowledgements

This study was generously supported by the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes for Health Research (K.-Z.L. and M.G.S.); by the Manitoba Medical Service Foundation (G.N.-F. and K.-Z.L.) and Sao Paulo State Foundation FAPESP (J.A.S. and L.A.G.C.).

### References

6. Shibli JA, Melo L, Ferrari DS, Figueiredo LC, Faveri M, Feres M. Composition of supra- and subgingival biofilm of subjects...


Oxygen in Wound Healing
Nutrient, Antibiotic, Signaling Molecule, and Therapeutic Agent

David E. Eisenbud, MD

INTRODUCTION

Common observations made many decades ago by mountain climbers who noted the inability to clear skin infections at high altitude, and Jacques Cousteau’s deep sea divers who noted that their work wounds healed fastest when they were diving, brought general appreciation of the importance of oxygen in healing. Recent years have brought an increased and more detailed scientific appreciation of the diverse roles that oxygen plays in normal physiology and disease states. As the individual steps of the wound healing cascade have become elucidated in greater detail, the involvement of oxygen at nearly every stage has become evident. More oxygen is not always better; nature seems to have adapted us to respond constructively to the relative hypoxia that characterizes the healing edge of many wounds.

There remain many gaps in understanding of the biochemical events of healing. Some of the current knowledge regarding oxygen, growth factors, and other mediators is seemingly contradictory, and classification of molecules as promoters or inhibitors of healing (eg, oxygen is good, tumor necrosis factor α [TNF-α] is bad) is simplistic. However, it seems possible to reach a unified understanding of healing that reconciles most of the thousands of basic science investigations into individual steps in the chain, and oxygen is central to this. This article summarizes oxygen physiology in wound biology, and discusses the supporting literature.

Given the central role of oxygen in healing, there is the potential to manipulate the wound

The author has no financial conflicts of interest to declare in relation to the writing and publication of this article.

Millburn Surgical Associates, 225 Millburn Avenue, Suite 104-B, Millburn, NJ 07041, USA

E-mail address: dave@njdave.net

doi:10.1016/j.cps.2012.05.001
0094-1298/12/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
environment by treatment with supplemental oxygen. Oxygen therapy in various forms has been used to ameliorate many medical conditions for centuries. However, clinical results have been varied, and frequently disappointing. There has been an indiscriminate use of oxygen treatments in the past, and there is still an aura of quackery associated with this area of medicine. However, in the face of deeper scientific understanding of oxygen physiology, and with support from randomized, prospective clinical investigations, the judicious, individualized use of oxygen therapy in wound management may now be considered mainstream. This article reviews the current rationale, regimens, and preclinical and patient data regarding various oxygen treatments that have been used to improve the outcomes of dermal wounds. See Box 1 for a summary of roles of oxygen in wound healing.

**Oxygen Delivery**

In normal conditions, oxygen delivery to peripheral tissues is the net result of:

- Cardiac output
- Peripheral vascular resistance
- Oxygen saturation of hemoglobin (usually 90% or greater).

**Oxygen in serum**

Minimal amounts of oxygen are dissolved in the serum. Release of oxygen is governed by the hemoglobin dissociation curve. Serum \( P_{O_2} \) is typically about 100 mm Hg. Once released at the capillary level into normal tissue, oxygen can diffuse up to 64 \( \mu m \).\(^4\) Given normal capillary density, this diffusion ability is sufficient to nourish and support the viability of the skin. Hunt\(^1\) emphasized the often-neglected but relevant point that oxygen delivery can sometimes be increased significantly by reversing the local vasoconstriction that may result from pain, cold, or other noxious stimuli.

**Intact skin as barrier to oxygen**

The keratin layer of intact epithelium is a barrier to oxygen diffusion; probes designed to exclude air detect only 0 to 10 mm Hg on the skin surface. Warming of the skin makes this layer more permeable and enables \( P_{O_2} \) to increase substantially, although it does not reach the ambient level. Stripping the stratum corneum with tape enables free diffusion of oxygen into the upper layers of dermis, and the \( P_{O_2} \) there closely matches the oxygen tension in the environment. However, within about a day, exudation of serum and accumulation of inflammatory cells lead to formation of a soft eschar that again prevents oxygen diffusion into the skin. Therefore, the oxygen tension in subcutaneous tissue and dermis of intact skin depends on delivery through the underlying circulatory system.

**Inadequate Oxygen Delivery is a Causal Factor in Many Chronic Wounds**

Each of the most common categories of chronic wounds (arterial, venous, diabetic, pressure) become established or are perpetuated because of factors that limit oxygen delivery to the wound bed. Scheffield\(^5\) noted that chronic wounds have a \( P_{O_2} \) in the range of 5 to 20 mm Hg, compared with 35 to 50 mm Hg measured in normal tissue.

In the case of venous leg ulceration, the essential disturbance is abnormal venous hypertension, which is propagated back to the capillary level. The capillary-tissue pressure gradient is increased, causing water to diffuse out of the intra-vascular space and into the interstitium; large molecules such as fibrinogen, albumin, and \( \alpha_2 \)-macroglobulin are also forced out of the vascular system, and pericapillary cuffs are formed that can be noted histologically.\(^6\) These cuffs and the local edema impair oxygen diffusion and render the cells furthest from the capillary hypoxic.

Lower extremity arterial and diabetic wounds, are prone to suffer macrovascular and/or microvascular occlusive disease, limiting blood flow and therefore oxygen delivery to the lesion. Pressure wounds that are not properly off-loaded become ischemic (and therefore also hypoxic) when capillary closing pressure is exceeded by...
the weight of the body part pressing against a support surface.

**Reperfusion**

In addition to ischemia/hypoxia, another mechanism of injury has been shown to play a major role in a variety of chronic wounds: reperfusion. Patients with impaired arterial inflow or venous return have repeated episodes of ischemia and reperfusion related to leg elevation or dependency. Restoring circulation induces endothelial stickiness, which draws white cells into the lesion; the already established proinflammatory environment established by ischemia intensifies as ROS flood the wound and cause further tissue destruction. Repeated episodes of ischemia and reperfusion are more detrimental to wound healing than are prolonged phases of uninterrupted ischemia.

**Inflammatory cycle**

It is a popular current concept that many chronic wounds are stuck in a self-perpetuating inflammatory cycle. Hypoxia may contribute to this pathophysiology in many cases. Under significant hypoxic conditions, mitochondrial adenosine-triphosphate (ATP) production ceases and ATP-dependent transmembrane transport systems such as sodium/potassium ATPase or calcium ATPase fail. Intracellular accumulation of calcium promotes release of proinflammatory cytokines such as TNF-α and interleukin (IL)-1, which attract neutrophils and macrophages. Endothelial adhesion molecules are overexpressed in hypoxia, and enable white blood cells to localize to the wound. The net effect is a self-perpetuating inflammatory vicious cycle in which tissue destruction leads to increased white cell recruitment and release of proinflammatory mediators and ROS, which leads to even more tissue destruction. Bacterial colonization, a nearly universal feature of chronic wounds, adds to the inflammatory burden by attracting and activating leukocytes. Although inflammatory cells are capable of producing ROS at low oxygen tensions, the antidotes to ROS (the most potent of which is nitric oxide) require higher oxygen tension for their synthesis.

**Measurement of Wound Oxygen**

Accurate, repeatable measurements of wound oxygen are central to many in vivo investigations into the role of oxygen. Although numerous investigators have refined research-grade systems, measurement of oxygen at the tissue/cellular level in routine clinical practice is difficult and imprecise. There is a vast literature on oxygen measurement and a detailed review is beyond the scope of this article. Most methods are indirect and measure oxygenation of periwound skin rather than the wound bed.

**Transcutaneous oximetry**

Perhaps the most popular of these techniques, transcutaneous oximetry (TcPO₂) is subject to high variability related to fluctuations in vasomotor tone at the site of measurement, light penetration of skin, and hemoglobin level. Even perfectly performed TcPO₂ typically overestimates wound Po₂ because the skin is warmed to the point of maximal local vasodilatation, which is not representative of the ordinary state of the local vascularature. In addition, there can be significant oxygen consumption along the path from periwound intact skin to the healing tissue edge in the center of the wound. Thus, arterial blood Po₂ is ordinarily about 100 mm Hg; the Po₂ of dermal wounds ranges from 60 mm Hg at the periphery to 0 to 10 mm Hg centrally. There are many reports supporting the usefulness of TcPO₂ in determining levels of amputation healing, but many practitioners have found that the method is cumbersome and yields results that have poor repeatability. Measurements at 1 point in time and only a limited number of skin sites may not accurately portray the wound microenvironment, because wounds are not uniform and vasomotor tone may change from moment to moment.

**Luminescence imaging**

Luminescence lifetime imaging has recently shown significant advantages compared with earlier methods of wound oxygen estimation. A phosphorescent indicator is held in place on a plastic matrix; quenching of the phosphorescence is proportional to the amount of oxygen present. The technology provides a noninvasive, painless, reliable, sensitive estimate of wound PO₂. Although offering potential for widespread clinical adoption, the technology is not yet available commercially in a format and at a cost that is compatible with ordinary clinic operations and economics.

**Role of Oxygen in the Essential Steps of Dermal Wound Healing**

**Collagen synthesis**

Extracellular matrix deposition is inadequate in many chronic wounds because of poor fibroblast production and inadequate remodeling of collagen, which are both oxygen dependent, and because of excessive degradation of extracellular membrane oxygenation (ECM) by matrix metalloproteinases (MMPs). Molecular oxygen is required for the hydroxylation of proline and lysine during collagen synthesis and for the maturation of procollagen into stable triple-helical collagen.

---

Oxygen in Wound Healing
the absence of sufficient oxygen, only protocolla-
gen, which does not have the functional abilities
of collagen, can be made. Collagen synthesis
proceeds in direct relation to P O₂ over the range
of 25 to 250 mm Hg. Prolyl hydroxylase under
20 mm Hg O₂ functions at 20% of maximal speed;
the enzyme requires more than 150 mm Hg to
reach 90% of maximal speed.

Angiogenesis

Many authorities have noted an apparent incon-
sistency in well-known observations of wound
healing, whereby hypoxia is noted to increase
VEGF production from fibroblasts and macro-
phages, but angiogenesis seems to proceed more
successfully under normoxic or even hyperoxic
conditions. In one set of instructive experi-
ments, mice underwent subcutaneous injection
of a gel alone, gel with VEGF, or with anti-VEGF
antibodies. The animals were then maintained in
various environments of 13% to 100% oxygen
at 1 absolute atmosphere (ATA) to 2.8 ATA to
simulate hypoxia, normoxia, and hyperoxia. The
explanted gel plugs were then sectioned and
graded for the degree of angiogenesis. Angiogen-
esis was significantly decreased in the hypoxic
animals (P ≤ 0.001) and increased in those who
were rendered hyperoxic (P < 0.05). Addition of
VEGF to the implanted gel did not prevent the
deleterious effect of hypoxia. In contrast, the
beneficial effect of hyperoxia was blocked by
anti-VEGF antibody. These findings suggest that
both adequate oxygen and the presence of
VEGF are required for angiogenesis.

Sen noted that all developing vascular buds
require a sheath of ECM, mainly consisting of
collagen and proteoglycans, to guide tube forma-
tion and resist the pressures of blood flow. His
investigations have confirmed that optimal angio-
genesis requires high P O₂; hypoxia, by retarding
synthesis of the collagen to support developing
vessels, decreases angiogenesis.

Fibroblast growth

Oxygen consumption by cells has been studied by
measuring changes in oxygen tension in superna-
tant media covering cells in tissue culture. Fibro-
blast cellular proliferation is directly correlated with
ambient oxygen level:

- Freshly harvested cells only grow in an
  environment that includes 15 mm Hg O₂,
  or more
- Initially, after 72 hours exposure to 1% 
  oxygen, fibroblast proliferation increases
  by 71%
- During this period, fibroblast secretion of
  transforming growth factor (TGF)-β1
  increases 9-fold, in turn causing upregula-
  tion of the procollagen gene.

This adaptation to hypoxia is only transient, and
chronic oxygen deprivation severely diminishes
fibroblast activity. The surface of a poorly healing
wound can be mapped with oxygen tension
measurements, and fibroblast growth is progres-
sively diminished until the hypoxic center of the
lesion is reached, where proliferation is minimal
or nil. By contrast, under hyperoxic conditions,
fibroblasts are induced to differentiate into myofi-
broblasts, which are critical to wound closure
through contraction.

Epithelial

Numerous studies have shown that keratinocytes
and fibroblasts migrate faster under hypoxic con-
ditions. Keratinocytes express more lamelli-
podia proteins and collagenase, and decrease
lamellin-5 (motility brake) under hypoxia. This
finding is expected, considering the mechanisms
of natural dry wound healing, in which new
skin regenerates underneath a stable eschar.
The microenvironment through which the healing
edge advances is protected from ambient oxygen,
and the lack of blood vessels inside the eschar
engender local hypoxia. In order for the epithelial
dge to migrate the keratinocytes must carve
a path through the tissue/eschar interface using
collagenase and other enzymes.

Energy generation and use

Fundamental biochemical events such as molec-
ular synthesis and transport cannot occur without
a source of energy, and the ubiquitous source of
energy in the human body is the coenzyme ATP.
ATP is synthesized in mitochondria and stores
chemical energy to fuel diverse biochemical pro-
cesses in the body. The process by which ATP is
created is known as oxidative phosphorylation
and is critically dependent on the availability of
molecular oxygen. About 90% of the oxygen
consumed by tissues goes to ATP synthesis.
Other energy stores generated through the citric
acid cycle and the breakdown of fatty acids are
also highly oxygen dependent. The requirement
for energy and, hence, the need for oxygen, is
accentuated in healing tissue because cellular
activities such as collagen synthesis, cell migra-
tion, and bacterial defense are heightened.

Influence of age

Another critical dimension to oxygen’s role in heal-
ing relates to the age of the wounded host. The
demographics of chronic nonhealing wounds indicate a strong relationship between incidence
and advanced age, with most cases occurring in
patients more than 60 years of age. Aging fibroblasts show: 6,7,20
- Reduced proliferative ability
- Diminished capacity to respond to growth factor stimulation
- Increased production of destructive enzymes such as MMPs.

Advanced age induces greater sensitivity to the negative effects of hypoxia. The migratory response of fibroblasts to TGF-β1 stimulation is blunted in older patients, compared with younger ones. Mustoe and colleagues6 compared the effects of hypoxia on young (age 24–33 years) human dermal fibroblasts in tissue culture with fibroblasts from older (age 61–73 years) donors. Under 1% oxygen, there was greater decrease in TGF-β1 receptor expression in the aged cells (decrease of 12% in young fibroblasts vs 43% in old fibroblasts).

Responsiveness to TGF-β1 stimulation was reduced by advanced age:
- Activity of p42/p44 mitogen-activated kinase increased 50% in young cells versus decreasing 24% in the aged cells
- Unstimulated fibroblast migration increased 30% in young cells exposed to hypoxia, whereas the aged cells showed no change
- When TGF-β1 stimulation was added to the migration assay, young cells increased activity by 109% versus only 37% in the aged cells.2

Mendez and colleagues20 studied fibroblasts harvested from the venous ulcer wound beds and used, as controls, cells taken from normal skin of the thigh.

- The 7 patients who were evaluated (mean age 51; range 36–67 years) had suffered wounds for a mean of 12.7 months (range 11–17 months)
- Chronic wound fibroblast growth rates were only one-third of those observed in the control cells ($P = .006$).
- β-Galactosidase (β-GAL) activity was used as a sign of cellular senescence, a state of irreversible arrest of proliferation despite maintenance of metabolism.
- Six of 7 controls had no senescence associated (SA)-β-GAL activity
- All samples from the chronic wounds had measurable activity (mean 6.3%, median 2%)
- Level of SA-β-Gal correlated inversely with cellular growth rate ($R^2 = 0.77$).

The issue of cellular senescence may be more complex than mere chronologic age of the wounded patient. It may be the number of cell divisions that fibroblasts have undergone, rather than the age of the host, that defines the age of a cell. Many cell lines, including fibroblasts, are capable of only a finite number of cell divisions. Chronic wounds in young individuals may contain fibroblasts that have already reached the limit of their proliferative ability and are prematurely senescent.7 Thus, although the lessons learned about the roles of oxygen in wound physiology are generally applicable, the degree to which patients may react to hypoxia and hyperoxia with the predicted responses may vary according to the physiologic age of the host cells.

**Nitric oxide generation**
Nitric oxide synthetase metabolizes the amino acid l-arginine into nitric oxide using oxygen as a substrate. Although nitric oxide is well known for its diverse, generally beneficial, effects on inflammation, angiogenesis, and cell proliferation, a full discussion of the putative benefits of enhanced nitric oxide is beyond the scope of this article.21

**Role of Oxygen in Infection Control**
During the initial phase of wound healing, activated leukocytes enter the wound and engulf bacteria. In the presence of adequate oxygen, an oxidative burst ensues, in which oxygen consumption increases as much as 50-fold compared with baseline conditions, and persists for hours, creating ROS that destroy the invaders.22 ROS include:
- Peroxide anion (HO$_2^-$)
- Hydroxyl ion (OH$^-$)
- Superoxide anion (O$_2^-$)
- Hydrogen peroxide (H$_2$O$_2$).

About 98% of oxygen consumption by leukocytes is related to this respiratory burst, which is facilitated by phagocyte (neutrophil, eosinophil, monocyte, and macrophage) cell membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase23:

$$\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{H}_2\text{O}_2 + \text{H}^+$$

Glucose provides the energy to drive the reaction, generating substantial amounts of lactate in the process.1

ROS, and especially superoxide, are toxic and kill bacteria, then are rapidly degraded to H$_2$O$_2$ and other by-products.10,24 The kinetics are such that the killing process works at 50% of maximal
speed in the presence of oxygen at 40 to 80 mm Hg, and as much as 400 mm Hg are required to increase the velocity to 90%. Neutrophils therefore lose most of their ability to kill bacteria at less than 40 mm Hg. Patients afflicted with chronic granulomatous disease, which is characterized by defects in the genes that encode NADPH oxidase, have increased susceptibility to infection, and also show impaired wound healing.

The results of many investigations suggest that, in a reduced PO2 wound environment, the ability of leukocytes to generate ROS is substantially impaired, and therefore the ability to ward off colonization/infection is lowered. In vitro studies of leukocytes obtained from venous blood show that neutrophil oxygen consumption increases as ambient oxygen concentration increases, and suggest that increasing ambient oxygen to more than physiologic levels may induce even greater ROS synthesis. However, there is the theoretic potential that excess ROS may be destructive and counterproductive to wound healing. The body has a robust system for removal of ROS by superoxide dismutase, catalase, and reduced glutathione, and the wound concentration of ROS is the net result of synthesis and destruction. ROS clearance requires an adequate circulatory supply.

The potential to increase local wound oxygenation to supraphysiologic levels and cure or prevent infection was evaluated in 30 patients with chronic diabetic wounds. Patients were randomized to receive standard care (wound dressings; antibiotics guided by culture results) alone or in combination with 4 hyperbaric oxygen therapy (HBOT) treatments given during a 2-week period. In the control group, cultures grew significant colony counts of 16 different isolates at baseline, and 12 at the end of the observation period; in the patients receiving HBOT, isolates were reduced from 19 before treatment to only 3 afterward (P<.05). HBOT seemed particularly effective in controlling Pseudomonas and Escherichia coli. Seven patients in the control group required major lower extremity amputation (5 for spreading infection) versus 2 in the HBOT-treated patients (P<.05).

**Redox Signaling**

The traditional view of ROS has been that they are destructive to bacteria and host cells, necessary for wound hygiene in the early phase of healing, but otherwise counterproductive to the normal healing cascade. This view has prompted numerous clinical trials testing the role of various antioxidants in ameliorating different disease conditions, and these trials typically have shown disappointing results.

The late 1990s brought a growing appreciation that, at very low levels, ROS (particularly H2O2) serve as signaling messengers, a role independent of bacterial killing. NADPH oxidase exists not only in neutrophils but also in nonphagocytic wound cell lines, and there is a continuous low-level production of ROS that is unrelated to leukocytes, oxidative burst, or response to wound colonization or debris. ROS bind to proteins and can alter their conformation, leading to increases or decreases in their functional abilities. H2O2 functions primarily by oxidizing cysteine moieties. The molecule has a potent effect in recruiting leukocytes to the wound site, and the concentration of endogenous hydrogen peroxide increases significantly at the wound margins within the first few minutes of injury. The functions of important growth factors such as VEGF, platelet-derived growth factor (PDGF), keratinocyte growth factor (KGF), and TGF-β are inhibited in the absence of such signals. Thus, overexpression of catalase, which removes H2O2 from the wound, is associated with impaired angiogenesis and delayed wound closure. Cellular functions like migration and leukocyte recruitment are also inhibited in the absence of H2O2. Micromolar levels of peroxide can be measured in normal wound fluid. Research in oncology indicates that low-level ROS foster angiogenesis, and overexpression of extracellular super oxide dismutase inhibits tumor vascularization in mice.

**Note**

The low physiologic concentration of H2O2 must be distinguished from the 3% v/v strength that is available commercially and often used to clean and disinfect wounds; at such high concentrations, severe oxidative damage to wounds is noted, and this is contraindicated in modern wound management. However, at 0.15% H2O2, topical angiogenesis is favorably influenced.

The state of overabundance or underabundance of ROS is reflected in the redox potential of the wound microenvironment. The ratio of NADH to NAD+ has been used as a redox index, and transcription of various genes important in wound repair seems to be responsive to this ratio. Every phase of the wound healing cascade (hemostasis, inflammation, proliferation, epithelialization) has been shown to have key steps that require NADPH oxidase action. Individuals with mutations in either p47phox or Rac2, which are each associated with action of this enzyme, show impaired wound healing.

**Hypoxia**

Many of our conclusions about the role of oxygen in wound repair come from observations of the
defects in healing that are associated with hypoxia. Absolute hypoxia is usually defined as an oxygen level less than 30 mm Hg. However, hypoxia is more typically a relative term, indicating insufficient oxygen for the tissue and physiologic situation under consideration. For example, with infection or an open wound, oxygen demand increases and levels of delivery that might be adequate for intact, uninfected dermis may be deficient. Cells challenged with hypoxia must reduce activity and rely on anaerobic metabolism, or die. Acute and mild/moderate hypoxia usually leads to cellular adaptation and survival; prolonged and more extreme hypoxia may result in cellular death.

Hypoxia is a more frequent issue in wound repair than is commonly appreciated. Wound bed oxygenation depends on:

- Pulmonary uptake
- Hemoglobin level
- Cardiac output
- Vascular patency
- Capillary density
- Factors that deplete oxygen such as parenchymal consumption and inflammatory cell activity.

Decreased wound oxygen tension occurs not only from macrocirculatory issues but also because oxygen is consumed by metabolically active and proliferative cells located along the path from capillaries to the healing edge of tissue. Box 2. As much as a 150-μm distance from the nearest capillary to the healing edge may need to be nourished by oxygen diffusion. This effect is magnified in the presence of heavy bacterial colonization or infection, further consuming oxygen and decreasing the available oxygen to the healing wound. A vicious cycle may ensue in which bacterial oxygen consumption reduces the ability of leukocytes to synthesize ROS, enabling further bacterial proliferation, and so on. The centers of even seemingly well-oxygenated wounds may be hypoxic because of high oxygen extraction along the path of diffusion from capillaries. Although circulating blood may contain a PO₂ of 100 mm Hg, the periphery of a dermal wound may be 60 mm Hg and, at the center of the wound, readings can be as low as 0 to 10 mm Hg. Many chronic wounds suffer local hypoxia; in one study, normal, nonwounded tissue showed oxygen tensions of 30 to 50 mm Hg, whereas measurements of PO₂ in nonhealing chronic wounds in the same patients were in the range of 5 to 20 mm Hg.

At present, it is not easy to reconcile all the scientific knowledge about effects of hypoxia and hyperoxia on wound tissues and to synthesize all the evidence into a coherent story. Hypoxia has been traditionally regarded as an important stimulus to fibroblast growth and angiogenesis. Hypoxia encourages angiogenesis by increasing levels of hypoxia-inducible factor 1 (HIF-1), which in turn binds to the promoter segment of the VEGF gene and activates transcription, leading to higher synthesis of VEGF, the principal angiogenic growth factor in human physiology. Paradoxically, multiple studies have shown that hyperoxic conditions induce greater angiogenesis, perhaps by increasing local ROS.

Acute hypoxia induces temporary increases in cellular replication (3-fold increase under 5 mm Hg compared with 150 mm Hg). This has been associated with a 6.3-fold increase in the expression of TGF-β1 and enhanced procollagen synthesis. However, these increases in proliferation and metabolic activity are short lived and, when these conditions are maintained for more than a week, cellular growth and synthetic activity decrease to significantly less than baseline physiologic levels. The situation is reversible; restoration of normal oxygen levels restores typical proliferation rates, and a second bout of hypoxia induces a second temporary burst of fibroblast activity. In chronic hypoxia, the production and secretion of the most important cytokines and chemokines central to healing (including TNF-α, TGF-α, TGF-β1, KGF, EGF, PDFG, and insulin growth factor) require oxygen and are reduced or absent.

There seem to be at least 2 ways to reconcile the older concept that hypoxia is beneficial with modern understanding that healing proceeds more quickly with increased oxygen delivery.

1. The true primary stimulus to VEGF secretion, angiogenesis and collagen deposition may be lactate, more than hypoxia. Even in well-perfused and properly oxygenated wounds, lactate may accumulate because leukocytes, fibroblasts, and endothelial cells lack mitochondria and rely on anaerobic glycolysis for energy production; a principal by-product of this glycolysis is lactate. Thus, lactate levels may still be increased in hyperoxia, albeit less so compared with hypoxia.

Box 2
Clinical pearl: wound hypoxia

Clinical pearl: wound hypoxia is under-recognized and may occur in even normally perfused lesions because of high oxygen extraction along the diffusion path from the feeding capillary to the edge of healing tissue.
2. It is possible that the \( \text{P}O_2 \) gradient between the capillary and the most distant cell may drive angiogenesis more than the absolute level of \( \text{P}O_2 \). The immediate effect of hyperoxygenation of the blood may be to increase this gradient, and increased oxygen diffusion to areas of low \( \text{P}O_2 \) may follow.

**Therapeutic Oxygen Supplementation**

Because of the high incidence of wound hypoxia and the knowledge of the deleterious effects of inadequate oxygen on healing, it is natural to consider the potential of oxygen supplementation to improve wound repair **Box 3**. In some instances, the most effective way to improve wound oxygenation may involve measures to enhance blood flow rather than changing the oxygen content of the blood. Such maneuvers may include hydration and relief of vasoconstriction using local warmth and analgesics. Because hemoglobin is nearly saturated in most individuals, enhancing inspired oxygen may only modestly increase peripheral delivery. Other techniques for hyperoxygenation of wounds include breathing oxygen under supranormal pressures (hyperbaric oxygen [HBO]) or bathing the wound topically with an enhanced oxygen environment (topical oxygen therapy [TOT]). The remainder of this article focuses on various attempts to supplement oxygen for the benefit of healing acute and chronic wounds.

**Enriched inhaled oxygen to prevent surgical site infections**

Under normal circumstances, hemoglobin is almost completely saturated while breathing room air under normobaric conditions, so the opportunity for increased oxygen delivery with enhanced forced inspiratory oxygen (\( \text{FiO}_2 \)) is limited. However, by increasing the serum \( \text{P}O_2 \), the tissue diffusion gradient is increased. For example, in the rabbit ischemic ear model, breathing 100\% \( \text{O}_2 \) at 1 ATA increases blood \( \text{P}O_2 \) from 90 to 450 mm Hg. At less than 20\% \( \text{O}_2 \), most of the oxygen is consumed within 70 \( \mu \)m of the nourishing capillary, but, after breathing 100\% \( \text{O}_2 \) for 45 minutes, there is measurable increase in \( \text{P}O_2 \) even 150 \( \mu \)m away.\(^5\)

Knighton and colleagues\(^37\) in 1984 showed that higher inspired concentrations of oxygen lowered the extent of infection induced by intradermal injection of bacteria. Guinea pigs were given intradermal injections containing \( 10^8 \) *E coli*, then were treated with either 12\%, 21\%, or 45\% inhaled normobaric oxygen. The end point, the size of the resulting lesion, was substantially reduced with increased oxygen (\( P<.005 \) for either 21\% or 45\% vs 12\%; \( P<.01 \) for 45\% vs 21\%). The therapeutic effect was attributed to improved leukocyte killing with higher oxygen tension.

Conversely, it has been noted that the rate of surgical site infection (SSI) in surgical patients increases as systemic subcutaneous oxygen tension decreases.\(^38\)

- Upper arm subcutaneous \( \text{P}O_2 \) measurements were conducted in 130 patients who underwent major general surgery (mostly abdominal) on the day of surgery and during postoperative days 1 and 2.
- A total of 24 patients developed infection at the surgical site.
- There was a strong correlation between decreased \( \text{P}O_2 \) and the development of infection (\( r = 0.91; \ P<.001 \)).

The potential for supplemental inspired oxygen to lower the rate of SSI has been studied extensively. Grief and colleagues\(^39\) randomized 500 adult patients undergoing colorectal resection to breathe 30\% or 80\% oxygen during, and for 2 hours after, the procedure. After surgery, patients were evaluated daily until hospital discharge, and then in the clinic 2 weeks later. Although the anesthesiologists knew the treatment to which each patient was randomized, the surgeon conducting the operation and the postoperative evaluation was blinded to this assignment. Wounds were considered infected if there were clinical signs and symptoms of infection, and if fluid or pus expressed from the wound cultured positive.

- Thirteen patients in the 80\% oxygen group developed wound infection versus 28 patients in the 30\% group (5.2\% vs 11.2\%; \( P = .01 \)).
- Six patients in the 30\% group had signs of infection but cultured negative, compared with 4 in the 80\% group.

The investigators concluded that supplemental inspired oxygen was effective in lowering the perioperative wound infection rate.
A similar prospective, randomized trial of 30% versus 80% inspired oxygen was conducted by Belda and colleagues.\textsuperscript{40} 

- Three hundred patients were randomized to yield 291 who were evaluable
- The rate of SSI was unusually high (57 patients [39.3%]) and there was no clear explanation for this finding
- The incidence of SSI was reduced significantly in response to higher inspired oxygen: 24% versus 15% infected for patients treated with 30% and 80% oxygen, respectively ($P = .04$).

Chura and colleagues\textsuperscript{41} reviewed the literature and conducted a meta-analysis on the value of supplemental oxygen in preventing SSI in colorectal surgery. Among thousands of articles that emerged from a keyword search on “surgical site infection” and “perioperative oxygen” only 4 studies met criteria for sufficient scientific rigor to warrant analysis. Although there was significant heterogeneity among these studies, the investigators concluded that there was sufficient evidence to support the assertion that perioperative oxygen supplementation reduces SSI. The aggregate number of patients in these studies was 943 (477 who received supplemental O\textsubscript{2} and 466 who were controls). The pooled relative risk for SSI with perioperative O\textsubscript{2} supplementation was 0.68 (95% confidence interval, 0.49 to 0.94).

Only 1 major study failed to show reduction of SSI with supplemental oxygen. Meyhoff and colleagues\textsuperscript{42} studied 1400 Danish patients undergoing laparotomy at 14 hospitals. Patients and observers were blinded to the random assignment to 30% or 80% O\textsubscript{2}. The study end point was superficial or deep wound infection, or intra-abdominal infection, using the Centers for Disease Control and Prevention definitions. The low-oxygen group had an SSI rate of 20.1% versus 19.1% for 80% O\textsubscript{2} (not significant [NS]). Most patients received perioperative antibiotic prophylaxis with cefuroxime and metronidazole or benzylpenicillin and gentamycin; perhaps this accounts for the apparently discrepancy between this study and the others cited earlier.

On balance, it seems that increasing the concentration of inhaled oxygen is likely of benefit. In 2008, the UK National Institute for Health and Clinical Excellence concluded that, “The mechanism for improved blood oxygen carriage due to increased FIO\textsubscript{2} is physiologically not clear. However, this simple, cheap intervention deserves further investigation.”\textsuperscript{43}

### Systemic HBOT

HBOT is conducted in single-person or multiple-person chambers that completely envelop patients in an environment of 2 to 2.5 atmospheres (atm) and 100% oxygen.\textsuperscript{44} Sessions are typically delivered for 90 to 120 minutes daily (sometimes twice daily), and therapeutic courses lasting 2 to 8 weeks are typical. Because most patients under room air conditions show hemoglobin oxygen saturations of 90% or greater, the incremental binding of oxygen to hemoglobin induced by HBOT is marginal. However, independently of the normal mechanism of oxygen transport to tissues via hemoglobin binding, HBOT dissolves substantial amounts of oxygen directly into serum, much as carbon dioxide is dissolved under pressure into carbonated beverages.

- Serum P\textsubscript{O}2 can reach 1200 to 2000 mm Hg during treatment sessions.\textsuperscript{35}
- Total blood oxygen content, which is ordinarily in the range of 20 volume %, increases to about 27 volume % during 100% oxygen breathing at 3 ATA pressure.
- Because oxygen delivery from capillaries into tissue is driven by diffusion along a gradient, large increases of capillary P\textsubscript{O}2 result in substantial increases in oxygenation at the cellular level. When the P\textsubscript{O}2 is increased to 2000 mm Hg, oxygen may diffuse as far as 246 μm.
- As shown by the classic report of Boerema and colleagues\textsuperscript{45} in 1960, sufficient oxygen can be dissolved in the bloodstream to maintain life and vital organ function even in the absence of hemoglobin.
- Following a session of HBOT, skin oxygen tension remains increased for a period of 30 minutes to 4 hours.

The details of nearly 400 years of hyperbaric medicine have been described by Kindwall.\textsuperscript{46} The history of HBOT is checkered. On a naïve level, hyperoxygenation of tissue seems to be potentially beneficial in a wide variety of pathologic conditions. Given the sensitivity of peripheral and central nervous tissue to hypoxia, for example, HBOT has been attempted in a range of neurologic diseases and conditions. In past decades, hyperbaric operating rooms were used to conduct procedures such as carotid endarterectomy, in which temporary brain ischemia was anticipated. In general, results of HBOT for these and many other conditions were disappointing.

The combination of overly optimistic expectations and disappointing outcomes led to the perception that HBOT was not useful in any
medical condition, and the therapy was relegated to the realm of quackery in the opinion of many practitioners since the 1970s. Nevertheless, an increase in interest and effort to understand the physiology of wound healing in the past 20 years has generated a large body of evidence both in favor of the results of HBOT and to explain the mechanisms by which HBOT is beneficial. Thus, opinion has returned to a middle ground in which HBOT is understood to be a valuable adjunctive wound healing therapy but not a panacea. Nevertheless there are still skeptics of HBOT who think that, for most patients, the therapy is unnecessary and that oxygenation of most wounds can be achieved in other ways, such as by improving local perfusion.¹

**HBOT: growing scientific basis**

Medical societies, government agencies, and health insurers now agree that there is a legitimate role for HBOT in a variety of conditions, including many aspects of chronic open wounds. The American Diabetes Association endorsed HBOT for treating recalcitrant diabetic foot ulcers in 1999; 3 years later, the Centers for Medicare and Medicaid Services announced its concurrence and its policy to reimburse for such treatments in patients who had failed to heal with a month of standard care.¹⁴ Professional societies such as the Undersea and Hyperbaric Medical Society and the Wound Healing Society have included adjunctive HBOT in suggested algorithms of care for diabetic foot ulceration.

There is a large and growing body of scientific information to indicate the potential physiologic benefits of HBOT in wound healing. Typical HBOT increases $P_{O_2}$ to 1200 mm Hg and increases $O_2$ diffusion distance from 60 to 250 μm.⁴⁷ Fibroblast proliferation is improved by this treatment. Hehenberger and colleagues ²² described a series of experiments in which fibroblasts harvested from normal patients undergoing reduction mammoplasty were compared with cells derived from specimens of diabetic foot ulcer beds. Cells were plated in tissue culture medium and placed inside a monoplace hyperbaric oxygen (HBO) chamber, then subjected to air (as control, at 795 and 1875 mm Hg) and 100% oxygen at various pressures from 795 to 2250 mm Hg. Total cell count was determined by measuring DNA content in the specimens immediately before treatment and after 24 hours. Although pressurized air did not induce fibroblast growth, fibroblasts proliferated more as $P_{O_2}$ was increased, and maximal proliferation rate was observed at 1875 mm Hg (2.4 ATA).²² HBOT was able to restore fibroblast growth in diabetic ulcer cells to the level seen in the normal control cells. Other investigators have noted that HBOT induces fibroblasts to differentiate into myofibroblasts, which are responsible for wound contraction.⁴⁸

See Box 4 for a summary of HBOT wound healing indications.

**HBOT treatment and wound vascularity**

A common observation among hyperbaric physicians is the large increase in wound vascularity after patients are treated for several weeks. HBOT has been shown to increase VEGF mRNA levels in endothelial cells and macrophages, and increased VEGF is noted in wound fluid of patients receiving this treatment.⁷ Much of this effect may be mediated by increased nitric oxide.⁴⁹,⁵⁰ HBOT induces endothelial progenitor cells (EPCs) to migrate out of bone marrow, circulate, and settle in the peripheral wound, forming vascular buds.⁵¹,⁵² Circulating EPCs in the peripheral blood are diminished in diabetes mellitus; HBOT reverses this. The HBOT-induced steep oxygen gradient between capillary and hypoxic wound bed prompts macrophage migration and release of angiogenic growth factors. There is a distinction between angiogenesis (proliferation and migration of resident endothelial cells supported by fibroblasts) and vasculogenesis (a de novo process whereby EPCs enter a wound, differentiate into endothelial cells, and create a new vascular network). HBOT facilitates both these regenerative processes by increasing wound VEGF, basic fibroblast growth factor, and TGF-β1.

Patients undergoing 20 HBO treatments in preparation for dental procedures related to osteoradionecrosis of the mandible were found to have a 5-fold increase in EPCs. Under inhibition of nitric oxide synthetase, this effect is blocked. Maximal stimulation of peripheral nitric oxide synthetase requires up to 2.8 ATA.¹²

**HBOT and hypoxia/hyperoxia**

We have noted that both hypoxia and hyperoxia have certain salutary effects on healing. Perhaps
by fortune more than design, the current regimen of HBOT makes use of both stimuli: during HBO treatment, and for about 4 hours afterward, the supplemental oxygen facilitates fibroblast growth and collagen deposition and maturation, whereas during the remaining 20 hours before the next treatment, relative hypoxia stimulates angiogenesis.

**HBOT and antibiotics**

HBOT also increases the susceptibility of various bacteria to antibiotics, but is this strain specific and the effect cannot be generalized to all pathogens, whether aerobic or anaerobic.5,7

**HBOT and MMPs**

Sander and colleagues36 used a model of impaired healing created by depleting mice of macrophages to study the effects of HBOT. Healing was restored to normal pace in HBOT-treated animals, even though the wounds were not ischemic or hypoxic. The author proposed that the benefit of HBOT was mediated through contradictory effects on MMPs. The activity of tissue inhibitor of metalloproteinase-1 (TIMP-1) was increased by HBOT, increased TNF-α-mediated tissue breakdown necessary for neovascularization and supply of MMPs available for selective tissue breakdown necessary for neovascularization and epithelial migration.36

**HBOT and granulation tissue**

In the rabbit ischemic ear model, HBOT increased granulation tissue and epithelial regrowth; the response was accentuated when growth factors were added to the HBOT.53 Two of the 3 arteries supplying the external ear were ligated to create tissue ischemia, then 6-mm, full-thickness dermal wounds were created (n = 42). Wounds were treated with PDGF-BB, TGF-β1, or buffered saline; animals underwent HBO for 90 min/d at 1, 2, or 3 ATA 100% O2 for up to a week. Treatment at 1 ATA did not alter epithelial advance or granulation tissue. Hyperoxygenation at 2 and 3 ATA increased granulation and increased wound P O2 to as high as 300 mm Hg, increasing tissue volume by 100% (P = .03) but not influencing epithelial advance.54 In combination with topical growth factors, granulation tissue increased by 200% (P = .0001) and epithelial advance increased significantly.

**Obstacles to defining benefits of HBOT**

A major obstacle to precise definition of the benefits of HBOT is the paucity of scientifically rigorous clinical studies. In 2005, Roeckl-Wiedmann and colleagues55 performed an extensive search through some 24 electronic databases as well as a manual search through texts to gather 78 articles that purportedly offered clinical evidence on the role of HBOT in wound management. Of these, only 21 were deemed to be suitable human clinical trials, and 15 were excluded because they were not randomized, not clearly focused on 1 wound cause, used TOT instead of HBOT, or reported data that had already been published elsewhere. Six publications of at least moderate scientific merit were left: 5 on diabetic foot ulcers and 1 on venous leg ulcers.

**Kessler and colleagues’ study on diabetic foot ulcers**

Kessler and colleagues56 noted that HBOT doubled the rate of wound healing (P = .037) compared with controls during 2 weeks of treatment, but, once the twice-daily HBOT treatments were discontinued, the rate of healing in the control and HBOT groups equalized.56 Twenty-eight patients were randomized, 15 to HBOT and 13 to control therapy, including off-loading. Patients were excluded if they suffered significant arterial occlusive disease or serious contraindications to HBOT (emphysema, claustrophobia, proliferating retinopathy). The groups were similar in terms of demographic factors and indicators of diabetic complications (renal, ocular, vascular), and all suffered with neuropathy. Lesions ranged from Wagner grade I to III. HBOT was delivered as 100% oxygen at 2.5 ATA pressure, giving two 5-minute air breaks during each treatment session. Only 1 patient had barotrauma to the ear and had to discontinue participation. Periwound TcP O2 increased from a mean of 22 to 454 mm Hg with first treatment, and 26 to 550 mm Hg after 20 treatments (P < .001). At day 15 (completion of HBOT), the surface area of ulcers treated with HBOT was reduced substantially more than the healing that was observed in control subjects (41.8% ± 25.5% surface area reduction with HBOT vs 21.7% ± 27.3% in controls; P = .037). By day 30, the control group had caught up: respective surface area reductions were 48.1% (± 30.3%) and 41.7% (±27.3%; NS).

**Faglia and colleagues’ study on ischemic diabetic wounds**

HBOT reduced lower amputation rates significantly in patients with ischemic diabetic wounds.57 Seventy diabetic patients who were hospitalized for severe foot wounds were considered appropriate candidates for HBOT. They consented to randomization to standard therapy with or without HBOT. Two patients dropped out (1 died of a stroke shortly after hospital admission; the other withdrew consent for treatment). Of the remaining patients, 35
underwent HBOT and 33 did not. There were no differences in the treatment groups in terms of demographic features, depth of foot ulceration, other diabetic complications, circulatory status, or duration of hospitalization. In the treatment groups, Wagner grade III and IV lesions predominated (25.7% and 62.8% in the HBOT group, and 24.2% and 60.6% in the control groups, respectively). All patients underwent an initial aggressive excisional debridement shortly after admission to the hospital, then twice-daily dressing changes and antibiotics guided by frequent wound cultures. Patients receiving HBOT received an average of 38 treatments; daily in the beginning and then 5 days per week later on (2.5 ATA 100% O₂, 90 minutes). The end point of the study was the incidence of major amputation. Three patients receiving HBOT required below-knee or above-knee amputations, compared with 11 controls (8.6% vs 33.3%, respectively; \( P = .016 \)). TcPO₂ measurements were included in the study: at baseline the 2 patient groups had similar readings (23.2±10.7 mm Hg vs 21.3±10.7 mm Hg, respectively; NS). However, at hospital discharge, the values had increased to 37.3 (±16.1) mm Hg and 26.3 (±13.5) mm Hg, respectively. The greater improvement in the HBOT group was highly statistically significant \( (P = .0002) \). The investigators concluded that the enhanced increase in TcPO₂ was related to improved angiogenesis in and around the wound, and they proposed that HBOT is effective in lowering the incidence of major amputation in diabetic wounds.

Baroni and colleagues’ study on diabetic foot ulcers
Baroni and colleagues⁵⁸ conducted a nonrandomized comparison of the outcomes of 18 hospitalized patients with diabetic foot ulcers treated with standard of care plus HBOT with another group of 10 patients treated identically but without HBOT. All patients had advanced diabetes, and most suffered retinopathy, neuropathy, and vascular occlusive disease. In the HBOT group, 16 patients healed versus 1 in the comparator group \( (P = .001) \). Two patients receiving HBOT and 4 comparator patients underwent amputation. The investigators concluded that there is major benefit in HBOT for amputation avoidance.

Abidia and colleagues’ study on ischemic lower extremity ulcers
Abidia and colleagues⁵⁹ conducted a small double-blind study in which patients with ischemic lower extremity ulcers were randomized to hyperbaric air or hyperbaric oxygen treatment. By 6 weeks, 5 of 8 wounds healed in the oxygen group versus 1 of 8 of the controls (NS because of small numbers). At 1 year, all the HBOT wounds remained healed but the wound that had healed in the control group reopened, and the difference in 1-year closure was significantly better in the treated group \( (P = .026) \). The investigators analyzed overall direct patient treatment costs (fees for dressings, clinic visits, and hospitalizations) and found that HBOT was also able to show cost-effectiveness (based on 100 UK pounds per HBO treatment session). At 6 weeks, median wound surface area closure was 100% in the HBOT group and 52% in controls \( (P = .027) \). Although the study included few patients, the randomization was strict and the methodology was rigorous.

Kranke and colleagues’ study on diabetic foot ulcers
Kranke and colleagues⁶⁰ found only 5 publications that met the standards for inclusion in a Cochrane Review of the evidence in favor of HBOT for chronic wounds. The investigators concluded that HBOT significantly reduces the chance of amputation and may increase the chance of healing at 1 year for diabetic foot ulcers. However, they concluded that there is a need for further substantiation because the number of patients on which these conclusions were based is small, and the trial methodologies imperfect. They could not justify the use of HBOT in the management of other wound causes based on current evidence. The report estimated that 4 diabetic ulcers needed to be treated with HBOT to prevent 1 amputation.

Lin and colleagues’ study on diabetic foot lesions
Lin and colleagues⁶¹ randomized 29 patients with Wagner 0, 1, and 2 foot lesions to standard care with and without HBOT. Although patients started the study with similar values, after 30 HBO treatments, the patients receiving HBOT had significantly reduced HgA1c (6.6±1.7 vs 9.3±4.3) TcPO₂ (57.5±20.7 vs 35.8±21.2), and laser Doppler perfusion scan flux (35.6±7.0 vs 25.8±4.1; all 3 comparisons were statistically significant at the \( P<.05 \) level).

Predictive ability in HBOT
A frequent objection to HBOT is the inability to predict accurately which patients will receive benefit. Because there are certain indications that sometimes have good responses, patients are usually treated empirically for several weeks, assessed for response, and then continued or terminated according to clinical parameters. Perhaps because of well-intentioned optimism, many patients are treated for weeks or even
months, at great expense ($1000 per treatment at our center) but with minimal tangible improvement. Fife and colleagues\textsuperscript{34} found only moderate predictive ability of TcPO\textsubscript{2} to distinguish patients who would benefit from HBOT, whether measured under room air, breathing 100% oxygen at sea level, or in the chamber. Barnes\textsuperscript{47} noted that, for diabetic lesions, the extent of the wound according to the Wagner scale correlated well with response to HBOT, with Wagner 3, 4, and 5 lesions responding well 77%, 64%, and 30% of the time, respectively. In the same report, patients who achieve a periwound TcPO\textsubscript{2} of 200 while breathing 100% oxygen at 2.5 ATA were likely to heal.

Complications in HBOT

HBOT generally is safe and comfortable; nevertheless, there are well-known complications that may occur. One set of risks relates to maintaining the patient under supranormal external pressure. About 60% to 70% of patients experience measurable, but reversible, myopia; patients with presbyopia may experience temporary improvement in visual acuity during the course of therapy. The incidence of otic barotrauma has been quoted between about 2% and 20%.\textsuperscript{35,47,55} In my experience, the incidence is even higher, and fully one-quarter of my patients require temporary tympanostomy tubes to equalize pressures in the middle ear and prevent permanent damage. The tubes are easily removed (or sometimes fall out spontaneously) and the tympanic membranes typically heal within days afterward. Other barotrauma is less frequent; the incidence of pneumothorax has been cited at 1 in 1 million treatments. HBOT imposes increased afterload on the left ventricle and patients with New York Heart Association class 3 and 4 congestive heart failure, with ejection fraction less than 35%, should not be treated, because their cardiac situation may worsen.

The other set of potential complications relates to the effects of oxygen as a medicine. Perhaps the most feared complication is oxygen toxicity seizure, the risk of which is about 0.01% to 0.03%. These seizures, which are usually over within the brief time it takes to switch the breathing mixture from 100% to air and to decompress the HBO chamber, do not cause permanent brain damage and are random events that do not necessarily preclude further HBOT. Regimens dictating a 90-minute treatment and limiting pressure to about 2.5 ATA are based on known tolerance of patients to high levels of oxygen for given periods of time. The risk of seizure may be lowered by giving a 5-minute period of air breathing, rather than 100% oxygen, every half hour while in the chamber. There are other theoretic risks of excessive oxygen, such as pulmonary toxicity and of enhancing tumor growth, but these have not been borne out in everyday practice.

Indications for HBOT

At present, the generally approved wound-related indications for HBOT include moderate and deep diabetic foot/leg lesions, necrotizing soft tissue infections, radiation damage to soft tissue and/or bone (osteoradionecrosis), selected problem wounds (Figs. 1 and 2), compromised skin grafts and flaps, crush injury, clostridial myositis, gas gangrene, and refractory osteomyelitis. Perioperative treatments have been particularly useful in patients with osteoradionecrosis of the mandible who are undergoing dental extractions or implants; typical regimens call for 10 preoperative treatments and then postprocedure treatment until full healing is observed. Similarly, in anticipation of skin grafts or flaps that may be compromised because of tension or because the tissue to be closed has previously been irradiated, a brief period of preoperative treatment followed by resumption of HBOT as soon as practical after surgery optimizes graft/flap take.

The American Diabetes Association has since 1999 endorsed the used of HBOT as an adjunctive therapy for severe diabetic wounds that fail to respond to standard therapy.\textsuperscript{14} The Centers for Medicare and Medicaid Services in 2002 approved the therapy for treatment Wagner III to IV ulcers. Professional organizations such as the Undersea and Hyperbaric Medical Society and Wound Healing Society have included HBOT in their suggested algorithms of care. More than 90% of wounds closed with HBOT remain healed 4 years later. Although health economic aspects of treatment are beyond the scope of this article, a case can be made that the expense of HBOT is justifiable with cost-effectiveness data.\textsuperscript{47}

TOT

TOT involves enclosing a body part (usually an extremity) in a portable chamber that circulates pure oxygen over the wound surface. Potential advantages to this approach, compared with HBOT, include lower cost, greater convenience, therapy not limited to 2 hours per day, and success not dependent on the macrocirculation and microcirculation to the wound bed.\textsuperscript{7} Potential complications of HBOT, such as oxygen toxicity to tissues/organs, may be avoided by using TOT.\textsuperscript{35}

TOT Penetration of Wound Surface

Perhaps the most controversial issue is the ability of TOT to achieve meaningful penetration of the
wound surface and reach the cells/tissues that would benefit from hyperoxygenation. This issue has been addressed both with direct measurement of tissue oxygen levels and, indirectly, by showing the biochemical and biologic effects of TOT. Fries and colleagues \(^{35}\) showed that, at a depth of 2 mm into the wound bed, \(P_{O_2}\) increased from its baseline of 5 to 7 mm Hg to 40 mm Hg.

---

**Fig. 1.** Multiple-recurrent ventral hernia had recently been repaired with fascial flaps and mesh, but the wound became infected and dehisced shortly after surgery. The patient was returned to surgery and the mesh was removed. (A) Exposed abdominal organs thinly covered with collagen mesh. (B) After 20 HBO treatments, there is substantial in-growth of vessels and soft tissue. (C) After nearly 40 HBO treatments, the wound has partially contracted and the soft tissue coverage has thickened. (D) Perioperative HBOT was used to support the take of partial-thickness skin grafts, achieving definitive closure of the wound.

**Fig. 2.** Open transmetatarsal amputation. (A) Early postoperative period. Cut ends of metatarsals #2 to 5 are exposed. (B) After approximately 40 HBO treatments. Bone ends are covered. Healthy granulation tissue fills the wound.
after 4 minutes of TOT. Standardized full-thickness wounds on the backs of pigs were treated with either topical air or topical pure oxygen. By the second day of treatment, the surface area of the oxygen wounds was significantly \( P < .05 \) reduced compared with the controls, and the acceleration in healing was maintained to 3 weeks \( P < .005 \). Healed wounds were subjected to 3-mm punch biopsy and histologic evaluation; TOT wounds were more mature than the controls, in terms of epithelial architecture, expression of keratin-14, and expression of VEGF and \( \alpha \)-smooth muscle actin. Under resting conditions, wound site tissue PO\(_2\) levels were 4 times higher in the TOT versus the control wounds (42 vs 11 mm Hg; \( P < .05 \)).

In another report, db/db diabetic obese mice were subjected to standardized full-thickness wounds and then randomized to TOT or control dressings.\(^{62}\) TOT was delivered as pure oxygen at low flow (3 mL/h) over the wounds. The surface area of the TOT wounds was significantly smaller at 6 and 10 days \( (P = .022 \) and \( P = .008 \), respectively). Histologic sections of the wounds showed more extensive collagen deposition in the TOT-treated lesions.

**TOT and Increased VEGF**

Gordillo and colleagues\(^{63}\) showed that TOT increased VEGF up to 20-fold in diabetic foot ulcers. A large clinic population was screened to enroll 57 patients who suffered a variety of chronic wounds. The criteria by which these patients were selected were not described in detail. These patients were assessed for HBOT but patients who had contraindications were offered TOT; this yielded a population of 32 HBO-treated and 25 TOT-treated patients. Patients who consented to serial biopsies underwent wound edge biopsy just before initiation of treatment and again at 7 and 14 weeks into therapy. Tissue levels of mRNA expressed by the genes for VEGF, TGF-\( \beta 1 \), and COLIA1 were measured. In patients who experienced complete wound healing, VEGF expression was substantially increased by TOT but not by HBOT; expression of the other 2 genes did not show a significant difference with either treatment. The issues with study design limit the interpretation of these results, but demonstration of increased VEGF expression implied that TOT succeeded in penetrating the wound with oxygen to a meaningful extent.

**TOT Versus Silver Alginate**

Blackman and colleagues\(^{64}\) conducted a prospective, but not randomized, comparison of patients treated with TOT versus a silver alginate dressing. TOT treatments were conducted for 60 minutes, 5 times each week. Fourteen of 17 patients (82.4%) healed in the TOT group, compared with 5 of 11 (45.5%) in the alginate group \( (P = .04) \). Median time to closure was also improved by TOT (56 vs 93 days, respectively). The results were particularly impressive considering that the wounds in the TOT group were significantly larger (mean 4.1 vs 1.4 cm\(^2 \); \( P = .02 \)) and of greater duration (6.1 vs 3.2 months, \( P = \text{NS} \)). Although subject to all the flaws inherent in a nonrandomized and nonblinded trial, these results are provocative.

**TOT Versus Standard Wound Care**

Heng and colleagues\(^{25}\) reported a prospective study comparing TOT and standard care (wet-to-dry or hydrocolloid dressings) in the care of wounds of various causes, including 39 diabetic foot ulcers and 40 wounds of other types (mostly pressure ulcers). Many patients suffered significant comorbidities. The TOT was delivered at 1.03 atm, 4 h/d, 4 d/wk up to 4 weeks. Complete wound healing was noted in 90% of the TOT group versus 22% of controls. Four of 7 stage IV wounds healed with TOT versus none of 5 in long-term follow-up. The methodology used clouds the results: many patients were not randomized and most patients had more than 1 study wound, making the statistical analysis and interpretation of the data difficult. Wound biopsies were conducted and showed substantial increase in the density of blood vessels and collagen in the TOT wounds. Although acknowledging the limitations of this report, the results argue in favor of TOT compared with standard wound care alone.

Tawfick and Sultan\(^{65}\) published a nonrandomized comparison of venous leg ulcers treated with TOT and others who were managed using standard therapy \( (n = 83) \). At 12 weeks, 80% of patients receiving TOT were healed (median 45 days) compared with 35% of controls (median 182 days; \( P < .0001 \) for both incidence of healing and time to healing). Patients were allowed to choose whether to be treated with TOT or standard of care. TOT was given in an inpatient setting, with 3-hour treatments twice daily, \( O_2 \) at 10 L/min with continuous humidification. Patients were treated until full healing or 12 weeks, whichever came first. Standard of care consisted of wound cleaning, debridement, and Profore (Smith and Nephew, London, United Kingdom) compression dressings 1 to 3 times weekly. Initial patient and wound characteristics were not significantly different between the 2 groups. All wounds were at least 2 years old, and most were larger than 10 cm\(^2 \). The methodology raised the question of whether the
therapeutic effect came more from mandatory leg elevation 6 hours per day, or from TOT. In 32 of 46 TOT-treated ulcers, granulation tissue developed first in the center of the wound, and then extended peripherally to cover the surface of the lesion.

Clinical Evidence for TOT

Although there is sufficient penetration of TOT to achieve meaningful physiologic effects within the wound bed, the current clinical evidence in favor of this modality needs to be supplemented with more rigorous investigations. In choosing between HBOT and TOT, the benefits for TOT of lower cost and avoidance of potential complications are outweighed by the less potent clinical effect achieved with this approach. Particularly in the case of diabetic foot and leg ulceration, in which there is substantial risk of amputation, the preponderance of data seems to support HBOT in preference to TOT.

HOW MUCH OXYGEN IS OPTIMAL?

Given that correction of wound hypoxia is beneficial to many aspects of healing, it does not necessarily follow that more is better, or that hyperoxygenation of normally nourished wounds confers enough benefit to justify the risks. It is possible that, for some individuals, imposing oxidative stress and excessive ROS may be more harmful than helpful.2,12 The rate of production of toxic radicals is directly proportional to local oxygen tension.4 Heng and colleagues25 make important points about potential negative influences of excess oxygen on wound repair. Unphysiologically high levels of oxygen can react with NADP and be metabolized to ROS in the cytoplasm without the usual catalysis by the mitochondrial cytochrome system, which ordinarily also controls the release of ROS. In this event, the ROS created may be injurious to the host cell (fibroblast, endothelium) rather than serving a useful role in killing foreign organisms. This may be one mechanism by which oxygen exerts brain toxicity, occasionally causing seizures in patients under HBOT.66 In addition, bypassing the cytochrome system sacrifices production of ATP, so the extra oxygen is wasted. There is the potential for cell cycle arrest and genotoxicity from exposure of cells to pure oxygen.10,67 Cellular senescence is accelerated in hypoxic conditions.12

Cellular Adaptation to Ambient Oxygen Level

Cells seem to accustom themselves to chronic hypoxia or hyperoxia. For example, the PHD family of proteins, which are known to suppress cellular proliferation, modulate the cell cycle and encourage apoptosis, is expressed when hypoxia is sensed.68 Cells cultured under 20% oxygen and then subjected to a 5% oxygen environment synthesize higher levels of PHDs; over longer periods of time, PHDs return to baseline levels. Similarly, cells accustomed to culture under 30% oxygen overexpress PHDs when suddenly put under 20% oxygen.12 Thus there is a hypoxia set point that is tunable. In theory, the optimal oxygen treatment strategy may be to restore hypoxic areas of the wound bed to normal oxygen tension without overtreating and risking oxidative stress.

Individualized Oxygen Dosing

In common practice, nearly all patients receiving HBOT are dosed similarly: 90-minute sessions, once daily at 2.0 to 2.5 ATA. It is possible that this one-size-fits-all approach to dosing might not be appropriate for all patients; some wounds are more hypoxic than others, therefore some may be overdosed and others underdosed. Everyone has endogenous genes that encode antioxidant molecules to protect them against oxidative stress; however, individuals may vary in the levels of expression of these genes and therefore some patients may be less capable than others of dealing with the oxygen load. Real-time wound PO₂ mapping assist understand of the degree of hypoxia in each wound, and could be the basis for more individualized therapy.

REFERENCES


How honey kills bacteria

Paulus H. S. Kwakman,* Anje A. te Velde,† Leonie de Boer,* Dave Speijer,‡ Christina M. J. E. Vandenbroucke-Grauls,*§ and Sebastian A. J. Zaat*†

*Department of Medical Microbiology, Center for Infection and Immunity Amsterdam, †Laboratory of Experimental Gastroenterology and Hepatology, and ‡Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam; and §Department of Medical Microbiology and Infectious Diseases, Vrije Universiteit Medical Center, Amsterdam, The Netherlands

ABSTRACT With the rise in prevalence of antibiotic-resistant bacteria, honey is increasingly valued for its antibacterial activity. To characterize all bactericidal factors in a medical-grade honey, we used a novel approach of successive neutralization of individual honey bactericidal factors. All bacteria tested, including Bacillus subtilis, methicillin-resistant Staphylococcus aureus, extended-spectrum β-lactamase producing Escherichia coli, ciprofloxacin-resistant Pseudomonas aeruginosa, and vancomycin-resistant Enterococcus faecium, were killed by 10–20% (v/v) honey, whereas >40% (v/v) of a honey-equivalent sugar solution was required for similar activity. Honey accumulated up to 5.62 ± 0.54 mM H2O2 and contained 0.25 ± 0.01 mM methylglyoxal (MGO). After enzymatic neutralization of these two compounds, honey retained substantial activity. Using B. subtilis for activity-guided isolation of the additional antimicrobial factors, we discovered bee defensin-1 in honey. After combined neutralization of H2O2, MGO, and bee defensin-1, 20% honey had only minimal activity left, and subsequent adjustment of the pH of this honey from 3.3 to 7.0 reduced the activity to that of sugar alone. Activity against all other bacteria tested depended on sugar, H2O2, MGO, and bee defensin-1. Thus, we fully characterized the antibacterial activity of medical-grade honey.—Kwakman, P. H. S., te Velde, A. A., de Boer, L., Speijer, D., Vandenbroucke-Grauls, C. M. J. E., Zaat, S. A. J. How honey kills bacteria. FASEB J. 24, 2576–2582 (2010). www.fasebj.org

Key Words: antibacterial agents • drug resistance • isolation and purification • methicillin-resistant Staphylococcus aureus • peptides

Honey has been renowned for its wound-healing properties since ancient times (1). At least part of its positive influence is attributed to antibacterial properties (2, 3). With the advent of antibiotics, clinical application of honey was abandoned in modern Western medicine, although in many cultures, it is still used (4). These days, however, abundant use of antibiotics has resulted in widespread resistance. With the development of novel antibiotics lagging behind (5), alternative antimicrobial strategies are urgently needed.

The potent in vitro activity of honey against antibiotic-resistant bacteria (6, 7) and its successful application in treatment of chronic wound infections not responding to antibiotic therapy (3) have attracted considerable attention (8–10).

The broad spectrum antibacterial activity of honey is multifactorial in nature. Hydrogen peroxide and high osmolality—honey consists of ~80% (w/v) of sugars—are the only well-characterized antibacterial factors in honey (11). Recently, high concentrations of the antibacterial compound methylglyoxal (MGO) were found specifically in Manuka honey, derived from the Manuka tree (Leptospermum scoparium) (12, 13). Until now, no honey has ever been fully characterized, which hampers clinical application of honey.

Recently, we determined that Revamil medical-grade honey, produced under standardized conditions in greenhouses, has potent, reproducible bactericidal activity (14). In the current study, we identified all bactericidal factors in the honey used as source for this product and assessed their contribution to honey bactericidal activity.

To accomplish this, we used a novel approach of successive neutralization of individual honey bactericidal factors combined with activity-guided identification of unknown factors.

MATERIALS AND METHODS

Honey

Unprocessed Revamil source (RS) honey was kindly provided by BFactory Health Products (Rhenen, The Netherlands). RS honey has a density of 1.4 kg/L and contains 333 g/kg glucose, 385 g/kg fructose, 73 g/kg sucrose, and 62 g/kg maltose. To study the contribution of the sugars to the bactericidal activity of honey, a solution with a sugar composition identical to that of the honey was prepared.

1 Correspondence: Department of Medical Microbiology, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands, E-mail: s.a.zaat@amc.uva.nl doi: 10.1096/fj.09-150789
Microorganisms

Bactericidal activity of honey was assessed against the laboratory strains Bacillus subtilis ATCC6633, Staphylococcus aureus 42D, Escherichia coli ML-35p (15), and Pseudomonas aeruginosa PAO-1 (ATCC 15692), and against clinical isolates of methicillin-resistant S. aureus (MRSA), vancomycin-resistant Enterococcus faecium (VREF), extended-spectrum β-lactamase-producing E. coli (E. coli ESBL) and ciprofloxacin-resistant P. aeruginosa (CRPA).

Determination of \( \text{H}_2\text{O}_2 \) concentration in honey

Hydrogen peroxide concentrations in honey were determined quantitatively using a modification of a method described previously (16). Undiluted and 10-fold diluted samples of honey (40 μl) were mixed in wells of microtiter plates with 135 μl reagent, consisting of 50 μg/ml Odiansidine (Sigma, St. Louis, MO, USA) and 20 μg/ml horseradish peroxidase type IV (Sigma) in 10 mM phosphate buffer (pH 6.5). Odiansidine and peroxidase solutions were freshly prepared from a 1 mg/ml stock in demineralized water and from a 10 mg/ml stock in 10 mM phosphate buffer (pH 6.5), respectively. After 5-min incubations at room temperature, reactions were stopped by addition of 120 μl 0.2 M NaOH, and absorbance at 540 nm was measured. Hydrogen peroxide concentrations were calculated using a calibration curve of 3.37 mM was determined using the extinction coefficient of SLG of 0.01 mM of MGO.

Reduced glutathione (Sigma) was added to diluted honey to a final concentration of 15 mM, and conversion of MGO to S-α-lactoyl-glutathione (SLG) was initiated by addition of 0.5 mg/ml glyoxalase I (Sigma). The amount of MGO converted was determined using the extinction coefficient of SLG of 3.37 mM⁻¹ at 240 nm (17). Thus, we determined that up to 10 mM of exogenous MGO added to 40% honey was completely converted, and that undiluted RS honey contained 0.25 ± 0.01 mM of MGO.

Antibee defensin-1 polyclonal antibody

An affinity-purified polyclonal antibee defensin-1 antibody was purchased from Eurogentec (Seraing, Belgium). The N-terminal part of bee defensin-1 is hydrophobic and contains 3 disulfide bonds, whereas the hydrophilic C-terminal region lacks cysteine residues (18). Therefore, rabbits were immunized with a synthetic peptide corresponding to the C terminus of bee defensin-1 (CRKTSFKDLW DKRF), and antibodies were subsequently affinity-purified using this peptide coupled to AF-Amino Toyopearl 650 M resin (Toso, Tokyo, Japan).

Liquid bactericidal assay

Bactericidal activity of honey was quantified in 100-μl volume liquid tests, in polypropylene microtiter plates (Costar Corning, New York, NY, USA). For each experiment, a 50% (v/v) stock solution of honey was freshly prepared in incubation buffer containing 10 mM phosphate buffer (pH 7.0) supplemented with 0.03% (w/v) trypsin/case soy broth (TSB; BD Difco, Detroit, MI, USA). Bacteria from logarithmic phase cultures in TSB were washed twice with incubation buffer and suspended at a final concentration of 1 × 10⁷ CFU/ml, based on optical density. Plates were incubated at 37°C on a rotary shaker at 150 rpm. At indicated time points, duplicate 10-μl aliquots of undiluted and 10-fold serially diluted incubations were plated on blood agar. Bacterial survival was quantified after overnight incubation at 37°C. The detection level of this assay is 100 CFU/ml.

To assess the contribution of \( \text{H}_2\text{O}_2 \) to the bactericidal activity of honey, bovine liver catalase (Sigma) was added to a final concentration of 600 U/ml. A catalase stock solution was prepared according to the manufacturers’ instructions in 50 mM phosphate buffer (pH 7.0). The addition of 0.25% (w/v) of this catalase stock solution reduced the amount of \( \text{H}_2\text{O}_2 \) to undetectable levels at all honey concentrations tested and did not affect bacterial viability.

Sodium polyanetholsulfonate (SPS) (Sigma) was added to neutralize cationic bactericidal components (19) at a final concentration of 0.025% (w/v). The incubation buffer did not affect the pH of the concentrations of honey used in our experiments. A 1 M NaOH solution was used to titrate honey solutions to pH 7.0.

Agar diffusion assay

To assess antibacterial activity of fractionated honey, an agar diffusion assay was used (20). In brief, a B. subtilis inoculum suspension was prepared as described for the liquid bactericidal assay. Bacteria (10⁴ CFU) were mixed with 20 ml nutrient-poor agar [0.03% (w/v) TSB in 10 mM sodium phosphate buffer (pH 7.0) with 1% low EEO agarose (Sigma)] of 45°C, and immediately poured into 10-×10-cm culture plates. Wells of 1 mm diameter were punched into the agarose, and 2.5-μl samples were added to the wells and allowed to diffuse into the agarose for 3 h at 37°C. Subsequently, the agarose was overlaid with 20 ml of double-strength nutrient agarose [6% TSB and 1% Bacto-agar (BD Difco), 45°C], and plates were incubated overnight at 37°C. Clear zones around the wells indicated antibacterial activity.

Ultrafiltration of honey components

Fifteen milliliters of 20% honey was centrifuged in a 5-kDa molecular weight cutoff Amicon Ultra-15 tube (Millipore, Bedford, MA, USA) at 4000 g for 45 min at room temperature. The <5-kDa filtrate was collected, and the >5-kDa retentate was subsequently washed 3 times in the filter tube with 15 ml of demineralized water and concentrated to 0.4 ml.

Bacterial overlay assay

Native cationic proteins were separated by acid urea polyacrylamide gel electrophoresis (AU-PAGE) (21). Gels were either stained with PAGE-Blue (Fermentas, St. Leon-Rot, Germany) or washed 3 × 8 min in 10 mM phosphate buffer (pH 7.0) for a bacterial overlay assay. After washing, the gel was incubated for 3 h on B. subtilis-inoculated nutrient-poor agarose (see Agar Diffusion Assay). After removal of the gel, the agarose was overlaid with double-strength nutrient agarose and treated as described for the agar diffusion assay.

Immunoblotting

Proteins were separated by tris-tricine SDS-PAGE, as described previously (22), and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). Membranes were subsequently blocked with 5% nonfat dry milk (Bio-Rad, Veenendaal, The Netherlands) plus 0.5 M NaCl and 0.5% (v/v) Tween-20 in 10 mM Tris-HCl, pH 7.5 (rinse buffer), for 1 h. Blocked membranes were incubated with affinity-purified antibee defensin-1 antibody at 1.4
μg/ml in rinse buffer for 2 h. After incubation with primary antibody, membranes were washed 2× for 15 min in rinse buffer, incubated with horseradish peroxidase-labeled goat-anti-rabbit secondary antibody (Jackson ImmunoResearch West Grove, PA, USA) at 0.4 μg/ml in rinse buffer for 1 h, and washed again for 10 min in rinse buffer and 5 min in PBS, respectively. The membrane was developed using a DAB liquid substrate kit (Sigma).

**Purification of antibacterial peptide from honey**

An amount of >5-kDa honey retentate equivalent to 13 ml of honey was dissolved in loading buffer (3M urea in 5% acetic acid with methyl green as tracking dye) and loaded on a preparative acid-urea PAGE, as described previously (21) with slight modifications. A cylindrical gel (3.7 cm diameter, 6 cm height) in a model 491 Prep Cell (Bio-Rad) was prepared, prerun at reversed polarity for 3 h at 150 V in 5% acetic acid at 4°C, and protein was electrophoresed at 40 mA with reversed polarity. Protein was eluted in 5% acetic acid at 0.5 ml/min and collected in fractions of 2 ml. Fractions were assessed for protein composition by tris-tricine SDS-PAGE and for antibacterial activity by bacterial overlay assay. Fractions containing purified antibacterial protein were pooled, concentrated, dialyzed against 0.01% acetic acid in a 3.5-kDa molecular weight cutoff MINI Slide-A-Lyzer tube (Pierce, Rockford, IL, USA), freeze-dried, and dissolved in demineralized water.

**Protein identification by V8 digestion with subsequent mass analysis**

Duplicate fractions (estimated to contain ~2 μg of protein each) were adjusted to 50 mM sodium phosphate (pH 7.9) and 5% (v/v) acetonitrile. Approximately 0.5 μg of endopeptidase Glu-C (Fluka) was added per fraction and incubated at 25°C overnight. The resulting peptide mixtures were purified and concentrated with the aid of C18 ziptips (Millipore) and eluted in 10 μl 90% (v/v) acetonitrile and 1% (v/v) formic acid. The samples were checked for the presence of nonautodigest peptides with a reflectron MALDI-TOF mass spectrometer (MALDI; Waters, Milford, MA, USA). Next, samples were analyzed with ESI-tandem mass spectrometry (MS/MS). Data were acquired with a QT of 1 (Waters) coupled to an Ultimate nano-LC system (LC Packings Dionex, Sunnyvale, CA, USA). One microliter of peptide mixture was diluted in 10 μl of 0.1% TFA. The peptides of both samples were separated on a nanoanalytical column (75 μm i.d. × 15 cm PepMap; LC Packings Dionex) using a standard gradient of acetonitrile in 0.1% formic acid. The flow of 300 nl/min was directly electrosprayed in the QT of 1 operating in data-dependent MS and MS/MS mode. The resulting MS/MS spectra were analyzed with Mascot software (Matrix Science, Boston, MA, USA). In both fractions, a doubly charged ion (VTCDDLSSFKGQVN, mass 1537.8) with a sequence corresponding to the mature N terminus of bee defensin-1 could be identified (MOWSE scores >73).

**RESULTS**

Hydrogen peroxide is produced by the *Apis mellifera* (honeybee) glucose oxidase enzyme on dilution of honey. RS honey diluted to 40 to 20% accumulated high levels of H$_2$O$_2$ 24 h after dilution, with a maximum of 5.62 ± 0.54 mM H$_2$O$_2$ formed in 30% honey (Fig. 1A). The addition of catalase reduced H$_2$O$_2$ to negligible

---

**Figure 1.** Contribution of H$_2$O$_2$, sugars, and MGO to the bactericidal activity of honey after 24 h. A) Mean ± se hydrogen peroxide accumulation in different concentrations of honey, without catalase (squares) or with catalase added (asterisks). B) Bactericidal activity against indicated laboratory strains (top row) and against clinical isolates of vancomycin-resistant *E. faecium* (VREF), methicillin-resistant *S. aureus* (MRSA), extended-spectrum β-lactamase-producing *E. coli* (E. coli ESBL), and ciprofloxacin-resistant *P. aeruginosa* (CRPA) (bottom row). Bacteria were exposed to various concentrations of honey (squares), honey with catalase added (asterisks), or to honey-equivalent sugar solutions (circles). C) Killing of *B. subtilis* by honey in incubation buffer without addition (squares), with catalase (asterisk), with glyoxalase (small solid circles), or with catalase and glyoxalase I (inverted triangles), added to neutralize H$_2$O$_2$ and MGO, respectively, or by a honey-equivalent sugar solution (circles). Data are mean ± se log-transformed bacterial concentration (CFU/ml).

---

2578 Vol. 24 July 2010 The FASEB Journal · www.fasebj.org KWAKMAN ET AL.
levels (Fig. 1A) and markedly reduced the bactericidal activity against all bacteria tested, except *B. subtilis* (Fig. 1B). However, H$_2$O$_2$-neutralized honey exerted stronger bactericidal activity than equivalent sugar solutions (Fig. 1B). This indicates that H$_2$O$_2$ is important for the bactericidal activity of honey, but that additional factors must also be present. As *B. subtilis* was the most susceptible bacterium for nonperoxide bactericidal activity, we used it for identification of additional bactericidal factors.

The honey bactericidal compound MGO can be converted into 5-glutathione (SLG) by glyoxalase I, and this product can be measured spectrophotometrically. RS honey contained 0.25 ± 0.01 mM MGO. We aimed to apply glyoxalase I to neutralize the bactericidal activity of MGO in honey. This required that SLG, the reaction product of MGO, would be nonbactericidal. Indeed, the activity of up to 20 mM MGO was neutralized by conversion into SLG (Supplemental Fig. 1), indicating that SLG up to high concentrations did not kill the bacteria. Neutralization of MGO or H$_2$O$_2$ alone did not alter bactericidal activity of RS honey, but simultaneous neutralization of MGO and H$_2$O$_2$ in 10% honey reduced the killing of *B. subtilis* by 4-logs (Fig. 1C). At higher concentrations of honey, the bactericidal activity was not affected by neutralization of H$_2$O$_2$ and MGO (Fig. 1C), indicating that still more factors were involved.

As a first step to characterize the unknown bactericidal factors, we size-fractionated honey by ultrafiltration with a 5-kDa molecular weight cutoff membrane. Unfractionated honey produced a small zone of complete bacterial growth inhibition and a larger zone with partial growth inhibition in an agar diffusion assay with *B. subtilis* (Fig. 2A). After ultrafiltration, the factors that caused complete and partial bacterial growth inhibition were separated and were present in the >5-kDa retentate and the <5-kDa filtrate, respectively (Fig. 2A).

Ion exchange chromatography of the retentate indicated a cationic nature of the antibacterial factors. Indeed, the polyanionic compound SPS abolished the antibacterial activity of the retentate (Fig. 2B). Moreover, pepsin treatment also abolished this activity (Fig. 2B). Together, this implies that cationic antibacterial proteins were present.

We separated cationic proteins in the retentate using a native acid-urea PAGE gel, and allowed the separated components to diffuse from this gel into a *B. subtilis*-inoculated agar to identify antibacterial proteins. This yielded a single zone of bacterial growth inhibition that corresponded to a protein band in a Coomassie-stained gel run in parallel (Fig. 2C). This protein was purified from a larger amount of retentate using preparative acid-urea PAGE (Fig. 2D), and identified by peptide mass analysis as bee defensin-1.

To specifically assess the contribution of bee defensin-1 to the bactericidal activity of honey, an antibee defensin-1 antibody was raised (Fig. 2E). Like SPS, this antibody negated all bactericidal activity of the >5-kDa retentate against *B. subtilis* (Fig. 3A). The <5-kDa filtrate had only minor bactericidal activity (Fig. 3A), but this was not due to cationic compounds, since SPS failed to neutralize this activity (Fig. 3A). Thus, bee defensin-1 was the only cationic bactericidal compound present in RS honey.

Next, we assessed the contribution of bee defensin-1 to the bactericidal activity of nonfractionated honey.
Roles of bee defensin-1 and pH in bactericidal activity of honey against *B. subtilis*. A) Contribution to bactericidal activity of cationic components in general and of bee defensin-1 specifically was tested by neutralization with SPS or with antibee defensin-1 antibody (α-bd), respectively, at concentrations of retentate equivalent to 20% honey (open bars) and 40% honey (solid bars); ctrl. indicates survival without neutralization. B) To assess the contribution of bee defensin-1 to bactericidal activity of unfractonated honey, *B. subtilis* was incubated in various concentrations of honey in incubation buffer (squares), or with catalase and glyoxalase I added either without (triangles) or with SPS (diamonds), or in a honey-equivalent sugar solution (circles). C) To assess the contribution of the low pH to the bactericidal activity of honey, *B. subtilis* was incubated in various concentrations of honey in incubation buffer (squares), or with catalase, glyoxalase I, and SPS added either without (triangles) or with neutralization to pH 7 (diamonds), or in a honey-equivalent sugar solution (circles). After 24 h, numbers of surviving bacteria were determined. Data are mean ± SE log-transformed bacterial concentration (CFU/ml).

against *B. subtilis*. As previously observed, ≥20% honey retained bactericidal activity when H₂O₂ and MGO were neutralized. Additional neutralization of bee defensin-1 strongly reduced the bactericidal activity of 20% honey but did not affect the activity of 30 and 40% honey (Fig. 3B). So, bee defensin-1 contributed to the bactericidal activity of honey, but other bactericidal factors were involved.

Honey has a low pH, mainly because of the conversion of glucose into hydrogen peroxide and gluconic acid by glucose oxidase. This low pH might also contribute to the bactericidal activity of honey (23). Titration of the pH of 40–10% RS honey from 3.4–3.5 to 7.0, combined with neutralization of H₂O₂, MGO and bee defensin-1, reduced the bactericidal activity of honey to a level identical to that of a honey-equivalent sugar solution (Fig. 3C). Thus, with this experiment, we succeeded in identifying all bactericidal factors in RS honey responsible for killing of *B. subtilis*.

The contribution of the identified bactericidal factors to activity against antibiotic-susceptible and -resistant strains of various species was tested with honey diluted to 20%, since this killed the entire inocula of all bacteria tested independent of sugar (Fig. 1). Simultaneous neutralization of H₂O₂, MGO and bee defensin-1 negated all activity (Fig. 4), showing that these were the major factors responsible for broad spectrum bactericidal activity of honey.

We studied the contribution of the honey bactericidal factors in more detail by neutralizing the factors individually or combined. Neutralization of H₂O₂ alone strongly reduced the bactericidal activity against all bacteria tested except *B. subtilis* (Fig. 4). Neutralization of MGO alone strongly reduced killing of *E. coli* and *P. aeruginosa* strains (Fig. 4). Neutralization of bee defensin-1 alone reduced killing of VREF, but not of the other bacteria tested (Fig. 4). When compared to neutralization of MGO alone, the additional neutralization of bee defensin-1 reduced killing of all bacteria tested, except *E. coli* ESBL (Fig. 4). In summary, H₂O₂, MGO, and bee defensin-1 differentially contributed to the activity of honey against specific bacteria, and their combined presence was required for the broad-spectrum activity.

**DISCUSSION**

All bacterial species tested were susceptible to different combinations of bactericidal factors in honey, indicating that these bacteria were killed via distinct mechanisms. This clearly demonstrates the importance of the multifactorial nature of honey for its potent, broad-spectrum bactericidal activity.

Some factors had overlapping activity. For instance, the activity of bee defensin-1 against most bacteria was only revealed after neutralization of MGO. This clearly demonstrates the importance of neutralizing known bactericidal factors in honey to reveal the presence of additional factors. Similarly, the contribution of the low pH for activity of honey against *B. subtilis* was only revealed when H₂O₂ was reduced by neutralization of MGO. This was only revealed when H₂O₂, MGO, and bee defensin-1 were simultaneously neutralized.

In other situations, bactericidal activity depended on the combined presence of different factors. Thus, the activity of honey against *E. coli* and *P. aeruginosa* was markedly reduced by neutralization of either H₂O₂ or MGO. Alternatively, the activity of certain bactericidal factors likely is more potent in the context of honey than as pure substances. This is most clearly illustrated by the activity of MGO. When tested in a buffer, ≥0.3 mM MGO was required for activity against *B. subtilis* (Supplemental Fig. 1). In contrast, as little as 0.05 mM MGO, the concentration in 20% RS honey, was sufficient to substantially contribute to the bactericidal activity. This suggests that the presence of the other bactericidal factors in honey enhanced the effect of
MGO. It is not possible to quantify the contribution of the different factors to honey bactericidal activity since, as we have shown, these factors may have redundant activity, be mutually dependent, or have additive or synergistic activity depending on the bacterial species targeted.

We have demonstrated for the first time that honey contains an antimicrobial peptide, bee defensin-1, and that this peptide substantially contributes to the bactericidal activity. Bee defensin-1 was previously isolated from royal jelly (24), the major food source for bee queen larvae (and then referred to as “royalisin”), and was identified in honeybee hemolymph (18). Royal jelly is produced by young worker bees and contains their hypopharyngeal and mandibular gland secretions (25, 26). Bee defensin-1 mRNA has been identified in the hypopharyngeal gland of young worker bees (18), suggesting this gland is involved in production of bee defensin-1 found in royal jelly (24). When worker bees age, they become the major producers of honey. Major differences develop in morphology and protein expression of their hypopharyngeal glands (27, 28), e.g., several important carbohydrate-metabolizing enzymes, including glucose oxidase are expressed (29). The bees add the secretion from their hypopharyngeal glands to the collected nectar. The carbohydrate-metabolizing enzymes then convert sucrose to glucose and fructose, and glucose oxidase converts the glucose to hydrogen peroxide and gluconic acid. These latter compounds presumably are involved in prevention of microbial spoilage of unripe honey (11). Since we have found bee defensin-1 in honey, this suggests that after the transition in hypopharyngeal gland function of the worker bees with age, the gland still produces bee defensin-1. This peptide, therefore, likely contributes to protection of both royal jelly and honey against microbial spoilage.

It remains to be established whether bee defensin-1 is also present in other honeys. In Manuka honey, no evidence was found for the presence of antimicrobial peptides (30). For several other honeys, proteins were reported to contribute to the antibacterial activity (31, 32), but their identity remains unknown. Using our ant bee defensin-1 antibody, we aim to assess the role of bee defensin-1 for the antibacterial activity of other honeys.

Previous studies regarding the effect of low pH to antibacterial activity of honey have yielded conflicting results (11). In our study, the contribution of the low pH for activity against B. subtilis was only revealed on inactivation of all other bactericidal factors. So, in other studies, which did not employ an approach of neutralization of bactericidal factors in honey, the contribution of the low pH of honey may easily have been overlooked.

Much effort has been put into identification of phenolic antibacterial components in honey (11). Several of these compounds have been isolated from honey, but as they were tested at concentrations far exceeding those in honey, no conclusions can be drawn regarding their contribution to honey bactericidal activity (11). Our data do not show a role of phenolic compounds in RS honey bactericidal activity.

Our approach of selectively neutralizing individual bactericidal factors present in a medical-grade honey allowed us to unravel the multifactorial bactericidal activity of a honey for the first time. We presently use the same approach to assess the contribution of these factors to activity of other honeys, and simultaneously to screen for novel bactericidal factors. Such honeys, or isolated components thereof, may serve as novel agents to prevent or treat infections, in particular those caused by antibiotic-resistant bacteria.

The authors thank Jorn Blom and Sadira Thomas for their help with purification of bee defensin-1; Henk Dekker for expert nano ESI-ms/ms experiments; and Ton Bisseling, Ben Berkhout, Mark van Passel, and Brendan McMorran for critically reviewing the manuscript.

REFERENCES


Received for publication November 18, 2009.
Accepted for publication February 4, 2010.
Honey: An immunomodulator in wound healing

Juraj Majtan, PhD¹,²

¹. Institute of Zoology, Slovak Academy of Sciences, and
². Department of Microbiology, Faculty of Medicine, Slovak Medical University, Bratislava, Slovakia

Reprint requests:
Dr. J. Majtan, Institute of Zoology, Slovak Academy of Sciences, Dubravska cesta 9, 845 06 Bratislava, Slovakia.
Tel: +421 2 59302647;
Fax: +421 2 59302646;
Email: juraj.majtan@savba.sk

Manuscript received: April 5, 2013
Accepted in final form: August 28, 2013
DOI:10.1111/wrr.12117

ABSTRACT

Honey is a popular natural product that is used in the treatment of burns and a broad spectrum of injuries, in particular chronic wounds. The antibacterial potential of honey has been considered the exclusive criterion for its wound healing properties. The antibacterial activity of honey has recently been fully characterized in medical-grade honeys. Recently, the multifunctional immunomodulatory properties of honey have attracted much attention. The aim of this review is to provide closer insight into the potential immunomodulatory effects of honey in wound healing. Honey and its components are able to either stimulate or inhibit the release of certain cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-6) from human monocytes and macrophages, depending on wound condition. Similarly, honey seems to either reduce or activate the production of reactive oxygen species from neutrophils, also depending on the wound microenvironment. The honey-induced activation of both types of immune cells could promote debridement of a wound and speed up the repair process. Similarly, human keratinocytes, fibroblasts, and endothelial cell responses (e.g., cell migration and proliferation, collagen matrix production, chemotaxis) are positively affected in the presence of honey; thus, honey may accelerate reepithelization and wound closure. The immunomodulatory activity of honey is highly complex because of the involvement of multiple quantitatively variable compounds among honeys of different origins. The identification of these individual compounds and their contributions to wound healing is crucial for a better understanding of the mechanisms behind honey-mediated healing of chronic wounds.

Honey has been used as a traditional medicine for centuries by different cultures for the treatment of various disorders including burns and chronic wounds. Honey offers broad spectrum antimicrobial properties and promotes rapid wound healing.¹ The antibacterial potential of honey has been considered the exclusive criterion for its wound healing properties. Therefore, the antibacterial activity of honey from different floral sources has been intensively studied over the past few decades. Recently, defensin1, one of the major antibacterial factors in honey, was shown to be a regular but quantitatively variable component of each honey.² One reason for the varying contents of defensin1 in different honeys seems to be constitutive but variable defensin1 expression in individual honeybees in bee populations.³ It has also been found that some types of honey derived from specific floral sources become more potent than others because of the presence of phytochemicals with antibacterial properties.³–⁶ These potent natural honeys, such as manuka (Medihoney, Comvita NZ Ltd., Te Puke, New Zealand) and RS honey (Bfactory Health Products B.V., Rhenen, The Netherlands) (honey with unknown origin used as a source for Revamil), are currently being used as medical-grade honeys in clinical applications. Medical-grade honey is being incorporated into sterile devices that are applied topically to wounds. However, honeys may also contain bee- or plant-derived substance(s) with immunomodulatory effects that can positively affect the wound healing process. Therefore, the antibacterial potential of honey may not be the sole criterion for selecting medical-grade honeys.

It has been assumed that the antibacterial action of honey has its main impact on the healing process of chronic wounds. Honey eliminates pathogens from wounds and provides an appropriate moist environment for proper wound healing. As the direct antimicrobial effects of honey were fully characterized in vitro, research has also focused on identifying the substances responsible for its immunomodulatory effects.⁷–⁸

---

COX-2
Cyclooxygenase-2

IL
Interleukin

LPS
Lipopolysaccharide

MM6
Mono Mac 6

MMP-9
Matrix metalloproteinase 9

MRJP1
Major royal jelly protein 1

mRNA
Messenger ribonucleic acid

MW
Molecular weight

NO
Nitric oxide

ROS
Reactive oxygen species

TNF-α
Tumor necrosis factor-α
Some promising candidates with immunomodulatory properties have been identified in honey (Table 1), but further research is necessary to prove these immunomodulatory properties.

The aim of this work is to review the immunomodulatory effects of natural honey on immune and cutaneous cells that participate in the wound healing process and to elucidate the different mechanisms of honey-induced immunomodulation.

## HONEY AND CYTOKINE PRODUCTION

Besides providing a structural barrier, the skin contains several types of immune cells that can be activated by skin damage. One of the most important groups of immune cells involved in wound healing are macrophages, which exhibit different immunological functions in the skin, including phagocytosis and antigen presentation. Tissue macrophages are cells derived from peripheral blood monocytes. In injured tissue, monocytes migrate through the vessel wall; they release enzymes that fragment extracellular matrix proteins, creating space for monocytes to migrate to the wound bed. Macrophages can be activated either classically (by lipopolysaccharide [LPS] and interferon-γ) or alternatively (by interleukin [IL]-4 and IL-13). LPS-stimulated macrophages are capable of synthesizing and secreting inflammatory mediators, including tumor necrosis factor-α (TNF-α), nitric oxide (NO), and IL-6. IL-4-activated macrophages play important roles in wound healing and angiogenesis.

In addition to the above-mentioned properties, macrophages produce many other cytokines and growth factors that stimulate new capillary growth, collagen synthesis, and fibrosis.

In recent years, several groups have examined honey and/or its individual components in order to elucidate its wound healing properties. Macrophages/monocytes are a suitable model for monitoring the immunomodulatory activity of novel potential immunomodulators. Tions and coworkers suggested that the wound healing effect of honey may be partly related to the release of proinflammatory cytokines from surrounding cells, mainly monocytes and macrophages.

An immunomodulatory effect was showed by cytokine release from the monocytic cell line Mono Mac 6 (MM6) and human peripheral monocytes after incubation with 1% (w/v) honey. Several natural honeys were used in this study, including manuka and jelly bush honey. All types of honey induced or stimulated the release of TNF-α, IL-1β, and IL-6 from MM6 cells and peripheral blood monocytes when compared with the syrup control (artificial honey) and untreated cells. The MM6 cells treated with jelly bush honey showed a significantly higher above-mentioned cytokines release than cells treated with manuka or the other natural honeys. The authors of the study also claimed that the concentration of endotoxins in all natural honeys (from 56 to 690 pg/mL) is negligible, and that stimulation of MM6 cells is independent of endotoxins. However, it is important to note that MM6 cells are very sensitive to endotoxins, and it is very likely that the endotoxin content of honey could be responsible for its stimulatory effect. Endotoxins possess special characteristics. They are, to a large extent, heat stable, and their activity can be abrogated by the antibiotic polymyxin B. It has been shown that MM6 cells responded to an endotoxin with a detect limit as low as 3.1 pg/mL, and that robust release of IL-6 occurred when they were stimulated with 100 pg/mL endotoxin.

In a recent study, Timm et al. (2008) investigated the effect of four different honeys including manuka honey on the release of important proinflammatory cytokine (IL-6) from MM6 cells. Similar to previous studies, natural honeys induced maximal release of IL-6 after 18 hours of treatment. They reported that the substances in honey responsible for its immunomodulatory activity are (1) heat stable; (2) retained in their activity can be abrogated by the antibiotic polymyxin B, an inhibitor of endotoxin activity. All of these characteristics are in concordance with the properties of endotoxins. In contrast to these findings, Tons et al. demonstrated that heat treatment caused a significant reduction in the ability of honey to stimulate cytokine production in MM6 cells. Moreover, the cytokine-stimulatory effect of honey was assessed in the presence of polymyxin B. Similarly, the ability of New Zealand honeys to release TNF-α from the monocytic cell lines THP-1 and U937 has recently been characterized. The immunomodulatory activity of all the honeys was associated with a high MW (>30 kDa) component that was partially heat labile and inhibitable with polymyxin B.

A number of peptides and proteins from natural sources are known for their nonspecific immunostimulatory responses. Peptide and protein immunomodulators, in general, generate a physiological response in target cells via their specific receptors. Glycosylated proteins are known to induce TNF-α secretion from macrophages, and this cytokine is known to induce wound repair mechanisms. We have previously shown that a natural acacia honey is able to stimulate TNF-α secretion from murine macrophages, whereas deproteinized honey

<table>
<thead>
<tr>
<th>Specific factor(s)</th>
<th>Honey</th>
<th>Immunomodulatory activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinogalactans</td>
<td>Kanuka honey</td>
<td>Monocytes activation</td>
<td>Gannabathula et al.18</td>
</tr>
<tr>
<td>261 MW component</td>
<td>Jungle honey</td>
<td>Neutrophils activation</td>
<td>Fukuda et al.7</td>
</tr>
<tr>
<td>5.8 kDa component</td>
<td>Manuka honey</td>
<td>Monocytes activation</td>
<td>Tonks et al.17</td>
</tr>
<tr>
<td>MRJP1</td>
<td>Acacia honey</td>
<td>Macrophages activation</td>
<td>Majtan et al.20</td>
</tr>
<tr>
<td>MRJP1</td>
<td>Acacia honey</td>
<td>Keratinocytes activation</td>
<td>Majtan et al.8</td>
</tr>
<tr>
<td>Apigenin, Kaempferol</td>
<td>Honeydew honey</td>
<td>MMP-9 inhibition</td>
<td>Majtan et al.65</td>
</tr>
</tbody>
</table>

MMP-9, matrix metalloproteinase 9; MRJP1, major royal jelly protein 1; MW, molecular weight.
has no effect on the release of TNF-α. This suggests that the protein content of honey, primarily the 55 kDa glycoprotein major royal jelly protein 1 (MRJP1), which is the dominant protein in royal jelly as well as in honey, might be responsible for the immunomodulatory effects of honey. Our previous results also showed that the production of TNF-α from murine macrophages is actually increased after limited proteolytic digestion. We found that the N-terminal region of recombinant MRJP1 elicited marked release of TNF-α. The endotoxin content of acacia honey or of native and recombinant MRJP1 samples was not determined in our study. It is very likely that samples of purified MRJP1 contain endotoxins at a sufficient level to stimulate the release of TNF-α from murine macrophages. Therefore, we can assume that endotoxins in honey may play an important role in the activation of monocytes and/or macrophages depending on the individual honey. On the other hand, it has been reported that MRJP1, at concentration of 25 μg/mL, increased the level of TNF-α messenger ribonucleic acid (mRNA) expression twofold in primary cultures of epidermal keratinocytes. Similarly, an upward trend in mRNA expression of IL-1β and transforming growth factor-β was observed following treatment with MRJP1 in human keratinocytes.

Another promising immunostimulatory protein identified in honey belongs to the group of type II arabinogalactan proteins, with an MW of about 110 kDa. Type II arabinogalactan proteins from a range of sources have been shown to have immunomodulatory properties. They are able to stimulate the release of TNF-α from monocyte cell lines THP-1 and U937. Although honey is a natural product and rich in various phytochemicals and bee-derived compounds that may possess immunomodulatory activities, some researchers have postulated that the immunomodulatory effects of honey could be because of its endotoxin content. Sterilization of honey using gamma irradiation effectively eliminates bacterial spores and vegetative forms of any bacteria present; however, bacterial endotoxins may still remain present. Bacterial endotoxins (LPSs), major components of the outer membrane of Gram-negative bacteria, are complex glycolipids composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A. Endotoxins activate macrophages to produce proinflammatory cytokines. The production of these cytokines is tightly regulated as excessive production leads to amplified inflammatory responses and devastating illness characteristic of severe septic shock.

Therefore, one way to interrupt chronic inflammatory cycle is to remove ROS with antioxidants, and honey is known to contain antioxidants that scavenge free radicals. Various components of honey contribute to its antioxidant properties, including flavonoids, phenolic acids, catalase, peroxidise, ascorbic acid, and carotenoids, and products of the Maillard reaction. The quantity of these components varies according to the floral and geographical origin of each type of honey. Several studies have shown that phenolic compounds in honey are partially responsible for its antibacterial and antioxidant activities. It has been shown that ROSs mediate TNF-α-induced cytotoxicity, which can be blocked by specific free radical scavengers (e.g., flavonoids). In fact, Habtermariam demonstrated that phenolic compounds, such as caffeic acid, effectively inhibit TNF-α-induced cytotoxicity in L929 cells. In a very recent study, a honey methanol extract and a honey ethyl acetate extract were tested in vitro for their effect on NO production in the endotoxin- and IFN-γ-stimulated murine macrophage cell line RAW264.7. It was shown that both honey extracts were capable of inhibiting NO production in the macrophages. The concentration of NO was inhibited in a dose-dependent manner in the presence of the honey extracts. The honey ethyl acetate extract exhibited greater activity than the honey methanol extract. However, the methanol extract contained a higher concentration of phenolic compounds, where the majority of the phenolics were ellagic, gallic, and ferulic acids, myricetin, chlorogenic acid, and caffeic acid. Similarly, Woo et al. found that chrysin, a natural flavonoid found in many plant extracts, honey, and propolis inhibited cyclooxygenase-2 (COX-2) gene expression in LPS-stimulated cultured macrophages, and this effect was mediated through inhibition of the binding activity of nuclear factor IL-6. The fact that nuclear factor IL-6 is negatively regulated by chrysin is important because this transcription factor plays a critical role in the regulation of a variety of genes involved in inflammatory responses.

Another study, by Ahmad et al., supports the hypothesis that honey exhibits its anti-inflammatory activity through inhibition of activated macrophages. They found that honey treatment of rodent macrophages activated by bovine thrombin resulted in effective suppression of oxidative respiratory bursts. Interestingly, all honey samples from different origins showed effective suppression. Taken together, these findings are contradictory, and it is difficult to distinguish which molecule(s) in honey is fully responsible for its immunomodulatory effect. It is important to carry out further detailed research in order to explain the immunomodulatory effect of honey on macrophages.

Persistent neutrophil infiltration and release of ROS by neutrophils contribute to the pathophysiology of chronic wounds. A decrease in neutrophil superoxide production by honeys has recently been reported. An antioxidant activity of honeys was attributed to inhibition of ROS formation, either by inhibiting the respiratory burst of neutrophils or by direct ROS scavenging. Interestingly, a dose-dependent reduction in human neutrophils’ superoxide production by honeys did not correlate with the levels of known honey-based phenolic compounds, which are well-known free radical scavengers. This observation indicates that the antioxidant activity of honey is likely caused by inhibition of neutrophils’ respiratory burst.
Honey: an immunomodulator

Figure 1. The immunomodulatory action of honey on immune and cutaneous cells involved in wound healing. Honey is able to either stimulate or inhibit the release of certain factors (cytokines, MMP-9, ROS) from immune and cutaneous cells depending on wound condition. Honey induces secretion of proinflammatory cytokines and MMP-9 during the inflammatory and proliferative wound healing phase, respectively. On the other hand, when the wound inflammation is uncontrolled, honey abrogates prolonged wound inflammation and reduces the elevated levels of proinflammatory cytokines, ROS, and MMP-9. IL, interleukin; MMP-9, matrix metalloproteinase-9; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α.

In a very recent study, a compound with an MW of 261 Da isolated from jungle honey was found to elicit chemotactic activity in neutrophils. The authors of this study also investigated the mechanism of the antitumor activity of jungle honey, which seemed to be related to the production of ROS by activated neutrophils. The jungle honey was injected into tumor tissues in mice, and many neutrophils infiltrated necrotic areas in the tumor and produced ROS. The incidence and mean weight of the tumors decreased in jungle honey-injected mice.

Taking these results together, honey seems to either reduce or activate the production of ROS from neutrophils, depending upon the microenvironment (Figure 1).

ANTI-INFLAMMATORY ACTIONS OF HONEY

Reduced inflammation observed in the clinic following the application of honey is supported by histological evidence of reduced numbers of inflammatory cells present in wound tissue. Inflammation is a nonspecific response of mammalian tissue to a variety of hostile agents. There are many mediators of inflammation, such as endotoxins, some cytokines, and NO. Therefore, the inhibition of inflammatory mediators is one of the important steps in controlling inflammation.

Honey exhibits potent multiple anti-inflammatory effects. Clinically, there have been numerous observations reported of honey reducing edema and exudate, minimizing scarring and having a soothing effect when applied to inflamed wounds and burns (reviewed in Molan). The anti-inflammatory effect of honey may be explained by several mechanisms of action: (1) inhibition of the classical complement pathway; (2) inhibition of ROS formation; (3) inhibition of leukocyte infiltration; and (4) inhibition of COX-2 and inducible NO synthase expression. Finally, the inhibition of matrix metalloproteinase 9 (MMP-9), a major protease responsible for the degradation of matrix and cell growth-promoting agents in chronic wound fluids, in human keratinocytes has been reported very recently and represents another novel anti-inflammatory mechanism of honey action.

In a very recent study, we found that acacia honey at a concentration of 1% (w/v) significantly enhanced the expression of MMP-9 mRNA in primary cultures of human keratinocytes. Furthermore, incubation of human skin fragments with honey for 24 hours was associated with increased expression of MMP-9 protein in the epidermis near the basement membrane. Subsequently, we also found a decrease in the relative amount of collagen type IV in the basement membrane and around the blood vessels following incubation of the skin with honey for 24 hours. These results appear contradictory to the results presented in our very recent study where honey inhibited TNF-α-induced MMP-9 expression. Therefore, we assume that honey can act as an immunomodulator with both proinflammatory and anti-inflammatory properties (Figure 1). We speculate that honey stimulates the production of inflammatory cytokines and MMP-9 from keratinocytes when a low level of an inflammatory/stimulatory mediator is present. On the other hand, if an environment is infected and inflammation is in progress, honey suppresses the production of inflammatory cytokines and MMP-9. This hypothesis is very promising and could result in new therapeutic advantages for the treatment of skin inflammation in the future.

To date, the components including phenolic compounds and flavonoids responsible for the anti-inflammatory honey in vitro activities have been partially identified. However, it is not clear whether these components within honey exhibit anti-inflammatory activities in vivo.

CONCLUSION

Honey, at medical-grade level, is a high-quality wound care product, as supported by the sheer number of papers in the recent scientific literature. It has been found to be particularly effective where standard wound care is limited or unsuccessful. However, some wound-care professionals are still skeptical about the benefits of honey in wound care. As the antibacterial action of honey is well characterized, there is a need to fully elucidate the compounds/mechanisms responsible for honey’s immunomodulatory and anti-inflammatory properties in order to support a positive clinical outcome of using honey in wound management.

ACKNOWLEDGMENTS

Source of Funding: This work was supported by the Slovak Research and Development Agency under contract no. APVV-0115-11.

Conflict of Interest: Authors have no conflict of interest to disclose.

REFERENCES

Honey: an immunomodulator


Efficacy of honey to promote oral wellness

Dr Neelam Gupta,
Reader,
Deptt. of Prosthodontics,
PDM Dental College & Research Institute,
Bahadurgarh-124507,
Haryana,
India

Dr. M. Gulati
Lecturer,
Deptt. of Prosthodontics,
PDM Dental College & Research Institute,
Bahadurgarh-124507,
Haryana,
India

Dr. N. Kathuria
Lecturer,
Deptt. of Prosthodontics,
PDM Dental College & Research Institute,
Bahadurgarh-124507,
Haryana,
India

Keywords: Honey, plant origin, osmotic effect, anti bacterial.

Abstract

Honey activity that rapidly clears infection from wounds when applied topically, which may make it suitable for treatment of periodontal disease as well as for clearing infection in oral ulcers and wounds following surgery. The anti-inflammatory activity of honey along with its antioxidant content has been used as a medicine throughout the ages, and in more recent times, it has been rediscovered that it is effective in treatment of burns, infected wounds and skin ulcers. It possesses potent antibacterial may be beneficial in preventing erosion of periodontal tissues that occur as collateral damage from free radicals released as an inflammatory response to infection.

Introduction

In ancient times, honey was considered the food of gods and the symbol of wealth and happiness. It is a sweet syrupy substance produced by honeybee from the nectar of flowers and used by humans as a sweetener. Although very well known as food, it is one of the oldest medicines and has continued to be used as such throughout the ages [1]. Honey was used to treat the infected wounds as long ago as 2000 years before the bacteria were discovered to be the cause of infection. In 50 AD, Dioscorides described honey as being “good for all rotten and hollow ulcers” [2]. More recently, honey has been reported to have an
inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, gram-positives and gram-negative microorganisms [3]. An anti-fungal action has also been observed for some yeasts and species of aspergillus and pencillium, as well as all the common dermatophytes [4]. Large volume of literature appearing on the effectiveness of honey in treating the infected wounds and skin ulcers describes the features that indicate that honey has potential for the therapy of periodontal disease, oral ulcers, and other problems of oral health. One of the most important features that may be particularly beneficial in promotion of oral wellness is its antibacterial activity. The purpose of this article is to review the effectiveness of honey in promoting oral wellness.

**Composition[5] :**

Major portion of honey is comprised of carbohydrates. It contains many enzymes and free amino acids, of which most abundant is proline. Traces of vitamin B, minerals, and antioxidants like flavonoids and vitamin C are also present.

**Antibacterial potential [3,6] :**

The antibacterial property of honey was first recognized in 1892 by van Ketel [7]. Important factors influencing the antibacterial effectiveness of honey are as follow:

**Osmotic effect:**

Honey because of its high osmotic properties can extract water from bacterial cells thus leading to cell death. It has an osmolarity sufficient to inhibit microbial growth[8].

**Acidic pH:**

Honey is quite acidic, its pH being between 3.2 and 4.5, which is low enough to be inhibitory to many animal pathogens. The optimum pH for growth of these species normally falls between 7.2 and 7.4[9,10].

**Hydrogen peroxide:**

Majority of anti-bacterial potential in honey is found to be due to hydrogen peroxide produced enzymatically in honey[9].

For processing of the nectar during ripening into honey, bees add some enzymes. One of them is glucose oxidase, which results in formation of gluconic acid and hydrogen peroxide from the glucose.

This enzyme is inactive in ripened honey but is reactivated when honey is diluted. On dilution of honey the activity of enzymes increases, giving rise to an antiseptic action at a level which is anti-bacterial but not tissue damaging.

**Clinical significance in dentistry:**

The therapeutic features of honey seen in its usage in wound care elsewhere on the body indicate that it has the potential to be useful for prevention or treatment of infected wounds following tooth extraction or surgery, and for treating other oral infections. Elbagoury and Fayed in 1985 conducted a study describing a small clinical trial of placing honey in sockets before closure of wound, after surgical removal of impacted third molar and concluded that there was less pain, less incidence of postoperative complications and less swelling in honey treated group than in the untreated control group. The effectiveness of honey in such an application is likely to be limited as it has a tendency to dissolve quickly in saliva and thus does not have a longer therapeutic effect [11]. A novel wound dressing material consisting of gelled honey that has been developed (PCT patent application by the University of Waikato, December 2000) may be useful for this, and for the treatment of oral ulcers, as it adheres to the oral mucosa and is slow to dissolve in saliva. Clinical trials of this are yet to be run, but reports of its use indicate that it is likely to prove to be successful: additional to any antimicrobial effect, the honey gave a rapid soothing effect from pain as has been observed with wounds and burns elsewhere on the body. A similar rapid alleviation of pain was observed when the gelled honey was used in a case of erosion of the gum and jaw bone due to infection following surgery with bone grafting to repair damage to the jaw from traumatic injury that had been non-responsive to any
conventional treatment for more than six months. In this case, the gelled honey was moulded into the infected area and held in place by wearing a mouth guard over it. The infection cleared and the wound healed up within a month after starting the application of the gelled honey. Topical application in the form of spray (from bee products), can be also be used for mouth and upper respiratory tract disease treatment and prevention. The optimal technology of spray is prepared, and concentration of ethanol as extragent 70% and 15% of honey is determined; and the preparation is called propomel [12].

Honey, having an anti-inflammatory activity raises the possibility of it being useful as a therapeutic agent for periodontitis; the anti-inflammatory activity would block the direct cause of the erosion of the connective tissues and bone. In a study, eight honeys and three types of propolis were tested and the result proved them effective as an anticalculus agent in toothpastes and mouthwashes [13]. Ozan et al conducted a study in which a mouthrinse containing propolis was prepared at four different concentrations as 10%, 5%, 2.5%, and 1%. Besides, CHX was used as control group. The antibacterial effects of five solutions on oral microorganisms were tested and their cytotoxic effects on human gingival fibroblasts were evaluated by agar diffusion test. At these concentrations, effectiveness of mouthrinse containing propolis samples on oral microorganisms was not found as effective as chlorhexidine. On the contrary, samples found less cytotoxic effects on human gingival fibroblasts than chlorhexidine; the administration of propolis at appropriate concentrations might be effective on oral microorganisms and non-cytotoxic to gingival fibroblasts [14].

The anti-inflammatory action and stimulating effect on tissue repair of honey could possibly be of benefit for the relief of oral conditions resulting from radiotherapy and chemotherapy of cancer. Chiba et al in 1985 has also published report on using honey to ease the pain of stomatitis during radiotherapy [15].

Candy made with honey may also be useful for prevention of halitosis, as according to Molan honey has been observed to give rapid removal of malodour from infected wounds [16]. It would not be just the antibacterial action of honey involved, but Nychas, Dillon, and Board concluded that as bacteria would use the glucose in honey in preference to amino acids, and thus would produce lactic acid instead of bad-smelling amines and sulphur compounds [17].

There has been much debate in the past about whether honey is harmful to the teeth or not. More recently, research has been carried out to find the minimum inhibitory concentration of honey for species of bacteria believed to cause caries, using honeys with standardised antibacterial activity. It was found that the minimum inhibitory concentrations of honey for Strep. mitis, Strep. sobrinus and Lactobacillus casei were 7%, 7.5–8.5% and 8–12% respectively. The production of acid by these bacteria was also inhibited. It can be concluded therefore that although honey may be cariogenic because of its high content of fermentable sugars, with selected honeys that have higher levels of antibacterial activity, they have the potential to inhibit the growth of cariogenic bacteria. But the degree to which this occurs in the oral cavity would have to be determined by feeding experiments [18].

**Conclusion:**

The therapeutic properties of honey is evident in its usage in wound care which clearly give it potential for therapeutic use in field of dentistry, but there is need for the trials to be carried out before its usefulness is known. Risk of caries is reduced by selecting honey having a high level of antibacterial activity, but literature is sparse as far as the field of dentistry is concerned. Studies are on to measure the antimicrobial efficacy of honey in oral cavity, but trials need to be carried out to determine to what extent this is true.

**References**


Journal of Innovative Dentistry, Vol 1, Issue2, May-August 2011
A Comparative Evaluation of the Antibacterial Efficacy of Honey In Vitro and Antiplaque Efficacy in a 4-Day Plaque Regrowth Model In Vivo: Preliminary Results

S. Aparna,* S. Srirangarajan,* Veena Malgi,* Krishnanand P. Setlur,† R. Shashidhar,‡ Swati Setty,§ and Srinath Thakur§

Background: Honey has a potent broad-spectrum antibacterial action that may make it suitable for “anti-infective” treatment of periodontal disease. The aims of this study are as follows: 1) to evaluate the antibacterial efficacy of honey against oral bacteria and compare the same with 0.2% chlorhexidine; and 2) to compare antiplaque efficacy in vivo with chlorhexidine.

Methods: The study was conducted in two parts. In the in vitro part, the inhibitory effects of three test agents, 0.2% chlorhexidine gluconate, honey mouthwash, and saline, against six oral bacteria at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL were tested in duplicate. The minimum inhibitory concentration (MIC) was set as the lowest concentration of the agent that completely inhibited the growth of the test species. The in vivo part consisted of a double-masked parallel clinical trial based on a 4-day plaque regrowth model. Sixty-six volunteers, 20 to 24 years of age, participated in the study, and the plaque scores were compared at baseline and at the end of 4 days. The Kruskal-Wallis test was used for significance, and the Mann-Whitney U test was used for pairwise comparison of the groups. The mean plaque scores were 1.77 ± 0.86, 1.64 ± 0.90, and 3.27 ± 0.83 for groups 1, 2, and 3, respectively.

Results: The honey mouthrinse effectively inhibited the six tested microorganisms. The chlorhexidine gluconate rinse had the lowest MICs compared with honey and saline rinses for all test species examined. The in vivo results revealed that plaque formation was inhibited/reduced by chlorhexidine and honey rinses.

Conclusion: Honey has antibacterial action against tested oral microorganisms and also has antiplaque action. J Periodontol 2012;83:1116-1121.

KEY WORDS
Antibacterial agents; chlorhexidine; dental plaque; honey; mouthwashes, minimum inhibitory concentrations.

Periodontitis is an inflammatory disease, associated with pathogenic microorganisms colonizing tooth surfaces in a susceptible host. Understanding that specific oral microorganisms are the cause of periodontitis, antimicrobials have been used to treat periodontitis in the past and at present. Several reviews1-4 have concluded that antimicrobial agents as adjuncts to mechanical therapy improve therapeutic outcome. However, the emergence of antibiotic-resistant microorganisms is compelling researchers to look for alternative means to destroy these microorganisms. Although some success has been reported with antibiotic therapy, several limitations have become evident. Most of these limitations are attributable to the fact that periodontal infections result from the formation of a biofilm.5

Mouthrinses have been used for centuries for medicinal and cosmetic purposes and have gained popularity worldwide. The advent of mouthrinses containing chlorhexidine was a major breakthrough in the search for a chemical means to prevent periodontal disease. Since then and especially in the past 10 years, the number of formulations that claim to have antiplaque, anticalcium,
and anticaries activity has increased, and much emphasis has been placed on such substances as adjuncts to, or indeed to replace, conventional toothbrushing. Chlorhexidine is unequivocal in its effects on reduction of plaque and gingivitis, but major drawbacks lie in the taste and stain-producing problems.6

Complementary and alternative medicine (CAM) encompasses a diverse group of medical treatments, such as acupuncture, aromatherapy, massage therapy, meditation, hydrotherapy, and herbal therapy. The increasing interest in CAM by the public has encouraged dental professionals to investigate the existing science of CAM. An alternative medicine branch called apitherapy offers treatments for many diseases based on honey and other bee products.7-9

The first written reference to honey, a Sumerian tablet writing dating back to 2100 to 2000 B.C., mentions the use of honey as a drug and an ointment. In most ancient cultures, honey has been used for both nutritional and medical purposes.10 Honeys vary in taste and colors, depending on their botanical origin.11

Honey has been shown to have potential for the treatment of periodontal disease, mouth ulcers, and other problems of oral health.12 A trial has demonstrated a statistically significant difference between chewing gelled honey and plain chewing gum in decreasing the number of bleeding sites in patients with gingivitis.13 The remarkable antibacterial properties, easy availability, and economic feasibility make honey a prospective therapeutic agent. To the best of the authors’ knowledge, there have been no studies thus far exploring the antibacterial efficacy of honey against putative periodontopathogens or antiplaque action, making this the first study of its kind aimed at evaluating the potential action of honey in vitro on oral bacteria, including key periodontopathic bacteria, and comparing the antiplaque effect of a honey mouthrinse versus a commercially available chlorhexidine mouthrinse in a 4-day plaque regrowth model in vivo.

**MATERIALS AND METHODS**

**In Vitro Testing for Antibacterial Efficacy**

In vitro testing was conducted to determine the efficacy of honey mouthrinse compared with 0.2% chlorhexidine and a negative control of saline as determined by the minimum inhibitory concentration (MIC) against reference strains of six predominant oral bacterial species: 1) *Eubacterium nodatum*, 2) *Streptococcus mutans*, 3) *Campylobacter rectus*, 4) *Streptococcus sanguinis*, 5) *Aggregatibacter actinomycetemcomitans*, and 6) *Porphyromonas gingivalis*.

All MICs were performed in duplicate, and an agar dilution method was used to assess the inhibitory effect of the three test agents. Serial dilutions of each agent to provide final concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL were prepared, inoculated, and grown in blood agar plates under an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 35°C for 3 days. They were then transferred aseptically and suspended in 1 mL sterile mycoplasma broth. One hundred microliters of each strain suspension were placed in the wells of a sterile microtiter plate and incubated under an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 35°C and evaluated daily. The MIC was interpreted as the lowest concentration of the agent that completely inhibited the growth of the test species.

**In Vivo Testing for Antiplaque Efficacy**

The study was conducted in the Department of Periodontics, Bangalore Institute of Dental Sciences, Bangalore, India. Sixty-six of 70 screened individuals (40 males and 26 females, aged 20 to 24 years) were recruited for the study in October 2010. Written informed consent was obtained from the participants enrolled for the study, and approval was obtained from the Ethical Committee of the Institute. Participants had: 1) ≥22 natural teeth, 2) no removable or fixed prostheses or fixed and removable orthodontic appliances, and 3) no more than one full-coverage restoration. Participants were in good systemic health with no medical or pharmacotherapy histories that might influence the conduct of the study. Participants were randomly allocated to three groups and masked to the mouthrinse received. Rinsing preparations were filled in identical but coded bottles. Instruction for usage was written on the bottles. On day 1, the study participants received an oral soft tissue examination and then rendered plaque, calculus, and stain free by a thorough ultrasonic scaling and polishing of the teeth. Volunteers were then asked to refrain from all forms of tooth cleaning and to commence the rinsing regimen (i.e., 10 mL of respective mouthrinse twice daily for 30 seconds). The three test agents included the following: 1) 0.2% chlorhexidine mouthrinse (group 1); 2) processed honey (group 2); and 3) saline (group 3).

Processed honey was diluted with distilled water to a concentration of 1:1 (1.8 mg/mL honey) and dispensed as mouthrinse by a masked dispenser (SSr). The Turesky Gilmore Glickman modification of the Quigley Hein plaque index (PI)14 was used to verify the plaque status. All clinical examinations were performed by a single clinician (SA) who was masked to the study. At baseline, all participants had a score of 0, as confirmed by the use of a disclosing agent. On day 5, plaque scores were evaluated using disclosing solution, and results were tabulated.
**Statistical Analyses**
The statistical analysis for assessment of plaque was performed using the Kruskal-Wallis test for overall significance, and pairwise comparison was performed using the Mann-Whitney U test.

**RESULTS**
Table 1 summarizes the MICs with the three test agents. MIC was determined as the lowest concentration of honey that inhibited the growth of the tested microorganisms. The honey mouthrinse effectively inhibited the growth of the tested microorganisms. Although 0.2% chlorhexidine was the most effective and saline showed no inhibition, the honey mouthrinse proved to be efficacious.

**In Vivo Results**
Table 2 depicts the results of the PI taken on day 5 of the study. Participants were divided into three groups of 22 each (group 1 = chlorhexidine; group 2 = honey; group 3 = saline). No adverse effects were reported by the participants in any of the groups.

In vivo comparison of antiplaque efficacy revealed chlorhexidine to be the most efficacious, followed by honey mouthrinse. Saline showed the least antiplaque action. Intergroup analysis between chlorhexidine and honey showed that both agents significantly reduced plaque formation. The results were statistically significant at a P value of <0.001. The mean plaque scores were 1.77 ± 0.86, 1.64 ± 0.90, and 3.27 ± 0.83 for groups 1, 2, and 3, respectively. Comparison between chlorhexidine and honey was not statistically significant (P=0.670); comparison between groups 1 and 3 and between groups 2 and 3 were statistically significant (P<0.001) (Table 2).

**DISCUSSION**
The multifloral processed honey used in this study is from *Apis mellifera* honey bees species with a pH of 3.84, 29.17 meq/kg acidity, 17.07 moisture content, 0.21 mS/cm electrical conductivity, 8.01% sucrose, 27.29 hydroxymethylfurfural, and diastase number of 14.20.

Honey has been found to be beneficial in treating gingivitis13 and oral mucositis in cancer patients undergoing radiation therapy.15 There is sufficient evidence to prove its effectiveness against various aerobic bacteria16-19; however, no literature on the use of honey as an antibacterial agent against key periodontopathogens *A. actinomycetemcomitans* and *P. gingivalis* is available to date. Thus, we aimed to evaluate the efficacy of honey against six oral bacteria, including *A. actinomycetemcomitans* and *P. gingivalis*. Subsequently, the MIC was determined for the selected oral bacteria with processed honey, chlorhexidine, and saline as the negative control. A comparative clinical trial was later conducted to evaluate the antiplaque action of the three test agents.

The in vitro results revealed that the tested organisms were susceptible to chlorhexidine and honey. Chlorhexidine gluconate exhibited the lowest MIC compared with honey or saline. Honey was also efficacious against the tested bacteria and particularly against *A. actinomycetemcomitans* and *P. gingivalis*, which are the putative pathogens implicated in periodontitis. The in vivo results revealed that plaque inhibition was the highest with chlorhexidine, followed by the honey rinse. Saline had minimal to no effect on plaque inhibition. Although there was no statistically significant difference between chlorhexidine and honey, it would be presumptuous at this point to say that honey is as efficacious as chlorhexidine in its antiplaque action.

The 4-day experimental period length was chosen because plaque accumulation reaches measurable volumes after 4 to 5 days of no oral hygiene.20 As with other short-term plaque studies21,22 involving

### Table 1.

**MIC of Test Agents Against Oral Microorganisms**

<table>
<thead>
<tr>
<th>Microorganisms Tested</th>
<th>ATCC Reference No.</th>
<th>0.2% Chlorhexidine</th>
<th>Honey</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. nodatum</em></td>
<td>33099</td>
<td>1</td>
<td>1:2</td>
<td>—</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>25175</td>
<td>1:2</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>29523</td>
<td>4</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>33277</td>
<td>1:16</td>
<td>1:32</td>
<td>—</td>
</tr>
<tr>
<td><em>C. rectus</em></td>
<td>33238</td>
<td>1:2</td>
<td>1:16</td>
<td>—</td>
</tr>
<tr>
<td><em>S. sanguinis</em></td>
<td>10556</td>
<td>1:16</td>
<td>1:512</td>
<td>—</td>
</tr>
</tbody>
</table>

ATCC = American Type Culture Collection.
suspension of normal oral hygiene, the 4-day regrowth model provides important information in chemical plaque control methods. This model also enables the removal of the confounding variables, such as the Hawthorne effect, influence of pre-study prophylaxis, and the possible interaction between mouthrinse and toothpaste.23,24

Since the original findings for plaque-inhibitory properties of chlorhexidine, this antiseptic has been used as positive control by which other potential plaque-inhibitory agents or formulations are compared.25 From a professional medical perspective, honey used in wound care has proven antibacterial activity against many important pathogens, such as *Pseudomonas aeruginosa*,26 *Staphylococcus aureus*,27 and herpes simplex types 1 and 2.28 A laboratory demonstration of its antibacterial activity was first performed by Dold et al.29 Adock30 first suggested the possibility that hydrogen peroxide was responsible for the antibacterial activity of honey. When honey is diluted with water, reducing its high sugar content, it still inhibits the growth of many different bacterial species that cause wound infections.33-37 Thus, we can hypothesize that antibacterial and antiplaque actions of honey may possibly be attributable to the above-mentioned mechanisms.

Another mechanism may be related to the pH level of honey being low, 3.4 to 5.5; bacterial colonization or infection and recalcitrant wound healing situations are often accompanied by pH values > 7.3 in wound exudates.38 It has been demonstrated that acidification of wounds speeds healing and is attributed to the low pH, increasing the amount of oxygen off-loaded from hemoglobin in the capillaries.39 Honey also causes suppression of protease activity in wounds by getting away from the neutral pH that is optimum for the growth of microorganisms.40 Cariogenicity and demineralization of tooth surface would be of concern with honey having a high content of fermentable sugars and an acidic pH. However, honey has been found to inhibit growth as well as acid production by cariogenic bacteria.41 The present study also found that honey inhibits *S. mutans* growth, which is a cariogenic microorganism. The effect of

### Table 2.

**Comparison of Antiplaque Efficacy in Three Groups**

<table>
<thead>
<tr>
<th>PI</th>
<th>Group 1 (n = 22)</th>
<th>Group 2 (n = 22)</th>
<th>Group 3 (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No plaque</td>
<td>1 (4.5%)</td>
<td>2 (9.1%)</td>
<td>0</td>
</tr>
<tr>
<td>Separate flecks of plaque at the cervical margin of the tooth</td>
<td>7 (31.8%)</td>
<td>8 (36.4%)</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td>A thin, continuous band of plaque (≤1 mm) at the cervical margin</td>
<td>11 (50.0%)</td>
<td>8 (36.4%)</td>
<td>2 (9.1%)</td>
</tr>
<tr>
<td>A band of plaque wider than 1 mm but covering 1/3 of the crown</td>
<td>2 (9.1%)</td>
<td>4 (18.2%)</td>
<td>9 (40.9%)</td>
</tr>
<tr>
<td>Plaque covering ≥1/3 but &lt; 1/3 of the crown</td>
<td>1 (4.5%)</td>
<td>0</td>
<td>10 (45.5%)</td>
</tr>
<tr>
<td>Plaque covering &gt;2/3 of the crown</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.77 ± 0.86</td>
<td>1.64 ± 0.90</td>
<td>3.27 ± 0.83</td>
</tr>
<tr>
<td>Overall significance (Kruskal-Wallis test)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups 1 to 2</td>
<td>( Z = 0.426; P = 0.670 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups 1 to 3</td>
<td>( Z = 4.473; P &lt; 0.001 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups 2 to 3</td>
<td>( Z = 4.642; P &lt; 0.001 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bonferroni-corrected \( P \) value for significance is 0.016.

* Pairwise significance by Mann-Whitney \( U \) test.
acidic pH on human tooth enamel in vitro was observed by electron microscopy and microhardness measurements. No erosion of enamel by honey over a period of 30 minutes or deterioration of enamel structure was observed.42

Thus, in summary, honey, although less potent than chlorhexidine, is effective against putative periodontopathogens and also reduces plaque formation. Other properties of honey, such as anti-inflammatory action that reduces edema and the amount of exudate by downregulating the inflammatory process,43-46 stimulation of tissue growth,43,47 and collagen synthesis and angiogenesis,31,48 merit investigation from a periodontal perspective. Honey has shown excellent cytocompatibility with healthy tissue cell cultures49 and also inhibits fungal growth.50 No adverse effects have been reported thus far in the literature.

The varied benefits of honey, such as economic feasibility, easy availability, antibacterial and anti-inflammatory properties, and healing, make honey a potential therapeutic agent in periodontal therapy.

In vitro testing of the antibacterial action against other plaque microorganisms, salivary counts of bacteria, a larger sample population, a commercially standardized formulation of honey mouthrinse, as well as application of honey as a local drug and in regenerative procedures would provide more conclusive evidence of the various properties and possible applications of honey in periodontal therapy.

CONCLUSION

Within the limitations of the study, honey is effective against oral bacteria and reduces plaque formation.

ACKNOWLEDGMENTS

The authors thank Mr. K. P. Suresh, Biostatistician, National Institute of Animal Nutrition and Physiology, Bangalore, India. The authors report no conflicts of interest related to this study.

REFERENCES

26. Mullai V, Menon T. Bactericidal activity of different types of honey against clinical and environmental


34. Molan PC. The evidence supporting the use of honey as a wound dressing. Int J Low Extrem Wounds 2006;5:40-54.


Correspondence: Dr. S. Aparna, Department of Periodonotics, Bangalore Institute of Dental Sciences and Post Graduate Research Centre, Bangalore 560029, India. Fax: 080-41506025; e-mail: aparna_bds@yahoo.co.in.

Submitted August 06, 2011; accepted for publication November 10, 2011.
Reducing Risks of Bacterial Endocarditis from Dental Procedures With Oral Time-Released Xylitol

Written by Jeff Burgess, DDS, MSD
Monday, 30 January 2012 18:26

Introduction

In April 2007, the ADA and its Council on Scientific Affairs published a position paper that provides newly revised guidelines for the prevention of infective endocarditis (IE). These guidelines, endorsed by the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Heart Association (AHA), among others, reflected current research assessing dental procedure related bacteremia, endocarditis prevention, and the most common pathogens associated with the condition. The new guidelines reduce the classes of patients for whom antibiotic prophylaxis is recommended because the risk of morbidity resulting from antibiotic use outweighs its probable benefits. However, the guidelines also suggest that dental manipulation of any type may result in the introduction of potentially pathogenic bacteria into the bloodstream. This raises an important question whether less risky approaches to controlling oral bacteria prior to dental procedures and general oral care might be a useful preventative strategy for individuals previously defined as at-risk for IE and where antibiotics are not now recommended. Given this, the following discussion is directed toward a possible new approach for reducing the oral bacteria associated with blood born infection using xylitol incorporated into a time release disk that adheres to the gingiva and/or teeth.

Factors in the Pathogenesis of Infective Endocarditis

The pathogenesis of IE is complex and involves a number of different factors. There must be vessel turbulence leading to platelet deposition and damage to the endothelium of the cardiac valve or surgically constructed pulmonary shunt or conduit, combined with a bacteremia arising from oral trauma with microorganisms that are capable of adhering to the site (typically streptococci, staphylococci, and enterococci), and bacterial proliferation at the site of adherence.

Multiple studies suggest that procedures such as tooth extraction, periodontal surgery, tooth cleaning and scaling, rubber dam placement, and root canal therapy can cause a bacteremia. Even though there is evidence that bacteremia follows dental treatment and dental hygiene procedures, because of potential allergy, resistance, and cost-effectiveness, among other factors, the counsel restricted the classes of patients for whom short-term antibiotic prophylaxis before dental procedures are recommended. Prevention via systemic antibiotic application is generally discouraged because, in addition to the above, there is a lack of viable supportive research and further, there are contradictory results. Additionally, limited studies assessing topical antiseptic rinses or germicides as a strategy for reducing bacteremia arising from dental procedures suggest that they are not likely to be effective.

To control the risk of IE in patients for whom antibiotic prophylaxis is no longer recommended, the ADA recommends that individuals simply maintain good oral hygiene and avail themselves of professional dental care. The underlying assumption is that good oral hygiene will reduce overall bacterial counts leading in turn to a reduction in the number of bacteria introduced during dental or hygiene procedures. Good oral hygiene includes daily tooth brushing, flossing, and minimizing sugars that feed resident bacteria. While this proposal makes eminent sense, from a public health standpoint it is not all that clear that the majority of people will follow good preventative practices and present for routine dental care. In fact, based on the US Surgeon General’s Report in 2000, there remains a substantial discrepancy between American children, young adults, and older adults in terms of access to preventative and other dental care services based on ethnicity, income, education, and special needs status. It is estimated that the fastest growing populations in the US (eg, Black, Hispanic) have the highest disease rates and lowest access to dental care even in the face of current National Health Insurance Programs. In addition, there appears to be considerable disparity between states with respect to implementation of the State Child Health Insurance Program and comparisons of children in states with and without the plan suggesting that there is over a 24% difference in terms of likelihood of preventative care based on this variable alone. The issue concerning adults may even be worse. Thus, many people in the US will not receive the preventative training and skills development necessary to satisfy the ADA’s basic requirements for prevention of IE associated with routine home care.

Other Methods to Reduce Oral Bacterial Burden

Among many of the other continuing and unanswered questions in relation to the introduction of bacteria into the bloodstream and potential IE is whether there are low risk methods besides “good oral hygiene” for reducing the overall burden of oral bacteria prior to dental procedures, specifically the bacteria that are known to cause bacteremia and IE, and whether such a reduction in these disease causing bacteria might result in a drop in the incidence of IE and its associated morbidity or mortality, particularly in the aged and disabled. To date, there have been no prospective, controlled, randomized studies to assess these issues. In a review of IE and dental procedures, it is concluded that, given the current evidence regarding pathogenesis and prevention of endocarditis, novel strategies need to be developed as alternatives to current approaches using antibiotic prophylaxis.
Assuming, as has been suggested by the AHA’s report, that it might be helpful to reduce the oral bacterial burden prior to dental procedures in individuals where antibiotic prophylaxis is not recommended, the literature regarding xylitol, a sugar substitute that occurs widely in nature which can be delivered topically in the mouth, offers a potential means for achieving this end. Specifically, in a number of dental studies addressing control of caries, it has been found that delivery of xylitol in a controlled manner with chewing gum significantly reduces salivary Streptococcus (Streptococcus mutans) counts.16-18 This is of particular significance not only in the arena of public dental health and caries but also because recent research completed by a group of investigators from Japan, with their findings published by the Journal of Clinical Microbiology, has revealed that S mutans (presumed to be derived from the oral cavity) is the most numerous specie in heart valve and atheromatous plaque specimens.19

Use of xylitol chewing gum four or five times per day has been shown to reduce not only S mutans but also Streptococcus salivarius and Streptococcus sanguis,20 and regular consumption by mothers has been shown to reduce mother-child transmission.21 Further, a xylitol induced reduction in bacterial counts has been shown to continue for a prolonged period of time (age 3 to age 6 years) in children studied.22 Xylitol has also demonstrated efficacy in reducing counts of Streptococcus mitis, Lactobacillus rhamnosus, Actinomyces viscosus, Porphyromonas gingivalis, and Fusobacterium nucleatum that have been experimentally incorporated into model biofilms.23 In reducing counts of Porphyromonas aeruginosa in maxillary sinus specimens,24 in altering the viability of strep pneumococci responsible for acute otitis media,25 and in altering cytokine expression induced by P gingivalis (one of many bacteria suspected in periodontal disease),26 These as well as other studies provide a potential medical use for xylitol in reducing bacterial counts potentially involved in cardiac disease and suggest an additive approach in the management of IE and other infective diseases (eg, sinus infection, middle ear infection, gingivitis, and periodontitis).

Presently, xylitol is primarily delivered via chewing gum, 15,27-29 but it has also been incorporated into lozenges and syrup. In a study assessing the effect of xylitol on hunger, xylitol has been put into yogurt,30 and it is also found in other food products.31 However, one of the problems with its incorporation into foods is whether an adequate concentration can be maintained over a long enough time in the mouth to effectively reduce oral bacteria. Delivery of xylitol via gum appears to be effective in this regard. However, xylitol released from gum is dissipated in about 15 minutes,34 new 32 which requires that it be used 5 times or more per day to be effective and be used in larger quantities than desired.

Xylitol has also recently been incorporated into a time-release adhering disc called XyliMelts (OralHealth Corporation) that can be adhered to the teeth or adjoining gingiva to time-release 500 mg of xylitol. Xylitol delivered in this manner is very likely to be present in the mouth for longer periods of time (30 to 120 minutes—based on reports from subjects in a pilot study) and may be more tolerated by patients unwilling or unable to chew gum (eg, the edentulous, those with temporomandibular joint disorder or disabilities). XyliMelts can also be used while sleeping, when saliva flow is lowest, potentially increasing the effectiveness of the overall antibacterial action. A similar oral adhering disc called Oramoist (Quantum) designed to adhere to the roof of the mouth and release flavor to stimulate saliva also contains xylitol but the amount of xylitol in each disc is not published by the manufacturer and is likely insignificant.

Studies suggest that a dose of xylitol in the range of 6.5 g per day to 10.3 g per day delivered with chewing gum over 4 or 5 uses per day is sufficient to reduce streptococci levels.27 Studies of quantities of xylitol that must be delivered with the time release discs to achieve the same effect have not been completed. One would expect the required dosage for an equal effect to be significantly lower and the effect of an equal dosage to be significantly greater. A preliminary study (which cannot be cited because of proprietary issues) has apparently shown that only one gram per day delivered over time by XyliMelts can significantly reduce S mutans counts.

While providing the required dosage in gum can be “cumbersome” as one of the above authors has suggested,27 delivery in a dissolving disc adhered to a molar or gingiva in the buccal vestibule is easily accomplished and could make the process considerably more palatable. To achieve an effect equal to 1.3 g of xylitol chewing gum used 5 times per day (6.5 g per day), the manufacturer (OralHealth) of XyliMelts suggests that a time release disc containing one-half g of xylitol should be used at bedtime and “after each meal, at least 4 discs per day,” for a minimum of 2 g per day. Until studies are completed to determine whether this usage is sufficient to obtain optimal reduction of pathogenic bacteria, to be conservative, based on prior chewing gum studies, one could increase the usage to 4 g per day (8 discs). As previously noted, this approach to the delivery of xylitol prior to dental procedures would likely be particularly worthwhile in those with physical or mental disabilities and in frail older people where maintenance of good oral health is often problematic and where bacterial endocarditis can be a significant problem.32,33

New American Heart Association Recommendations

The AHA now recommends antibiotic prophylaxis before dental procedures only in patients with high risk of IE where that risk outweighs the risks of problems from the antibiotics. (The cardiac conditions that present this level of risk include: prosthetic cardiac valves; congenital heart disease including unrepaired cyanotic coronary heart disease (CHD), including palliative shunts and conduits; completely repaired congenital heart defect with prosthetic material or devices, whether placed by surgery or by catheter intervention during the first 6 months after the procedure, repaired CHD with residual defects at the site or adjacent to the site of a prosthetic patch or prosthetic device; and cardiac transplantation recipients who develop cardiac valvulopathy). Also included are individuals with a previous IE history. However, another problem associated with IE is the presence of comorbid disease. Hence, individuals with diabetes mellitus, immunosuppressive conditions, and kidney disease necessitating dialysis may have an increased risk of morbidity and mortality from IE. And as previously mentioned, disability and age may also confound the risk of developing the condition in susceptible individuals. However, antibiotics are no longer recommended for this cohort of patients.

New Questions for New Technology

Important questions raised by the new technology for time release of xylitol in the mouth are: (1) whether the XyliMelts discs should be used for several days or weeks before undergoing dental procedures; (2) whether this prophylaxis should be recommended for all dental procedures or only those where substantial bleeding is expected; (3) whether this prophylaxis should be recommended for all patients or only those with a moderate risk of IE; and (4) how patients with moderate risk are to be distinguished from patients with low risk. As an initial step in answering these questions, both in vivo and in vitro studies need to be pursued to assess the effect of xylitol delivered by time-release adhering disc on the oral carriage of the previously defined pathogens causing IE. Then additional prospective randomized controlled trials should be considered to assess the efficacy of this delivery system in preventing IE in select patient cohorts. Finally, it would also be useful to assess the effectiveness of xylitol delivered via adhering disc on other conditions identified as also being caused, in part, by the identified bacteria and where other xylitol-based applications have been found to prevent disease (eg, sinusitis, otitis media).29
Conclusions

Given what is now known regarding oral bacteria and their introduction into the bloodstream, until the above questions are answered, it would seem prudent for individuals with previously identified risk factors for IE who are not now prescribed antibiotics to use over-the-counter products including xylitol (eg, XylitolMints, xylitol gum) for as many days as practically achievable (one to 20 days) prior to dental procedures. These products might also be recommended by health care providers other than dentists for individuals who, for varying reasons, are not able to avail themselves of routine dental preventative care and where there is perceived risk.

ACKNOWLEDGEMENT

The author wishes to thank OralHealthCorporation for its support for this article.

References

Jeff Burgess received his DDS from the University of Washington school of Dentistry, Seattle, and his MSD in Oral Medicine from the University of Washington. He completed a 2-year post-doctoral fellowship in the Department of Anesthesiology and the University of Washington Medical Center and served 15 years as a Consultant/Attending at the Medical Center Pain Center. He also practiced general dentistry for 10 years and was a Research and Clinical Research Assistant Professor in the Department of Oral Medicine for 15 years. In addition, he had a private practice in Oral Medicine for 18 years. He has work experience with dental PACS and DICOM. He has been the co-investigator on numerous studies and authored and co-authored multiple chapters in medical and dental texts and articles in peer-reviewed journals.


Nufar Kiryati
(01.02.2012 (06:46:27))

Wow what are great article!
Jeff,

I am so happy that you shared with your Linkedin community your article on the use of Xylitol as a preventive treatment for high risk patients for IE - it will be very interesting to see the result of future studies - do you know if any research is going on or about to start in regards to this topic?

Donna Burdett
(30.01.2013 (07:14:54))

Feldman Orthodontics
I am so happy knowing the use of Xylitol as a preventive treatment for high risk patients for IE - it will be very interesting to see the result of future studies. Feldman Orthodontics
Lactoferrin – A Novel Anabolic Factor

Dorit Naot, PhD, Andrew Grey, MD, Ian R. Reid, MD, and Jillian Cornish, PhD

ABSTRACT

Lactoferrin is an iron-binding glycoprotein that belongs to the transferrin family. It is present in breast milk, in epithelial secretions, and in the secondary granules of neutrophils. In healthy subjects lactoferrin circulates at concentrations of 2–7 x 10⁻⁸ g/ml. Lactoferrin is a pleiotropic factor with potent antimicrobial and immunomodulatory activities. Recently, we have shown that lactoferrin can also promote bone growth. At physiological concentrations, lactoferrin potently stimulates the proliferation and differentiation of primary osteoblasts and also acts as a survival factor inhibiting apoptosis induced by serum withdrawal. Lactoferrin also affects osteoclast formation and, in murine bone marrow culture, lactoferrin potently inhibits osteoclastogenesis. In vivo, local injection of lactoferrin above the hemicalvaria of adult mice results in substantial increases in the dynamic histomorphometric indices of bone formation and bone area.

The mitogenic effect of lactoferrin in osteoblast-like cells is mediated mainly through LRP1, a member of the family of low-density lipoprotein receptor-related proteins that are primarily known as endocytic receptors. Using confocal laser scanning microscopy, we demonstrated that fluorescently labeled lactoferrin is endocytosed and can be visualized in the cytoplasm of primary osteoblastic cells. Lactoferrin also induces activation of p42/44 MAPK signaling in primary osteoblasts, but the two pathways seem to operate independently as activation of MAPK signaling, but not endocytosis, is necessary for the mitogenic effect of lactoferrin. We conclude that lactoferrin may have a physiological role in bone growth and healing, and a potential therapeutic role as an anabolic factor in osteoporosis.

Keywords: Lactoferrin, Bone development, Bone remodeling, Bone regeneration, Anabolic agents, LDL-Receptor Related Protein 1

Osteoporosis is defined as a reduction in bone mass and a change in microarchitecture, which increases the susceptibility to fracture.¹ The bone loss results from imbalance between the activities of osteoclasts and osteoblasts that leads to uncoupling of bone resorption and bone formation. Osteoporosis is a major cause of morbidity and health expenditure in aging populations.² Until recently, antiresorptive medications such as bisphosphonates and estrogens represented the main pharmacological treatment options for patients with osteoporosis. Despite their great therapeutic efficacy, these agents are limited in their ability to restore bone mass, and reduce the incidence of osteoporotic fracture by 50% at best.¹ ² Consequently, there is a great interest in the development of anabolic agents designed to increase bone mineral density by stimulating bone formation.⁵ Currently, only one anabolic agent, teriparatide, a recombinant formulation of a fragment of parathyroid hormone (PTH), has been approved for the treatment of osteoporosis,⁵ and there is an intensive search for other potential therapeutic compounds with anabolic effects in bone.

Milk is a rich biological fluid that contains many growth factors, and provides nutrition at a time of very rapid skeletal growth and development in the neonate. Therefore, it was considered as a possible source of factors with anabolic effects on bone. We found that a number of fractions of whey protein extracted from milk have growth stimulatory effects in primary cultures of osteoblasts. High performance liquid chromatography analysis of the major proteins identified the presence of the glycoprotein lactoferrin in most of these fractions.

Lactoferrin is an 80 kDa iron-binding glycoprotein produced by many exocrine glands and is also a major constituent of the secondary granules of neutrophilic leukocytes.² − ³ Serum levels of lactoferrin are predominantly neutrophil-derived and in healthy subjects range from 2 μg/ml to 7 μg/ml, but during inflammation its local concentrations are much higher.¹¹ Lactoferrin acts as an iron-chelator, which may contribute to its antimicrobial activity,¹¹ but it also has effects on cell growth and differentiation,¹² embryonic development,¹³ myelopoiesis,¹⁴ endothelial cell adhesion,¹⁵ cytokine,¹² and chemokine,¹⁵ production, regulation of the immune system,¹⁵ and modulation of the inflammatory response.²⁰ We investigated the effects and mechanism of action of lactoferrin in bone cells in vitro and in a local injection model in vivo.²¹,²² These studies established lactoferrin as a novel anabolic factor in osteoblasts, which also reduces osteoclast differentiation, causing an overall effect of increase in bone mass through the promotion of bone formation and the inhibition of bone resorption.

LACTOFERRIN STIMULATES PROLIFERATION AND DIFFERENTIATION OF OSTEOSTALK-LIKE CELLS

The effect of lactoferrin on osteoblast-like cell proliferation was measured using a thymidine incorporation assay.²¹ Lactoferrin purified from bovine milk produced a dose-related increase in thymidine incorporation in primary rat osteoblasts (figure 1A). Similar proliferative effects were observed in primary human osteoblasts and in the two cell lines: human osteoblast-like cell line, SaOS-2, and stromal cell line, ST2.²¹ The concentrations of lactoferrin that stimulated cell proliferation were within the physiological range. Recombinant human lactoferrin that was produced in mammalian cells also potently stimulated the proliferation of rat primary osteoblasts (figure 1B) excluding the possibility that the proliferative effect seen with the bovine lactoferrin was due to minute quantities of other growth factors co-purifying with lactoferrin or residual lipopolysaccharide (LPS) in the bovine lactoferrin preparation.

Figure 1.

Effects of lactoferrin on osteoblast proliferation, differentiation and survival. Thymidine incorporation in primary cultures of rat osteoblast-like cells treated for 24 hours with increasing concentrations of (A) bovine lactoferrin (bLF) or (B) recombinant (more ...)
The effect lactoferrin has on osteoblast differentiation was investigated using a bone nodule formation assay. Confluent cultures of primary rat osteoblasts were treated with lactoferrin for 17 to 18 days in media supplemented with L-ascorbic acid-2-phosphate and β-glycerophosphate that induce osteoblast differentiation. They were then fixed and stained by Von Kossa stain. The process of bone nodule formation involves the two bone-forming activities of the differentiated osteoblasts: bone matrix deposition and mineralization. Lactoferrin, at concentrations of 100 µg and above, significantly stimulated the number of nodules and increased the mineralized area (figure 1A). The effect of lactoferrin on apoptosis of osteoblasts was also studied, as the inhibition of cell death is an additional way to expand the population of osteoblasts. Apoptosis, induced in primary rat osteoblasts by a 24-hour period of serum deprivation, was inhibited 50% to 70% by lactoferrin as judged by the number of cells stained with deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (figure 1D). Thus, lactoferrin increases the number of osteoblasts capable of synthesizing new bone both through stimulation of proliferation and inhibition of cell death. It also enhances the ability of the osteoblasts to synthesize and mineralize bone matrix.

**LACTOFERRIN INHIBITS OSTEOCLASTOGENESIS**

Factors that stimulate osteoblast proliferation and differentiation can affect osteoclasts in different ways. Some coupling factors, such as PTH, accelerate bone turnover by inducing the activities of both osteoblasts and osteoclasts while other factors, such as amylin and interleukin (IL)-18, induce the bone forming activity of osteoblasts and inhibit bone resorption by osteoclasts thus promoting bone accrual. Several *in vitro* assays were used to investigate the effect of lactoferrin on osteoblast differentiation and activity. In a mouse bone marrow culture, the number of multinucleated cells staining positively for tartrate-resistant acid phosphatase (TRAP) after 8 days in culture is a measure of osteoclastogenesis. In these cultures, lactoferrin (added on day 2 and day 4) potently inhibited osteoclast formation in a dose dependent manner showing complete arrest of osteoclastogenesis at a lactoferrin concentration of 100 µg/ml (figure 2A). In another study of the effects of lactoferrin on bone resorption, Lorget et al. demonstrated that bovine lactoferrin reduces bone-resorbing activity in a rabbit mixed-bone cell culture. This effect appeared to be mediated by an inhibition of the development of mature osteoclasts.

![Figure 2](image1.png)

(A) Effects of bovine lactoferrin on osteoclast development in mouse bone-marrow cultures. The number of newly developed osteoclasts, assessed as multinucleated cells (MNC) staining positively for TRAP, was significantly decreased by lactoferrin in concentrations.

The effect of lactoferrin on the activity of mature osteoclasts was measured in an isolated osteoclast culture. In this assay, an osteoclast-rich suspension from rat long bones is cultured for 20 hours on bone slices. After counting the TRAP-positive multinucleated cells per slice, the number of pits excavated by these osteoclasts is determined. The number of pits per osteoclast is a measure of the mature osteoclast activity. Lactoferrin had no effect in these cultures at concentrations of up to 100 µg/ml (figure 2B). In a neonatal mouse calvarial organ culture prepared from mice that had been previously injected with 45 Ca, the release of 42 Ca into the media is a measure of mature osteoclast activity. The results from this experimental system were consistent with the isolated osteoclast assay, confirming that lactoferrin does not affect mature osteoclast activity. Taken together, these results indicate that although lactoferrin does not affect the activity of fully differentiated osteoclasts to resorb bone, it inhibits bone resorption by reducing the number of osteoclasts formed from precursor cells.

**LACTOFERRIN INCREASES BONE GROWTH IN VIVO**

A local injection model was used to study the effect of lactoferrin on bone *in vivo*. Lactoferrin was administered over the right hemicalvarium of adult male mice for 5 consecutive days while the fluorochrome labels, calcine and alizarin red, were injected subcutaneously on days 1, 5, and 14. These fluorochromes incorporate specifically into ossifying bone, and their sequential addition is used to mark the timing and location of osteoid deposition and mineralization *in vivo*. The animals were sacrificed 24 hours after the last fluorochrome administration. Sections from the calvaria of the lactoferrin treated mice were compared to calvaria from mice injected with albumin or with vehicle only. No changes were observed between the albumin and the vehicle-only groups, indicating that there was no nonspecific effect of protein injection. As shown in figure 3, the distance between the first fluorochrome label and the bone surface (i.e., the measure of new bone formation) was greatly increased by lactoferrin. Assessment of dynamic histomorphometric indices indicated significant increases in mineral apposition rate and bone formation rate induced by lactoferrin. The potent effect of lactoferrin on bone area *in vivo*, as demonstrated in figure 3, is most likely the result of the combined anabolic effects of lactoferrin on osteoblasts and the inhibitory effect on osteoclastogenesis that were seen *in vitro*.

![Figure 3](image2.png)

Photomicrographs of calvariae from animals treated with (A) vehicle and (B) lactoferrin (4 mg) for 5 days. Fluorochrome labels used: green=calcine, red=alizarin. Two calcine labels were given 13 days apart. The increased new bone growth in the 13-day...
The molecular mechanisms by which lactoferrin acts at the cellular level are largely unknown. Specific lactoferrin receptors have been described in mammalian cell types and tissues including monocytes, lymphocytes, platelets, liver, mammary epithelial cells, and intestine. Many of these receptors have not yet been fully characterized or cloned, but lactoferrin was able to bind in a reversible, saturable, and specific manner to all these cell types and tissues. Among the putative lactoferrin receptors are the low-density lipoprotein receptor-related proteins −1 and −2 (LRP1 and LRP2) which are multi-ligand members of the LRP family of endocytic receptors. Binding of lactoferrin to LRP1 and LRP2 was initially demonstrated during investigation of the inhibitory effect of lactoferrin on the hepatic clearance of chylomicron remnants. Later, lactoferrin was shown to stimulate a G-protein-dependent signaling pathway which regulated receptor mediated endocytosis via LRP. Recent evidence suggests that LRP1 functions both as an endocytic receptor and a signaling receptor.

The 39 kDa receptor-associated protein (RAP) has been shown to co-purify and bind in vitro with high affinity to both LRP1 and LRP2. Although early studies localized RAP to the cell surface, Willnow et al. showed that RAP is predominantly localized in the endoplasmic reticulum and suggested that it acts as a chaperone in the biosynthesis of LRP1 and LRP2, preventing premature ligand binding during receptor trafficking. RAP was a useful tool in the study that aimed to establish the role of LRP1 and LRP2 in lactoferrin signaling in osteoblasts, as it can be added externally to the cells and specifically inhibits ligand binding to both receptors.

**LACTOFERRIN IS ENDOCYTOSED BY OSTEOBLASTIC CELLS**

The expression of LRP1 and LRP2 in primary osteoblasts and in several osteoblastic cell lines was investigated by reverse transcription-polymerase chain reaction (RT-PCR). LRP1 and LRP2 mRNAs were present in primary neonatal rat calvarial osteoblasts, the osteoblastic cell lines UMR-106 and MC3T3-E1, and the ST2 stromal cell line. The osteoblastic SaOS-2 cell line expressed LRP1 but not LRP2.

LRPs and LRP2 have primarily been studied as endocytic receptors while lactoferrin was shown to be endocytosed by several different cell types. Therefore, the possibility that lactoferrin acts on osteoblasts through binding to LRP and entering the cell itself was investigated. Confocal microscopic analysis showed that Oregon-Green labeled lactoferrin could be visualized inside the cells 30 minutes after addition to cultures of primary rat osteoblasts. Co-staining with the membrane label DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) demonstrated co-localization of the cytoplasmic lactoferrin with intracellular vesicles. The appearance of labeled lactoferrin inside the cells was the result of a physiological process of endocytosis since under conditions that are known to inhibit endocytosis, the use of hypertonic medium or placing the cultures at 4°C, no lactoferrin could be detected in the cytoplasm. Pre-treatment of the cells with RAP also abolished the endocytosis of lactoferrin by primary osteoblasts demonstrating that lactoferrin is endocytosed by osteoblastic cells via a mechanism that involves functional LRP1 or LRP2.

**Figure 4.**
Osteoblastic cells endocytose lactoferrin in a RAP–sensitive fashion. Upper panel: Confocal micrographs showing single optical sections of primary rat osteoblastic cells labeled with (A) Oregon Green-conjugated lactoferrin for 30 minutes at 37°C. (more ...)

**MITOGENIC ACTIONS IN OSTEOBLASTS INVOLVE ACTIVATION OF P42/44 MAP KINASES**

Although members of the LRP family were mainly described as endocytic receptors, recent evidence suggests that they also act as signaling receptors. The study of possible signal transduction pathways involved in the mitogenic activity of lactoferrin in primary osteoblasts showed that lactoferrin induced the phosphorylation of p42/44 MAP kinases. This phosphorylation could be inhibited by the addition of RAP to the cell cultures indicating that the mitogenic signal is mediated via LRP1. The significance of p42/44 MAP kinase phosphorylation to the transduction of the mitogenic signal was established by the use of two specific inhibitors of MAP kinase kinase: PD-98059 and U-0126. Treatment of primary osteoblast cultures with either of these agents inhibited the mitogenic effect of lactoferrin in a dose-dependent manner implying that lactoferrin-induced osteoblast mitogenesis involves RAP-sensitive phosphorylation of p42/44 MAP kinases.

It is therefore apparent that two pathways are activated as a consequence of the interaction of lactoferrin with LRP1 or LRP2 on osteoblasts, endocytosis of lactoferrin, and activation of p42/44 MAP kinases. To investigate whether these pathways operate independently or downstream of one another, we studied the phosphorylation state of p42/44 MAP kinases when cells were treated with lactoferrin under three different conditions where endocytosis is inhibited: placing the cells at 4°C, using hypertonic medium, or using phenylarsine oxide, a pharmacological inhibitor of endocytosis. Each of these treatments inhibited endocytosis of lactoferrin but had no effect on MAP kinases activation. Therefore, we conclude that, in this biological system, endocytosis and phosphorylation of p42/44 MAP kinases are independent of each other and that endocytosis is not required for the transduction of the mitogenic signal.

**Figure 5.**
Endocytosis of lactoferrin is not required for activation of mitogenic signaling. Primary rat osteoblastic cells were placed at 4°C for 15 minutes and then treated with lactoferrin (100 µg/ml) for the indicated times. Whole cell lysates (more ...)

**MITOGENIC ACTIONS IN OSTEOBLASTS ARE MEDIATED BY LRP1**

The ability of RAP to inhibit lactoferrin-induced osteoblast proliferation indicates a role for LRP1/LRP2. RAP inhibition of cell proliferation is not attributable to cell toxicity, since addition of RAP alone to the cell cultures had no effect on thymidine incorporation. RAP inhibition also is not nonspecific since RAP did not inhibit the mitogenic actions of the phospholipid growth factor lysosphosphatidic acid (LPA) that signals through a G protein-coupled receptor. The use of RAP does not allow discrimination between the individual contributions of LRP1 and LRP2 because both receptors are expressed in primary rat osteoblasts and RAP inhibits binding of ligands to both receptors.

**Figure 6.**
Lactoferrin-induced osteoblast mitogenesis is LRP1-dependent. (A) Growth-arrested primary rat osteoblastic cells were treated for 24 hours with either 10 µg/ml lactoferrin or 10 µM lysosphosphatidic acid (LPA) in the absence (open bars)
Several experimental systems were used to differentiate between the contributions of LRP1 and LRP2 to the transduction of the mitogenic signal of lactoferrin. Pre-treatment of primary rat osteoblastic cells with an LRPs antibody that inhibits ligand binding blocks the proliferative response to lactoferrin (figure 6A). In addition, lactoferrin promotes mitogenesis in a dose-dependent manner in SaOS-2 cells, which express LRPs but not LRPs. Lactoferrin-induced proliferation of SaOS-2 cells is also blocked by co-treatment with RAP. Taken together, these data strongly suggest that LRPs functions as a mitogenic receptor for lactoferrin in osteoblastic cells.

The availability of a fibroblastic cell line established from LRP1-deficient mice (PEA13, LRP1-null) enabled a direct comparison of the mitogenic effects of lactoferrin in these cells to the effects in a fibroblastic cell line that expresses wild-type LRPs (MEF-1, LRP1-wt). As shown in figure 6B, LRP1-wt cells proliferate in response to lactoferrin, while the proliferation of the LRP1-null cells was significantly abrogated. Further, addition of RAP to cultures of LRP1-wt cells reduced the proliferative response to a level equivalent to that observed in the LRP1-null cells. These data provide direct evidence that LRPs functions as a mitogenic receptor for lactoferrin.

**DISCUSSION**

The studies reviewed here provide the first evidence that lactoferrin is a promoter of osteoblast growth that also acts as an inhibitor of osteoclastogenesis in vitro, and increases local bone formation in vivo. The effects of lactoferrin on proliferation and survival of osteoblasts exceed those of other established anabolic factors that were tested in the same in vitro assays including transforming growth factor-β, PTH, amylin, and insulin. The combination of increased osteoblast proliferation, survival, and differentiation together with inhibition of osteoclast formation are likely to contribute to the potent stimulation of bone formation seen in the in vivo model following the administration of lactoferrin. The bone growth resulting from local lactoferrin injection is considerably greater than what we have found previously in response to factors such as insulin, amylin, adrenomedullin, C-terminal PTH-related peptide, calcitonin, or calcitonin gene-related peptide in the same model. Further examination of the animals that were locally injected with lactoferrin showed increases in new bone formation on the intracranial aspect of the calvariae and on the contralateral, un.injected hemicalvariae. This observation provides further evidence that lactoferrin is a very potent bone growth factor. However, its effect on bone formation in a long-term, systemic model remains to be established.

It is possible that lactoferrin has a physiological role in skeletal development or homeostasis. Lactoferrin is expressed in the embryo and could play a role in the development and function of chondrocytes and osteoblasts in the fetal skeleton. Lactoferrin is present in very high concentrations in colostrum as well as in milk. There is some evidence that large proteins, such as lactoferrin, can cross the neonatal gut and enter the systemic circulation. Thus, it is possible that the anabolic actions of lactoferrin might continue into the neonatal period. In adult animals, although some groups studied the biological effects of orally administered lactoferrin, the effect on bone formation has not been determined. The main source of lactoferrin in the circulation is the secondary granules of neutrophils. A recent study suggests that mice have a large reservoir of functionally competent neutrophils in their bone marrow which implies that under certain physiological conditions bone cells could be exposed to a high local concentration of lactoferrin. Systemic levels of lactoferrin can reach concentrations as high as 200 µg/ml during inflammation. Therefore, in these inflammatory states, lactoferrin may play a role in counter-balancing the catabolic effects on the skeleton from some of the mediators of the inflammatory response. Lactoferrin plays an important immunomodulatory function decreasing the secretion of a number of osteolytic cytokines. Therefore, its direct effects on the activity and development of bone cells appear to be complemented by these cytokine-mediated effects.

The physiological role of lactoferrin has recently been studied in lactoferrin-deficient mice produced by homologous gene targeting. The mice were viable and fertile, developed normally, and displayed no overt abnormalities, although their skeletal phenotype has not been determined in detail. It is possible that in the absence of lactoferrin, as is sometimes seen in deficient mice models, other similar proteins can substitute and provide the missing functions.

The identification of LRPs as a functional lactoferrin receptor in osteoblasts provides a mechanistic explanation for the anabolic skeletal actions of lactoferrin and suggests a novel pathway through which bone anabolism might be regulated physiologically and/or pharmacologically. A recently published report also demonstrated the expression of LRPs in osteoblastic cell lines and suggested a role for LRPs in delivery of lipoproteins and vitamin K1 to bone. It is noteworthy, in this regard, that LRPs and LRPs (non-endocytic members of the LR receptor family that are structurally related to LRPs) have been recently identified as critical regulators of osteoblast function and skeletal mass. Whether there is overlap between the skeletal functions of these two receptors and that of LRPs is currently unclear.

The development of lactoferrin as a pharmaceutical or “nutriceutical” agent is still in the early stages. Different formulations of lactoferrin are being designed and tested to optimize its bioavailability. The animal studies need to be extended to include a systemic administration model, as well as models of increased bone resorption, such as ovariectomy. The mechanisms of action of lactoferrin on bone cells also require further investigation as the pathways through which lactoferrin acts to inhibit osteoclastogenesis are largely unknown. It would be interesting to determine changes in gene expression levels induced by lactoferrin in bone cells and to study the interactions of lactoferrin signaling with other pathways that are known to play a major role in bone physiology.

In summary, the naturally occurring glycoprotein lactoferrin, is anabolic to bone in vivo, an effect that is consequent upon its potent proliferative, differentiating and anti-apoptotic actions in osteoblasts and its ability to inhibit osteoclastogenesis. LRPs, the receptor that mediates the anabolic actions of lactoferrin in skeletal tissue, functions as a mitogenic signaling receptor in osteoblasts. Lactoferrin may have a physiological role in bone growth, and could be considered as a potential therapeutic agent for bone disorders, such as osteoporosis, and might have utility as a local agent to promote bone repair.

**NOTES**

**REFERENCES**

Go to:


33. Ziere GJ, van Dijk MC, Bijsterbosch MK, van Berkel TJ. Lactoferrin uptake by the rat liver. Characterization of the recognition site and effect of
Antiinflammatory Activities of Lactoferrin

Orla M. Conneely, PhD

Author Affiliations

Address reprint requests to: Orla M. Conneely, PhD, Department of Molecular and Cellular Biology, M511 A Debakey Bldg., Baylor College of Medicine, Houston, TX 77030. E-mail: orlac@bcm.tmc.edu

Abstract

Lactoferrin is a non-heme iron binding glycoprotein produced during lactation and by epithelial cells at mucosal surfaces. The protein is a prominent component of the first line of mammalian host defense and its expression is upregulated in response to inflammatory stimuli. In this paper, the antibacterial and immune modulatory properties of lactoferrin that contribute to host defense are reviewed. In addition, the results of recent preclinical and clinical studies demonstrating that lactoferrin acts as an inhibitor of dermal inflammatory cytokine production are summarized. The results indicate that lactoferrin may act as a potent anti-inflammatory protein at local sites of inflammation including the respiratory and gastrointestinal tracts.

Key teaching points:

• Lactoferrin is a multifunctional protein with antibacterial and immune modulatory activities and is a component of the first line of host defense.

• Lactoferrin can decrease dermal immune responses in both rodents and humans by inhibiting local production of cytokine mediators of inflammation.

• Lactoferrin also inhibits gut inflammation, most likely by similar mechanisms.

• Sufficient quantities of recombinant human lactoferrin have been produced for clinical testing of its efficacy in the treatment and prevention of gastrointestinal inflammatory conditions.

INTRODUCTION

This paper reviews the role of lactoferrin in mammalian host defense and, in particular, its mechanism of action with regard to regulation of immune responses in the first line of defense. Current information on the functions and mechanism of action of lactoferrin are reviewed. The production from filamentous fungi and testing of recombinant human lactoferrin for clinical studies and for nutritional and pharmaceutical use in humans are summarized. Also discussed are recent data obtained from preclinical and clinical studies that demonstrate the efficacy of lactoferrin as a potent anti-inflammatory agent in both dermal and gastrointestinal inflammatory conditions.

LACTOFERRIN: A MEMBER OF THE TRANSFERRIN FAMILY OF IRON BINDING GLYCOPROTEINS

Lactoferrin is an iron binding glycoprotein that consists of a single polypeptide chain of Mr = 78kD. It is the second most abundant protein in human milk (~1 g/L) [1,2] and is found in most exocrine secretions including tears, nasal secretions, saliva, intestinal mucus and genital secretions [3,4]. The protein also is expressed and secreted by the secondary granules of polymorphonuclear neutrophils [5]. The polypeptide structure of lactoferrin comprises two homologous domains that appear to have arisen by intragenic duplication [6]. The crystal structure of the protein has been resolved [7,8] and each domain binds one ferric and one carbonate anion. In addition, each domain contains one glycosylated site to which N-linked glycan residues are attached [9].
Lactoferrin is a member of the transferrin family of non–heme iron binding proteins [10]. The family includes transferrin, the major iron transporting protein in blood [11,12], the egg-white protein, ovotransferrin [13], the melanocyte protein, melanotransferrin [14] and a recently identified carbonic anhydrase inhibitor [15]. Members of the transferrin family are distinguished from other iron binding proteins by their unique anion requirement for binding of iron. The essential role of transferrin in regulating iron delivery to erythrocytes has been demonstrated by the characterization of Hpx mutant mice that carry a splicing defect in the transferrin gene resulting in very low levels of functional transferrin expression. These mice suffer from severe iron deficiency anemia, display a compensatory increase in intestinal iron absorption and develop tissue iron overload similar to patients with hemochromatosis [16]. With regard to its iron binding properties, lactoferrin differs from serum transferrin in its higher iron binding affinity and in its unique ability to retain iron over a broad pH range [17,18]. These differences, together with the differential tissue distribution of lactoferrin relative to other transferrins, contribute to the unique functional properties of lactoferrin.

Lactoferrin is a multifunctional protein to which several physiological functions have been ascribed (reviewed in [19,20]). These include regulation of iron absorption in the intestine [21,22], promotion of intestinal cell growth [23], protection against microbial infection [24–26], regulation of myelopoesis [26,27] and systemic immune responses [28,29]. This review focuses on the activities of lactoferrin that contribute to host defense.

**LACTOFERRIN: A KEY COMPONENT OF THE FIRST LINE OF HOST DEFENSE**

Although originally identified as an abundant protein in milk secretions, lactoferrin is expressed predominantly by surface epithelia and secreted into the mucosal environment. Thus, the protein is produced at high levels in nasal and tracheal passages and in gastric, genital and ophthalmic secretions. These secretions contain a variety of broad range antimicrobial and anti-inflammatory peptides and proteins that protect against chemical and microbial challenges. Finally, lactoferrin is also produced at high levels in neutrophils where it is stored in secondary granules and released during inflammation to contribute to their antimicrobial activity.

The expression of lactoferrin in the above regions responds rapidly and robustly to a variety of physiological and environmental challenges. The protein is strongly upregulated during airway [30,31] and gut inflammation [32–34] and in response to allergens [35] and other environmental insults including estrogens [36].

**ANTIBACTERIAL PROPERTIES OF LACTOFERRIN**

The antibacterial functions of lactoferrin have been substantiated by both in vitro [37–39] and in vivo [40,41] evidence. It appears that two different mechanisms involving two separate domains of the protein contribute to the antimicrobial functions of lactoferrin. The first mechanism is a bacteriostatic effect related to the high iron binding affinity of the protein that deprives iron–requiring bacteria of this essential growth nutrient [37,42,43]. Since the bacteriostatic properties of lactoferrin are due to its iron binding ability, the protein is capable of retarding the growth of a broad range of microorganisms including a variety of gram negative and gram positive bacteria and certain yeasts [42,44]. However, bacteriostasis is often temporary because some gram negative bacteria adapt to iron restrictive conditions by synthesizing low molecular weight iron chelators (siderophores) that can remove iron from lactoferrin. The efficacy of bacteriostasis is dependent on the iron status of lactoferrin and is overcome by saturation of lactoferrin with iron [45]. In this regard, it should be noted that lactoferrin expressed in mothers’ milk is only 5% to 8% saturated with iron and therefore would be expected to function as an efficient bacteriostatic agent.

The second antibacterial property of lactoferrin is due to a direct bactericidal function within the protein. Lactoferrin has a direct bactericidal effect against some gram negative and gram positive bacteria that cannot be attributed to simple iron deprivation. Apolactoferrin can cause a rapid loss of bacterial viability that cannot be reversed by the addition of exogenous iron to the growth medium [37,46]. At physiological concentrations, apolactoferrin directly damages the outer membrane of gram negative bacteria by causing the release of lipopolysaccharides (LPS) [47]. Recent results suggest that a cationic domain at the N-terminus of lactoferrin is responsible for its bactericidal properties. Synthetic peptides containing this cationic domain
A significant body of evidence has accumulated in recent years to support a role for lactoferrin in regulation of host immunity [28,29]. Lactoferrin is expressed in neutrophil secondary granules [5] and has been reported to have both positive [50] and negative [27,51–53] regulatory effects on myelopoiesis. Systemic infection with bacteria is accompanied by a rapid rise in serum levels of lactoferrin secreted from granulocytes [54] and a concomitant decrease in serum iron levels (hypoferremia). Studies have demonstrated that administration of lactoferrin can protect mice against a lethal dose of Escherichia coli when administered either intraperitoneally (i.p.) or intravenously (i.v.) [41,55] prior to i.p. or i.v. infection with the bacteria. Both in vitro and in vivo studies suggest that this prophylactic effect of lactoferrin involves an inhibition of production of several cytokines including tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) that are key mediators of the inflammatory response leading to death from toxic shock [28,29]. It has been proposed that this inhibition of TNF-α release by lactoferrin is due to its ability to act as an anti-endotoxin by binding to the lipid A moiety of LPS released from lysed bacteria thereby inhibiting subsequent binding of LPS to CD14 receptors on macrophages where it initiates a proinflammatory response [56]. However, the identification of receptors for lactoferrin on the surface of myeloblasts [57], monocytes [58], macrophages [59], and lymphocytes [60], in addition to epitope shifts involved in local production of TNF-α [20], suggests that lactoferrin may have a direct effect on regulation of cytokine production by these cells via receptor mediated signaling pathways.

LACTOFERRIN INHIBITS ALLERGEN INDUCED CUTANEOUS IMMUNITY

To test the hypothesis that lactoferrin may regulate local TNF-α dependent inflammatory responses in an LPS independent manner, we recently used a mouse model of allergen induced skin inflammation [61]. Previous studies indicate that solvent allergens act as potent stimulators of cutaneous immunity. Upon topical administration of a solvent allergen such as oxazolone, hapten-primed epidermal Langerhans cells (LC) undergo phenotypic changes and migrate from the epidermis via afferent lymphatics to the draining lymph nodes where they accumulate as immunostimulatory antigen presenting dendritic cells (DC) [62,63]. The response can be measured by the number of LC remaining in the epidermis and the number of DC accumulating in the draining lymph nodes before and after allergen treatment. The process is a critical event during the initial phase of skin sensitization and other forms of cutaneous immune response. The reason is that a proportion of the LC, which are stimulated to migrate from the skin, bear high levels of antigen and transport it in an immunogenic form to the lymph nodes where primary immune responses are provoked. Recently, it has been demonstrated that epidermal cytokines provide the molecular signals necessary for LC migration and DC accumulation in the lymph nodes. Effective mobilization of LC is dependent upon the availability of IL-1β, a constitutively expressed and inducible product of LC, and TNF-α, an inducible product of keratinocytes (kC). Administration of allergen to the skin surface stimulates release of IL-1β from epidermal Langerhan cells (LC), which in turn stimulates the synthesis of TNF-α by neighboring keratinocyte epithelial cells.

Previous experiments demonstrated that the effects of allergen on stimulation of LC migration and DC accumulation can be mimicked in mice if either TNF-α or IL-1β is administered intradermally in the absence of contact allergen [64–66]. Conversely, intravenous administration of antibodies to either cytokine is sufficient to inhibit LC migration and lymph node accumulation of DC. Thus, this is an extremely attractive model to determine the role of lactoferrin in modulating IL-1β and TNF-α dependent local inflammatory responses initiated in an LPS independent system.

A potential role for lactoferrin in regulation of cutaneous immunity was originally suggested by the observations that a) lactoferrin is produced at high levels in human individuals with skin allergic reactions [35], b) the protein is produced locally in the epidermis of normal skin and c) lactoferrin exhibits competitive binding to putative receptors on keratinocyte cells, thereby
indicating a potential to directly modulate the function of these epidermal cells [61, 67].

To address the role of lactoferrin in regulating cutaneous inflammation, mice were sensitized by topical administration of oxazolone, a potent contact allergen and administered recombinant murine lactoferrin [68] by either intradermal injection or topical administration in aqueous cream. To determine whether topical administration of lactoferrin would deliver the protein to epidermal keratinocyte cells, exogenous radiolabeled lactoferrin was applied topically to mouse skin in aqueous cream and transverse sections were analyzed for uptake of the labeled protein. Autoradiographic analysis of these sections indicated that the protein appeared to be absorbed through the hair follicles and is concentrated in keratinocyte cells in the follicular regions of the epidermis.

Analysis of the effects of lactoferrin on oxazolone induced cutaneous immune response demonstrated that administration of the protein by either route resulted in a dose dependent inhibition of LC migration and accumulation of DC within the draining lymph nodes. Surprisingly, the inhibitory effect of lactoferrin was independent of its iron saturation status suggesting that its immune regulatory activity may be independent of its iron binding function. The inhibitory effect was also observed when the inflammatory response was initiated in the absence of allergen by IL-1β but was not observed when TNF-α was used as the initiating stimulus. Taken together, these results indicate that lactoferrin functions downstream of IL-1β by interacting directly with keratinocyte cells to downregulate de novo production of TNF-α. Further, the findings demonstrate that lactoferrin can directly inhibit local inflammatory responses in vivo by a mechanism independent of its ability to bind LPS.

PRODUCTION OF RECOMBINANT LACTOFERRIN FROM FILAMENTOUS FUNGI

In order to produce sufficient quantities of the human protein for clinical testing in humans, we previously reported the characterization of recombinant human lactoferrin produced in the filamentous fungus, Aspergillus awamori [69, 70]. The protein has been produced at commercial scale and purified to homogeneity. The purified protein is indistinguishable from natural human lactoferrin in terms of its three dimensional structure [71] and biological activities, including iron and receptor binding functions and antibacterial activity [69, 72]. The protein has undergone safety and toxicity testing in animals and humans for both oral and topical administration and has shown no adverse indications at concentrations up to 5g/kg [72].

INFLUENCE OF LACTOFERRIN ON CUTANEOUS INFLAMMATION IN HUMANS

The preclinical studies summarized above predicted that topical administration of lactoferrin to humans may influence the development of cutaneous inflammatory reactions. In order to determine whether the inhibitory activity of lactoferrin on LC migration observed in the mouse also extended to human allergenic skin reactions, a similar study was carried out in human volunteers using purified recombinant human lactoferrin [73]. In this experiment, human volunteers were treated by topical administration of the contact allergen, diphencyprone (DPC), in the presence or absence of topically applied recombinant human lactoferrin [73]. As previously observed in mice, analysis of punch biopsies taken from these volunteers demonstrated a significant depletion of epidermal LC induced by DPC that was markedly inhibited by administration of lactoferrin. The application of DPC to these individuals resulted in a local inflammatory reaction that was characterized by erythema and an infiltration of leukocytes into the skin, while the administration of lactoferrin was associated with a reduction in erythema and a decrease in the magnitude of the leukocyte response. Thus, the inhibition of allergen induced LC migration by lactoferrin is associated with a decrease in the severity of local cutaneous inflammatory reaction.

ANTINFLAMMATORY ROLE OF LACTOFERRIN IN THE GASTROINTESTINAL TRACT

The observation that lactoferrin can inhibit local inflammation by inhibition of TNF-α mediated immune responses predicts that lactoferrin exerts a similar antinflammatory role at local sites of immune defense where the protein is expressed (e.g., the gastrointestinal tract, lung, uterus, etc). In this regard, the allergen induced cutaneous inflammation model used in the dermal studies
described above is mechanistically similar to that observed in Crohn’s disease in humans [74,75], and mimicked by TNBS induced colitis in mice [76,77] both of which are mediated by Th1 cell dependent inflammatory responses. Consistent with the hypothesis that lactoferrin may play an important role in modulation of gastric inflammation, the protein is expressed in the gastric mucosa of the stomach [78,79] and interacts with receptors localized on gastric intestinal epithelial cells. Further, the expression of lactoferrin is elevated in the feces of patients with inflammatory conditions including ulcerative colitis and Crohn’s disease [32–34]. Several recent studies carried out in mice have shown that administration of lactoferrin can reduce gastritis induced by Helicobacter felis [80] and protect gut mucosal integrity during lipopolysaccharide–induced endotoxemia [81,82]. While results from human clinical trials to address the efficacy of the protein in regulation of gut inflammatory conditions have not yet been reported, the availability of recombinant human lactoferrin, together with the recently obtained positive results regarding the safety of orally delivered recombinant protein in phase 1 clinical trials [72,83], indicate that efficacy testing is indeed imminent.

CONCLUSION

Lactoferrin is a prominent component of the mucosal defense system whose expression is upregulated in response to inflammatory stimuli. The protein contributes to mammalian host defense by acting as both an antibacterial and anti-inflammatory agent. The anti–inflammatory activity occurs through inhibition of binding of lipopolysaccharide endotoxin to inflammatory cells, as well as through interaction with epithelial cells at local sites of inflammation to inhibit inflammatory cytokine production.

Footnotes


Received April 26, 2001.

REFERENCES

44. Rafter R, Borek M. Staal FD. Inhibition of *Escherichia coli* by bovine colostrum and *colostral milk II. The bactericidal effect of lactoferrin on *Escherichia coli* and serum resistant strain of *E. coli*. *Immunology* 28: 83, 1975.  


Cumberbatch M, Kimber I: Dermal tumour necrosis factor-alpha induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunology* **75**: 257, 1992.  


innovation in implant care

It’s natural for everyone to want to look as good and healthy as possible and to want to present themselves attractively, with a great smile. And that’s precisely what you are doing now. You’ve been through a lot to get here. Your implants are a long-term solution and certainly a costly but worthwhile investment. So now you want to enjoy this for the rest of your life. That’s perfectly possible if you take good care of your teeth and implants. Bluem toothpaste is the best core product to ensure healthy teeth and implants.

Bluem toothpaste has been specially developed by a team of implantologists, oral surgeons and dentists under the leadership of Dr. Peter Blijdorp. Blijdorp is an authority in the field of implantology, having filled his first implants in 1976. He has since fitted tens of thousands of implants and painstakingly advised his patients, before, during and after treatment. Dr. Blijdorp has discovered that the use of natural oxygen has a positive effect on the healing process and on the maintenance of healthy gums, teeth and implants.

natural oxygen
Oxygen has been used in medicine for more than a hundred years. Pure oxygen is in fact a medicine. There is no other medicine that is so effective in promoting healing as oxygen. Oxygen is essential to health and to the upkeep of the cell structure in the human body. In addition to its healing action, oxygen also has a powerful anti-bacterial effect. Bluem toothpaste contains a high concentration of oxygen, in part derived from honey enzymes. The oxygen in the toothpaste penetrates deep into the tissues to where it is really needed. The pockets around teeth and implants are kept clean and free of harmful bacteria.

bluem toothpaste
The effectiveness of Bluem toothpaste is the result of the interaction between the various ingredients:
- Bluem toothpaste has a unique formulation. This means your teeth, implants and gums get the best possible cleaning and care.
- Bluem toothpaste contains active ingredients. Some of which come from honey enzymes. These have an antiseptic, disinfecting and anti-inflammatory effect.
- Bluem toothpaste fights the bacteria that cause plaque, keeping your gums in tip-top condition. Healthy gums are important to protecting your implants, with healthy teeth and fresh breath.
- Bluem toothpaste encourages the growth of new blood vessels. In addition it helps the damaged cells around the implants to recover. This prevents the occurrence of receding gums and restores the jawbone, ensuring that the implants remain firmly fixed.
- Bluem toothpaste ensures that your teeth stay white and that no color differences occur between the natural teeth, facings and crowns (this can be caused by coffee, tea, tobacco and red wine).
- Bluem toothpaste is also suitable for those who wish to prevent bacteria and/or infection occurring in or on the gums.
- Bluem toothpaste has a neutral pH value and contains no scouring ingredients. No damage can therefore be caused to the surfaces of teeth or implants.
- Bluem toothpaste contains no fluoride, thus reducing the risk that the implant might loosen. The high concentration of fluoride found in normal toothpaste attacks the titanium layer of the implant.

how to use it
You can use Bluem toothpaste in the same way as normal toothpaste. Brush your teeth and implants every day with Bluem toothpaste. Preferably this should be after every meal or at least twice a day, or as indicated by your implantologist, dentist or oral hygienist. Because Bluem toothpaste contains no fluoride it is only suitable, according to the law, for adults (18 years and older). Replace the cap properly after use.

ingredients
Glycerin, Aqua, Hydrated Silica, Mel, PEG-32, Aromo, Cocamidopropyl Betaine, Cellulose Gum, Xylitol, Sodium Citrate, Magnesium Sulfate, Luctoferrin, Methyl Salicylate, Sodium Perborate, Sodium Sacchorin, Sodium Methylporoben, Citric Acid, Cl 42090

bluem toothpaste:  
- Ultimate tooth & implant protection  
- Deep elimination of bacteria  
- Effective pocket reduction  
- Optimal gum conditioner  
- Powerful breath care  
- Immune system enhancement

For more information:
www.swallowdental.co.uk  
tel: 01535 656 312

Distributed by:
S Pillors Reseorch B.Y. Arnhem NL  
Mode in the Netherlands

Errors and omissions reserved
Manual: Endodontic therapy (provisional version, suggested improvements please mail to info@bluemcare.com)

1. Remove the infected pulp and drill the nerve out of the root canal.
2. File with the endofile.
3. After every filing rinse with bluem oxygen fluid (root canal rinse)
4. After the filling sessions fill the root canal with bluem oxygen fluid.
5. Thrill with ultrasonic cleaner for 10 seconds and refresh the bluem oxygen fluid.
6. Repeat step 5 three times.
7. After the last ultrasonic cleaning session irrigate the canal once more with bluem oxygen fluid.
8. After rinsing the canals are dried with paper points and filled.

We advice to use the Navitip from Ultradent for the irrigation of the canals. Use approximately 10 a 20 ml bluem oxygen fluid per canal.

Additionally: extreme pressure during irrigation or binding of the irrigation needle tip in the root canal with no release for the irrigant to leave the root canal coronally may result in contact of large volumes of the irrigant to the apical tissues. If this occurs, the excellent tissue-dissolving capability of sodium hypochlorite will lead to tissue necrosis. Bluem oxygen fluid is tissue friendly and will not lead to tissue necrosis.