Evaluation of the Antibacterial Activity and Toxicity of 2 New Hydrogels: A Pilot Study

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Wound bed preparation remains a very important issue in wound healing. To promote the production of granulation tissue, it is necessary to remove necrotic tissue and to control infection. Necrotic tissue may be removed using a hydrogel preparation. Flaminal® and Flaminal® Hydro (Flen Pharma, Belgium) are 2 new hydroactive colloid gel dressings with state antibacterial properties. These properties are attributed to an enzymatic complex in their formulation. In the study described in this report, the antibacterial effects of Flaminal and Flaminal Hydro were confirmed in an in vitro as well as an in vivo setting. It was also demonstrated that Flaminal and Flaminal Hydro are not toxic to keratinocytes in vitro using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test.

Key words: alginates, antibacterial, hydrogel, leg ulcers, toxicity, wound healing

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Wound repair involves a complex and temporal integration of cytokines, various blood components, extracellular matrix, and cells. The normal healing process can be impeded at any step along its path by a variety of factors.1,2 To promote the formulation of granulation tissue and, in turn, epithelialization, a satisfactory wound bed has to be available. Wound bed preparation includes the removal of necrotic tissue and the control of bacterial overload.3,4 A wide variety of agents are available for treatment of wounds, including ointments and dressings.5,6 Selection of agents suited to an optimal treatment process for contaminated or infected wounds continues to be controversial.7

Topical antimicrobial agents are essential in wound care management. However, their use has also been associated with cytotoxicity, delayed healing, the emergence of bacterial strains resistant to common antimicrobial agents, and the appearance of contact dermatitis.3,8 Systemic antibiotic therapy should be used judiciously; there is a lack of evidence in favor of giving systemic antibiotics to patients with chronic wounds. Given the clinical significance of contamination and infection, there is a need to explore alternative management approaches for contaminated or infected wounds.

The aim of this study was to compare the antibacterial activities and toxicity profiles of 2 new hydrogel formulations with other products commonly used in the preparation of the wound bed. Intrasite® (Smith and Nephew, UK) and Purilon® (Coloplast, Denmark) and DuoDERM® hydrogel (ConvaTec, UK) are hydrogels often used to remove necrotic tissue from the wound bed. Isobetadine® (Viatris, Switzerland) and Flammazine® (Solvay, France) are commonly used in wounds because of their antimicrobial activity.3,9 Flaminal® and Flaminal® Hydro (Flen Pharma, Belgium) are 2 new hydroactive colloid gel dressings that contain acidic chemical polymers based on acrylates. Flaminal has a high concentration of alginates and is used on exudating wounds, whereas Flaminal
Hydro contains a lower amount of alginate and is used for the treatment of slightly exuding wounds. The products induce lysis of necrosis and crusts due to hydration of necrotic tissue and autolytic processes. These hydrogels also contain an enzymatic complex, a combination of glucose oxidase and lactoperoxidase that could protect against microbial contamination, and ultimately against infection. It is speculated that alginate polymers with their strong absorption capacity absorb microorganisms into the gel and that a catalytic conversion process leads to an oxidative antimicrobial effect.

MATERIALS AND METHODS

Wound-Healing Products

The topical wound-healing products used in these experiments were Isobetadine, Flaminal and Flaminal Hydro, Intrasite, Purilon, DuoDERM hydrogel, and Flammazine.

Antimicrobial Activity In Vitro

The antimicrobial activities of Flaminal and Flaminal Hydro were tested in vitro, using such pure cultures as Escherichia coli (ATCC10536), Pseudomonas aeruginosa (ATCC10145), Proteus vulgaris (ATCC 33420), Staphylococcus aureus (ATCC25923), and Candida albicans (ATCC10231). The bacteria were incubated on Trypticase Soy Agar and the yeasts on Sabouraud Agar. The standardized plates were overlaid with 3 g of Flaminal or Flaminal Hydro and incubated for at least 48 hours at 37°C, for bacteria, and at 28°C, for yeast and molds. To test the stability of the antibacterial activity at higher temperature, plates were also incubated at 50°C for at least 48 hours. The numbers of CFU/mL (colony forming units), for each detected species, were calculated. A 3 g overlay of Flammazine, and a 3 g overlay of DuoDERM hydrogel were used as positive and negative controls, respectively.

Antimicrobial Activity In Vivo

In a clinical pilot study, we studied the in vivo antimicrobial activity on 7 chronic wounds on 4 patients. We evaluated, in vivo, the antimicrobial properties of Flaminal or Flaminal Hydro. The patients with chronic lower extremity wounds had attended the outpatient facility of the Department of Dermatology, Ghent University Hospital, Ghent, Belgium. Prior informed consent was obtained from all patients whose wounds were sampled in this study. The wounds were caused by different etiologies: venous disease, diabetes, and rheumatic disease. Patient characteristics are presented in tabular form in Table 1. All ulcers showed clinical signs of major contamination or colonization, such as increased exudation, foul odor, friable granulation tissue, discoloration, or augmentation of slough. The wounds differed in size, redness of the skin around the ulcer, degree of edema, and pain. There were no signs of systemic infection. The duration of the ulcers prior to first sampling was at least 4 weeks.

Specimens were taken using sterile swabs prior to treatment. The wound bed was first cleaned with sterile saline solution, and superficial slough was debrided using tweezers and scissors. Premoistening the swab with sterile saline was considered when the surface of the wound was dry. The tip of the swab was rolled on its side in a zigzag pattern for at least 1 full rotation. The swab was taken in the granulation tissue with the most obvious signs of bacterial presence (eg, a zone with friable granulation tissue).

The swabs were put in transport medium and were sent to the laboratory, where they were processed immediately. Standard methods for isolation and identification of aerobic and anaerobic bacteria were used. Flaminal (n = 3) or Flaminal Hydro (n = 4) was chosen, according to the type of ulcer, based on the degree of exudation. A 3 to 5 mm layer of gel was applied to the wound and sterile gauze and

<table>
<thead>
<tr>
<th>Wound Number</th>
<th>Patient Number</th>
<th>Age</th>
<th>Gender</th>
<th>Underlying Disease</th>
<th>Location of the Wound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>72</td>
<td>M</td>
<td>Diabetes</td>
<td>Heel</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>72</td>
<td>M</td>
<td>Diabetes</td>
<td>Big toe</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>72</td>
<td>F</td>
<td>Venous insufficiency and diabetes</td>
<td>Lower leg</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>72</td>
<td>F</td>
<td>Venous insufficiency and diabetes</td>
<td>Ankle</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>85</td>
<td>F</td>
<td>Venous insufficiency and diabetes</td>
<td>Ankle</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>71</td>
<td>F</td>
<td>Rheumatoid arthritis</td>
<td>Lower leg</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>71</td>
<td>F</td>
<td>Rheumatoid arthritis</td>
<td>Lower leg</td>
</tr>
</tbody>
</table>

Table 1. Patients Characteristics
bandages were secured. The gel was applied every 2 days. Remnants of Flaminal or Flaminal Hydro were wiped away from the ulcer site with gauze soaked in a sterile saline solution. On day 8, the ulcer area was sampled again. Antiseptics were not used on the wound prior to sampling. Sampling was done as described above.

**In Vitro Toxicity**

The culture media, fetal calf serum, and other additives used were purchased from Invitrogen (Merelbeke, Belgium) or Sigma (Bornem, Belgium). Human keratinocytes were isolated from skin biopsies obtained from healthy young donors undergoing elective plastic surgery (eg, breast reduction) with prior written informed consent. The use of human keratinocytes was approved by the Ethics Committee of the Ghent University Hospital.

The resulting keratinocyte suspension was seeded on a feeder layer made of irradiated 3T3 mouse fibroblasts and cultured at 37°C in a humidified atmosphere containing 10% CO₂ as described by Rheinwald and Green. At confluency, cells were trypsinized and the resulting cell suspension was cryopreserved in a DMSO (10%) containing medium. For this study, secondary keratinocyte cultures were set up, using thawed cells, which were seeded on a feeder layer as described by Rheinwald and Green.

When these secondary cultures were subconfluent, the keratinocytes were used in subsequent experiments. The different products to be tested were diluted 10% (weight/volume) in culture media and heated, if necessary, in a water bath at 37°C for maximum 1 hour to obtain solubilization. The solutions were filter sterilized (pressure filtration with a 0.2 µm diameter pore filter, Nalgene, Rochester, NY) and were then used as culture media for the subconfluent keratinocytes. After 24 and 48 hours of exposure to the different products, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tests were carried out as recommended by the manufacturer's instructions (Promega, Madison, WI). MTT tests are often used to evaluate the toxicity of topically applied skin products as described by Poon and Burd who studied the toxicity of topical antibacterial products using this technique.

In the in vitro tests, cell cultures of 2 different donors were evaluated. After trypsinization of the cultures, the cell suspensions were diluted to 1 × 10⁶ cells/mL. Cell respiration was assessed by the mitochondrial-dependent reduction of MTT (25 µL, 5 mg/mL). Blue formazan crystals were measured by spectrometry at 570 nm after dissolution of the crystals with stop/solubilization buffer (100 µL added). Each experiment was repeated thrice. On each occasion, 12 to 24 samples were analyzed for each condition of the experiment.
DATA ANALYSIS

The Wilcoxon signed-rank test was done using an SPSS 10 software package to compare the number of isolated species before and after treatment of the lower extremity wounds. This test was also used to compare the treated samples in the toxicity aspect of the experiment. \( P < .05 \) was accepted as the threshold of statistical significance.

RESULTS

Antimicrobial Activity In Vitro

Flaminal and Flaminal Hydro were found to be fully active against all microorganisms tested, irrespective of the incubation temperature. After incubation with the antimicrobial agent, silver sulfadiazine (Flammazine), no colony growth was observed. The plate overlaid by DuoDERM hydrogel did not show any effects of the gel on microbial population, and confluent growth of the bacteria and yeasts was observed. These data are shown in Table 2.

Antimicrobial Activity In Vivo

On day 0, a large number of different types of bacteria were detected in all lower extremity wounds. All wounds harbored more than one species. \( S \) \( aureus \) and \( P \) \( aeruginosa \) were isolated from almost all wounds. \( P \) \( vulgaris \) was less often isolated. The predominant flora, in addition to those mentioned above, were aerobes or facultative anaerobes such as \( S \) \( taphylococcus \) \( epidermidis \) and \( E \) coli. \( C \) \( albicans \) was found in more than half of the chronic wounds.

Eight days after the start of the treatment with Flaminal or Flaminal Hydro, wounds had become negative for several bacterial species and \( C \) \( albicans \). In the case of \( S \) \( aureus \) and \( S \) \( epidermidis \), the eradicating effect of Flaminal and Flaminal Hydro was not complete. These results are presented in Table 3. The number of different types of isolated species decreased significantly \( (P = .018) \) after the use of Flaminal or Flaminal Hydro.

In Vitro Toxicity

Results are presented in Figure 1 as mean values with standard deviation and level of significance. After 24 hours incubation of the keratinocytes with the above-mentioned products, only Intrasite and Isobetadine showed a significant reduction of the optical density \( (P < .01) \). The reduction of the metabolic activity in the case of Intrasite was modest (approximately 5% reduction in optical density). With Isobetadine, however, the optical density was reduced by approximately 60%. The Flammazine-treated samples could not be evaluated with the MTT test because the solution was too opaque to count the cells.

<table>
<thead>
<tr>
<th>Wound Number</th>
<th>Number of Different Microorganisms Identified on Day 0</th>
<th>Number of Different Microorganisms Identified on Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 ( S ) ( aureus ) ( E ) coli ( P ) ( aeruginosa ) ( E ) ( aerogenes ) ( S ) ( epidermidis ) ( P ) ( vulgaris )</td>
<td>2 ( S ) ( aureus ) ( S ) ( epidermidis )</td>
</tr>
<tr>
<td>2</td>
<td>6 ( S ) ( aureus ) ( C ) ( albicans ) ( P ) ( aeruginosa ) ( E ) coli ( E ) ( cloacae ) ( E ) ( aerogenes )</td>
<td>2 ( S ) ( aureus ) ( E ) ( cloacae )</td>
</tr>
<tr>
<td>3</td>
<td>7 ( S ) ( aureus ) ( E ) coli ( P ) ( aeruginosa ) ( E ) ( aerogenes ) ( S ) ( epidermidis ) ( E ) ( cloacae ) ( C ) ( albicans )</td>
<td>1 ( S ) ( aureus )</td>
</tr>
<tr>
<td>4</td>
<td>6 ( S ) ( aureus ) ( E ) coli ( E ) ( aerogenes ) ( S ) ( epidermidis ) ( C ) ( albicans ) ( S ) ( pyogenes )</td>
<td>2 ( S ) ( aureus ) ( E ) coli</td>
</tr>
<tr>
<td>5</td>
<td>4 ( S ) ( aureus ) ( E ) coli ( P ) ( aeruginosa ) ( E ) ( cloacae )</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>5 ( S ) ( aureus ) ( E ) coli ( C ) ( albicans ) ( S ) ( epidermidis ) ( P ) ( aeruginosa ) ( S ) ( pyogenes ) ( S ) ( agalactiae ) ( S ) ( pyogenes ) ( P ) ( aeruginosa )</td>
<td>1 ( S ) ( epidermidis )</td>
</tr>
<tr>
<td>7</td>
<td>4 ( S ) ( epidermidis ) ( S ) ( epidermidis ) ( S ) ( epidermidis ) ( S ) ( pyogenes )</td>
<td>1 ( S ) ( epidermidis )</td>
</tr>
</tbody>
</table>
After 48 hours of incubation of the keratinocytes with the different products, 3 products exhibited a significantly different optical density ($P < .01$) when compared to the controls. For the cultures incubated with Flaminal, a modest increase of the optical density was observed. A limited reduction in metabolic activity was observed with Purilon. For Isobetadine, an important decrease of the metabolic activity was observed (approximately 70% decrease).

**DISCUSSION**

Chronic wounds are almost always contaminated with microorganisms. In wound management, it is very important to distinguish between wound contamination, wound colonization, and wound infection. In this context, the number and kind of microorganisms present, their virulence, and host resistance are important parameters.$^4$,$^{12,13}$ Small numbers of microorganisms present in wound exudate and on the surface of chronic wounds do not necessarily delay wound healing. In contrast with this, a count of $10^7$ to $10^9$ microbial cells per mm² of wound surface or per gram of tissue is reported to correlate with a critical bacterial colonization inside the wound. This may hinder wound healing by local toxin release and by eliciting an inflammatory reaction.$^9$,$^{13}$ It is therefore important to keep the number of bacteria to minimal levels.

In this study, Flaminal and Flaminal Hydro demonstrated antimicrobial effects, both in vitro and in vivo. The effects are considered to be due to the enzymatic complex present in these hydrogels.$^8$ The same antimicrobial effects were observed at higher temperatures. The presence of organic material in the in vivo situation does not seem to weaken the antimicrobial effect.

Schultz et al described that organisms such as *Staphylococci* or *Streptococci* are difficult to eradicate without systemic antibiotics.$^{14}$ The results from this study showed that in 6 out of 7 patients those species could not be removed with the Flaminal or Flaminal Hydro treatments.

Furthermore, hydrogels with alginates such as Flaminal and Flaminal Hydro permit the topical to gellate and demonstrate autolytic debridement. In a small clinical study, the use of these hydrogels resulted in a surface and a volume reduction of wounds compared with controls.$^8$

In our study, an MTT test was used to evaluate the toxicity profile of the new hydrogels. We were unable to demonstrate major cytotoxicity in the keratinocytes, treated with the hydrogels without antibacterial activity. The results of the MTT test of keratinocytes cultured with Flaminal and Flaminal Hydro were comparable to the control samples, suggesting the absence of cytotoxic effects. However, our findings are consistent with reports of the cytotoxic effects of iodine-containing products.$^3$,$^{15,16}$ We must hasten to add that the observed in vitro cytotoxicity of iodine-containing products does not necessarily mean that these products also impede the wound-healing process in vivo.$^{16,17}$ On the contrary, the use of...
the same antiseptic products in a clinical setting has proven to be beneficial for the wound-healing process.\textsuperscript{18}

In conclusion, the results of this pilot study demonstrate that Flaminal and Flaminal Hydro exert antibacterial activity in vitro and in vivo, and using the MTT test in cultured keratinocytes, toxicity was not observed. These are preliminary observations that raise the need for appropriately designed clinical studies to demonstrate the lack of cytotoxicity in vivo and to evaluate inherent properties desirable to achieve wound healing.

**ACKNOWLEDGMENTS**

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**REFERENCES**