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Chapter 6

Cadexomer-iodine Ointment Shows Stimulation of Epidermal Regeneration in Experimental Full-thickness Wounds

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Abstract

The use of iodine in wound healing is still controversial. Both wound healing stimulating effects and toxic effects leading to impaired wound healing have been reported. In order to study the direct effects of iodine on wound healing without interference of infectious pathogens, we investigated wound-healing parameters in noninfected experimental full-thickness wounds in the pig. Topical iodine treatment with an ointment consisting of a combination of iodine and cadexomer (modified starch), was compared with cadexomer ointment, the vehicle without iodine, and with treatment with saline. Treatment lasted for 30 days, followed by 30 days of wound assessment. The rate of epithelialization, wound contraction, systemic iodine absorption and several immunohistochemical markers were evaluated.

All 36 wounds healed without macroscopic signs of wound infection and re-epithelialized within 21 days. During the first 9 days of treatment, wounds treated with cadexomer-iodine ointment showed significantly more epithelialization than the wounds treated with either cadexomer or saline. In addition, the epidermis of wounds treated with cadexomer-iodine ointment had significantly more epithelial cell layers from day 12 to day 30 and these wounds stained for chondroitin sulphate proteoglycans in the newly formed basement membrane zone, which was not observed with the other treatments. No negative effects of cadexomer-iodine ointment on the formation of granulation tissue, neovascularization or wound contraction were observed. During the treatment systemic iodine absorption was physiologically acceptable. These results showed that treatment with cadexomer-iodine containing ointment had positive effects on epidermal regeneration during the healing of full-thickness wounds in the pig compared with ointment alone or saline treatment.

Introduction

Cadexomer-iodine ointment is an hydrophilic modified-starch polymer with 0.9% iodine immobilized within the matrix, in an ointment base of polyethylene glycol and poloxamer. When applied on a wound, cadexomer-iodine ointment becomes moist by absorbing the wound exudate and gradually releases the incorporated iodine [13]. Several multicentre studies have found that in combination with compression therapy cadexomer-iodine ointment is an effective débrider and antiseptic agent for chronic wounds such as venous leg ulcers [17,18,25,31,35,42,43]. Used as a dressing it removes pus, debris and wound exudate from wounds and reduces bacterial counts. As a result,
wound healing is stimulated, which is indicated by a reduction in the healing time of chronic wounds.

In the treatment of open infected wounds the use of antiseptics is preferred to antibiotics because of the risk of development of bacterial resistance. Topical treatment with iodine has proven to be effective against a wide range of bacterial and fungal infections [3,5,14,15,28,29,37,46] and to our knowledge bacterial resistance against iodine has never been reported. Nevertheless, its use in wound care is still somewhat controversial owing to reports suggesting that iodine may retard wound healing [16,30]. In one study povidone-iodine used on experimental burn wounds did delay wound healing [21] and on large burns it has been seen as the cause of metabolic acidoses and renal insufficiency resulting from systemic iodine uptake [7,41]. Further studies on animals and on cells in vitro have confirmed that iodine indeed affects cellular viability in a dose-dependent fashion and delays wound healing, which reinforces the opinion that iodine should not be used in the treatment of open wounds [2,4,22,26,32].

However, results from other studies suggest that iodine applied at lower concentrations is nontoxic to wounds, and may even accelerate wound healing. For example, two clinical studies have shown that topical iodine treatment significantly stimulates the epithelialization of small burns [12,36] and similar findings have been described for cadexomer-iodine ointment treatment of chronic wounds [25,31,35] and of partial-thickness wounds in the pig [29]. Furthermore, animal studies using the rabbit ear chamber model have shown that povidone-iodine does not adversely influence epithelialization and granulation [6,8,34].

The contradictions in the previous findings are probably a result of differences in application to the wounds and local concentration of iodine in tissue. Furthermore, wound healing studies in patients are often difficult to evaluate. Standardization is hardly possible owing to the multiple variables related to wound aetiology and the absence of internal controls. To overcome these problems, we investigated whether cadexomer-iodine ointment or the cadexomer vehicle itself were able to stimulate healing of acute noninfected wounds in a pig wound model. Both treatments were compared with a treatment with saline-moistened gauzes. During the evaluation, biopsies were taken from each wound and several parameters of the healing process were assessed: cosmetic appearance by photography, wound contraction and epithelialization by planimetry, and basement membrane regeneration, vascularization and granulation tissue formation by immunohistochemistry.
Materials and methods

Materials and antibodies

The cadexomer-iodine ointment, Iodosorb<sup>R</sup>, consisted of a formulation of cadexomer, a hydrophilic starch polymer cross-linked with epichlorohydrin in which 0.9% (w/w) iodine was incorporated, polyethylene glycol and poloxamer. The cadexomer ointment used had an identical formulation to the cadexomer-iodine ointment but without iodine; both ointments were from Perstorp Pharma, Lund, Sweden. Opsite<sup>R</sup> (Smith & Nephew, Hull, U.K.) is an oxygen-permeable polyurethane adhesive transparent film. From DAKO A/S (Copenhagen, Denmark) we purchased monoclonal mouse antibodies anti-α smooth muscle actin (dilution 1:200), polyclonal rabbit-antibodies anti-human Von Willebrand Factor (1:1500), biotinylated swine-antibodies anti-rabbit immunoglobulins, biotinylated rabbit-antibodies anti-mouse immunoglobulins and streptavidin biotinylated horseradish peroxidase complex (StreptABcomplex/HRP). Monoclonal mouse antibodies anti-chondroitin-4- and -6-sulphate (1:400) and the peroxidase substrate diaminobenzidine (DAB) were obtained from Sigma Chemical Co. (St. Louis, MO). All primary antibodies were shown to cross-react with porcine antigens.

Surgical procedures and skin biopsies

The operation procedures were performed as described previously [9,10] and the protocol was approved by the Ethical Committee of Animal Welfare of the University of Amsterdam. In brief, female Yorkshire pigs, weighing approximately 20 kg at the start of the study, were used. A grid was tattooed on the back of the animals to facilitate the measurement of wound surfaces by planimetry. Six identical full-thickness wounds (3.0 cm x 3.0 cm and 0.24 cm deep) were created with an electrodermatome. Two wounds were dressed with approximately 5 ml of cadexomer-iodine ointment, two with 5 ml of cadexomer ointment and two with 5 ml of saline on 5 x 5 cm gauzes. These dressings were fixed with Opsite, covered with another layer of hydrophilic gauzes which were fixed with adhesive tape (Curafix, Lohmann, Almere, the Netherlands) and elastic stockings (Tubigrip, Seton Health Care, Oldham, UK). This procedure protected the wounds from mechanical trauma. During the next 30 days, the wounds were cleaned every 3 day with saline and the treatments were repeated. All wounds were treated with occlusion to create an optimal wound environment for cell migration and granulation tissue formation and to prevent rapid desiccation of the saline gauzes and the ointments.

Treatment application and evaluation were not blinded and evaluation of
epithelialization was based on colour differences of the wound surface (Fig. 1). Wound edges, epithelialized area and the tattooed grid were traced on transparencies every third day for 30 days and after 36, 44 and 58 days. Reepithelialization was calculated as the percentage of epithelialized wound area divided by the total wound area. Wound contraction was calculated as the reduction in original wound area and corrected for growth of the animal. Punch biopsies and photographs were taken of each wound every sixth day for 36 days and on days 44 and 58. The biopsies were fixed in a 4% formalin PBS solution for 8-12 h at room temperature, processed by routine histological procedures, and embedded in paraffin. Biopsies were used to visualize wound histology, e.g. epidermal regeneration (rete-ridges, number of epithelial cell layers, presence of chondroitin sulphate basement membrane proteoglycans), angiogenesis (staining for von Willebrand Factor), and granulation tissue formation and thickness (staining for α-smooth muscle actin).

**Immunohistochemistry**

Sections of 5-6 μm thickness were mounted on lysine-coated glass slides. The sections were deparaffinized in xylol and hydrated through a graded series of ethanol. All incubations were performed at room temperature unless stated otherwise. To remove endogenous peroxidase activity the slides were incubated for 30 min in a 0.3% H₂O₂/methanol solution, and washed with water and PBS. Non-specific binding of antibodies was avoided by 15 min preincubation with a PBS solution with 10% normal human AB serum. The sections were incubated for 1 hour with primary antibodies (diluted as indicated above) and washed three times in PBS before the appropriate biotinylated secondary antibody was applied (diluted 1:400 in PBS 10% AB serum). After an incubation of 30 min, sections were washed with PBS twice and incubated for another 30 min with the streptABcomplex/Hrp (diluted 1:200 in PBS). After extensive washing, the colour reaction was performed for 7 min with 50 mM Tris/HCl buffer (pH 7.8) containing 0.05% DAB substrate and 0.03% H₂O₂. Finally, the sections were counterstained with haematoxylin, mounted in glycergel and examined microscopically.

For the Von Willebrand staining, the sections were predigested with a 0.25% pepsin solution for 30 min at 37°C, in order to unmask antigens. Sections of normal human and pig skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same animal as the primary antibodies at the same dilution. No staining was observed in negative controls. Microscopic examinations were carried out by three experienced researchers. Photographs of representative histological stainings were taken with an Olympus SC35 camera with 64T (EPY-135) Ektachrome film (Kodak, Netherlands).
Measurement of plasma iodine levels

From each of the six animals studied, 10 ml blood was collected on heparin on every third day from day 0 until day 30, and on days 36, 44 and 58. After centrifugation, plasma samples were stored deep frozen until assay. After digestion of organic matter with nitric acid, the total iodine content was assessed using inductive coupled plasma analysis with mass spectrometric detection. The analysis was performed by Biospectron AB, Tägarp, Sweden.

Statistical analysis

A total of twelve wounds per treatment, i.e. two on each pig were evaluated. The location of treatments was varied in a balanced design. A significant difference between two treatments was defined as $p<0.05$, using one-tailed Student's $t$-test.

Results

Cosmetic appearance and epithelialization

During the first 12 days of treatment, all three treatments yielded red granulating wounds which closed by epithelialization and wound contraction. Wound closure was fastest for wounds treated with cadexomer-iodine ointment. Epithelialization measured by planimetry as the percentage of closed wound area showed that after 6 and 9 days wounds treated with cadexomer-iodine ointment had significantly more epithelialized wound area ($p<0.05$) compared with the wounds treated with both cadexomer and saline (Fig. 1). Figure 2 shows a representative example of the wounds 12 days post-wounding. At this time point, the cadexomer-iodine ointment treated wounds still showed significantly more reepithelialization ($p<0.05$) than saline treated wounds. At later time points wound closure was almost complete and the differences between the treatment were no longer significant.

![Figure 1. Rate of epithelialization. Results are expressed as mean percentage (±SD, n=12) of wound area covered after contraction. * After 6 and 9 days, cadexomer-iodine ointment treated wounds showed significantly more wound closure compared those treated with cadexomer or saline ($p<0.05$). ** After 12 days, cadexomer-iodine ointment treated wounds were significantly more epithelialized than the saline treated wounds ($p<0.05$)(per treatment n=12, ±SD).](image-url)
Iodine Stimulates Epidermal Regeneration

Figure 2. Cosmetic appearance of the wounds after 12 days. A. Cadexomer-iodine ointment treated wound showing red granulation tissue and epithelialization. B. The cadexomer treated wound only shows epithelialization at the wound-edges. C. The saline treated wound showed little epithelialization.

Figure 3. Haematoxylin and eosine stainings of wound sections 18 days post-wounding. A. Cadexomer-iodine ointment treated wound showing a thick epithelium with the presence of rete ridges and a thick stratum corneum. B, C. Cadexomer and saline treated wounds, respectively, showing a flat epidermis without rete ridges. Scale bar = 40 μm.

Epidermal regeneration

Histological analysis of sections routinely stained with Haematoxylin/Eosin showed that the epidermis was thicker for the cadexomer-iodine ointment treated wounds than for the other treated wounds (Fig. 3). Significant differences were found in the number of cell layers for cadexomer-iodine ointment treated wounds compared with the other wounds starting 18 days post wounding and lasting until the end of treatment (p<0.01)(Fig. 4). In addition to the higher number of cell layers of the cadexomer-iodine ointment treated wounds, they also showed more rete ridges and a thicker stratum corneum, both markers for epidermal differentiation and keratinocyte differentiation. The advanced epidermal differentiation was confirmed by
immunohistochemical analysis. Chondroitin sulphate basement membrane protein, normally present in the basement membrane of intact skin (Fig. 5a), was detected in the basement membrane zone of the cadexomer-iodine ointment treated wounds as early as 12 days after start of treatment, but not in that of the wounds treated with cadexomer or saline. Figure 5b-d illustrates this at day 24.

Figure 4. The mean number (n=12, ±SD) of epidermal cell layers at different time points after wounding. * After 18, 24 and 30 days, cadexomer-iodine ointment treatment induced significantly more epidermal cell layers than the other treatments (p<0.05). Wound biopsies 6 days after wounding showed no reepithelialization.

Figure 5. Chondroitin sulphate stainings of normal pig skin and the different wounds 24 days after treatment. A. Normal skin positive for chondroitin sulphate in the basement membrane (arrows). B. Cadexomer iodine ointment treated wound clearly showing rete ridges and chondroitin sulphate staining in the basement membrane zone (arrows). C, D. Cadexomer and saline treated wounds, respectively, are negative for chondroitin sulphate in the basement membrane. Scale bar= 80 μm.
Dermal regeneration

During the study, the average wound contraction was somewhat higher with saline treatment than with cadexomer-iodine ointment and cadexomer treatment although this difference was never significant. On day 60, the final average area reduction with the cadexomer-iodine ointment and cadexomer treatments was around 70% and for the saline treatment 75% (Fig. 6). Granulation tissue thickness was not significantly different between the different treatments.

The immunohistochemical stainings for von Willebrand Factor, α-smooth muscle actin and dermal chondroitin sulphate did not reveal any obvious differences between the treatments (results not shown). Vascular structures were already abundant 6 days after the start of the treatment and at the same time myofibroblasts positive for α-smooth muscle actin appeared in the lower dermis. They were abundant in the granulation tissue after 12 and 18 days and started to disappear after 24 days when the rate of wound contraction decreased. Chondroitin sulphate proteoglycans were abundant in the granulation tissue.

Systemic iodine absorption

After the start of cadexomer-iodine ointment treatment, the total iodide concentrations in plasma were significantly raised to approximately three times baseline values (p<0.05)(Fig. 7). After the end of the treatment, the levels returned to normal within one week.

Figure 6. The average wound contraction (n=12, ±SD) measured by planimetry during the evaluation period. Results are expressed as percentage reduction of original wound area and were corrected for growth of the animal (p>0.05).

Figure 7. Mean total protein-bound iodine plasma levels of the 6 pigs during the evaluation period (±SD). * p<0.05 vs baseline values.
Discussion

The reason for the use of iodine in wounds is its antimicrobial activity. One likely mechanism which enhances the antimicrobial capacity of granulocytes and macrophages is the stimulation of the oxygen-dependent myeloperoxidase-H$_2$O$_2$-halide system in phagosomes by iodine [23]. Nevertheless, iodine has been shown to be cytotoxic in vitro, and its use in wound therapy has been discouraged. However, the observed cell toxicity in vitro has been shown to be dose-dependent [26] and in consequence tissue toxicity is likely to be dependent on the frequency and formulation of the application used [12,19]. Two reviews on the effects of iodine on wound healing have indicated that povidone-iodine does not retard wound healing when it is applied in small quantities [12,27]. The povidone-iodine preparations used nowadays are a solution and an ointment containing 10% povidone-iodine with 1% available iodine, and a povidone-iodine cream containing 5% povidone-iodine with 0.5% available iodine. Cadexomer-iodine ointment contains 0.9% available iodine, but has a different carrier and release mechanism. The present study showed that iodine was released from its ointment at a sufficiently low level that angiogenesis, granulation formation and wound contraction were not inhibited and tissue toxicity was avoided.

Previous clinical studies on chronic wounds [25,31,35,42,43] have shown that cadexomer-iodine ointment has positive effects on wound healing. Our comparatively acute wound model showed that in the absence of wound infection the cadexomer-iodine ointment treatment stimulated epidermal regeneration when compared with the treatments with cadexomer ointment and saline gauzes. Stimulation of keratinocyte proliferation, migration and differentiation was demonstrated by significantly faster epithelialization, a significantly greater number of epidermal cell layers, the presence of rete ridges, and early deposition of chondroitin sulphate basement membrane proteins. These effects were most likely caused by the release of iodine from its ointment. Faster epithelialization of cadexomer-iodine ointment treated wounds has also been demonstrated in partial thickness wounds by Mertz et al.[29]. However, in their study wound closure was achieved by re-epithelialization from hair follicles and quantified as the time needed to achieve wound closure. In our study reepithelialization only occurred from wound edges and was quantified by planimetry. Additionally, we investigated basement membrane regeneration by immunohistochemistry for which we have shown previously that chondroitin sulphate
staining is an appropriate marker [24].

It is not clear whether the observed stimulation of keratinocytes was direct or not. In cell culture it has been demonstrated [33] that iodine is able to modulate cytokine production of macrophages towards a pro-inflammatory profile. The IL-6 production is decreased while the TNF-α production is increased. In addition, low concentrations of cadexomer-iodine ointment, but not of cadexomer ointment, have also been shown to stimulate proliferation of fibroblasts [40]. Whether iodine also directly modulates keratinocyte proliferation or exerts its effects by acting on cytokine production of macrophages or other cell types remains to be established.

Wound therapy with products containing iodine results in systemic iodine uptake which is related to the size and depth of the wound [19] and which may cause side effects related to changes in thyroid function [44,45]. However, normal subjects without pre-existing thyroid disease are able to tolerate systemic iodine uptake well without any physiological disturbance. In a study on burn patients, serum levels of up to 4900 μg/dl were recorded, compared with normal serum levels of 4-8 μg/dl, without evidence of systemic toxicity [19]. Other studies of increased iodine intake in humans, using doses of up to 350 mg I/day orally as well as different topical dose regimens, have shown no or minimal effect on thyroid function except an increased level of thyroid stimulating hormone, indicating physiological adaptation to increased plasma iodine levels [1,11,19,20,38,39,47]. In our study, the iodine dose applied to wounds was approximately 45 mg every 3 days resulting in iodine plasma levels of 25-30 μg/dl. This level was significantly higher than baseline values (7-9 μg/dl), but is unlikely to affect thyroid function or will lead to organ toxicity. After cessation of iodine therapy iodine plasma levels returned to normal as a result of rapid renal clearance [1,47].

In conclusion, the cadexomer-iodine ointment stimulated epidermal regeneration of acute full-thickness wounds in pigs. The combination of antimicrobial activity and stimulation of the wound healing process may be clinically useful.

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References


