Effects of hyaluronan and iodine on wound contraction and granulation tissue formation in rat skin wounds

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Summary

Background. Hyaluronan (HA) plays an important role in the repair of damaged skin and has been used for the treatment of wounds. Iodine is a mild topical antiseptic.

Aim. A mixture of high molecular weight HA with the iodine complex KI₃ (hyiodine) was reported to accelerate wound healing in patients with diabetes and patients after surgery. We investigated how this mixture affects wound contraction, granulation tissue (GT) and wound edges in excision skin wounds in rats.

Methods. Hyiodine was applied to full-thickness wounds made on the back of rats. The areas of the contracting wounds were calculated from digital photographs. The moving edges of the wound were studied by histological methods. The properties of GT were studied in wounds in which contraction was prevented by the insertion of plastic rings. The effects of hyiodine were compared with those of high molecular weight (1200 kDa) HA, low molecular weight (11 kDa) HA and KI₃ solution.

Results. Hyiodine accelerated wound contraction significantly in the first 5 days of healing. On day 3, hyiodine-treated wounds had reduced to 63% of the original area, whereas the wound area in saline-treated animals was 75% of the original size. The proliferating epidermis was thicker in hyiodine-treated animals on day 7. In the wounds with inserted rings, hyiodine caused little change in GT, but the weight of the crust/exudate formed on the top of the wound was increased by 351% compared with only minor changes caused by the hyiodine components alone.

Conclusions. Hyiodine supports wound healing by stimulating wound contraction and epidermal proliferation and by keeping the wound moist through increased exudation.

Introduction

Hyaluronan (HA), a linear polymer of D-glucuronic acid and N-acetyl-D-glucosamine, is a part of the extracellular matrix and affects cellular behaviour. It plays a number of roles in the healing of damaged tissues. It has a high level of hydration, and as a component of granulation tissue (GT), it facilitates migration of inflammatory cells and fibroblasts into the healing wounds. It has been implicated in angiogenesis and wound re-epithelization. Enhanced migratory activity of keratinocytes correlates with increased hyaluronan synthesis after adding keratinocyte growth factor to cultured cells. Overexpression of hyaluronan synthase 2 also results in increased keratinocyte migration. The content of HA changes during skin repair, and in the wound fluid of adults is at its highest 2–4 days after injury. The molecular weight of HA is a few million daltons, but in the process of tissue repair it may be depolymerized. HA fragments of lower molecular weight...
may accumulate and their biological effects may be different from those of the high molecular weight precursor.\(^6\)

HA is a natural product that lacks immunogenicity and can thus be used for the treatment of human diseases.\(^7\) Exogenous HA applied to skin wounds accelerates wound contraction and increases blood flow in wounds. The effects are dependent on the molecular weight.\(^8\) Grafts of HA crosslinked with glutaraldehyde greatly shortened the time to wound closure when they were inserted into full-thickness skin wounds in rats.\(^9\)

The number of cells in the epithelial layer was increased in the wounds in normal and alloxan-diabetic rats after topical application of hyaluronan solution.\(^10\) Reduced expression of the Hoxb13 gene in mice simultaneously enhanced wound healing and hyaluronan content in the epidermal and dermal layers of the skin.\(^11\)

Iodine has been used for many years as an antiseptic to treat wounds. It is active against many bacteria, viruses and fungi, and it may also increase the healing rate.\(^12\) A polyvinyl pyrrolidone (PVP)–iodine preparation in hydrogel was reported to improve healing by increasing wound moisture and by preventing bacterial infection. The epithelialization of meshed skin grafts was increased in human patients after hydrogel application.\(^11\) The epithelium of chronic venous ulcers grew faster after treatment with cadexomer iodine than with standard dressings.\(^14\) Besides supporting re-epithelialization, iodine preparations may influence cytokine production by macrophages and modulate the redox environment of wounds.\(^15\)

A mixture of HA and iodine complex KI\(_3\) (hyiodine) was used by Sobotka et al.\(^16\) to treat chronic nonhealing wounds in patients with diabetes. Clinical improvement was found in most patients. Accelerated healing of chronic wounds treated with hyiodine was also reported by Ajemian et al.\(^17\) Hyiodine therefore seems to be a novel and useful preparation for wound treatment. We undertook a study in rats to evaluate the effects of hyiodine on wound contraction.

**Methods**

**Animals**

Male Wistar rats (Biotest, Konarovice, Czech Republic) 9 weeks old and weighing 300–380 g were housed in individual cages and fed commercial pelleted diet *ad libitum*. They were maintained in an air-conditioned room at 22 °C. The experiments were approved by the ethics committee of the Faculty of Medicine, Hradec Kralove.

**Materials**

Both high molecular weight 1200 kDa HA (HA1200) and low molecular weight 11 kDa HA (HA11) were obtained from the 1500 kDa product of *Streptococcus* sp. (CPN Ltd, Dolni Dobrouc, Czech Republic). HA1500 was sterilized by autoclaving, which caused a decrease in molecular weight to 1200 kDa. HA11 was prepared from HA1500 by acid hydrolysis (Dr Z. Bezakova, CPN). The average molecular weights were determined by size-exclusion chromatography with multangle light scattering detection and high-performance liquid chromatography (Dr M. Hermannova, CPN). The endotoxin content was < 0.5 IU/mg HA. HA11 was sterilized by filtration. The concentrations applied to the wounds were 1.5% w/v.

The complex KI\(_3\) was prepared by dissolving iodine in a solution of potassium iodide (Riedel de Haen, Seelze, Germany) to final concentrations of 0.1% and 0.15%, respectively. The mixture of HA1200 and KI\(_3\) is commercially available as hyiodine (CPN).

**Induction of contractible wound**

Full-thickness excision wounds were made on the back of rats as follows. The skin on the back of anaesthetized rats was shaved and disinfected. A full-thickness circular wound, 19 mm in diameter, was made, as described by Rudas.\(^18\) The wound was bandaged with gauze (Figs 1a,c). The removed tissue included the panniculus carnosus. An aliquot (1 mL) of hyiodine or saline was applied to the wound immediately after the operation, on the following day and then every other day for a total of 15 days. The wounds were photographed each time with a ruler calibrated in millimetres next to them (Fig. 1d). Wound area was measured by ImageJ software (NIH, Bethesda, MD, USA) calibrated on the standard length, using the ruler. There were 7–8 rats in each group.

**Induction of permanent wound**

Wounds were induced as described above. A plexiglas ring was then inserted into each wound\(^18\) and sutured to its edges. The inner diameter of the ring was 20 mm and its depth 9 mm (Figs 1b,e). The ring was covered with a nylon mesh. An injection syringe was used to apply 0.6 mL of each of the tested solutions (saline, HA11, HA1200, KI\(_3\), or hyiodine) to the wound once a day for 7 days. After harvesting, granulation tissue (GT) was used for hydroxyproline determination and RNA extraction. The crust and
Exudate formed on the top of the GT were used for protein and uronic acid determinations. There were 14–15 rats in each group.

**Histological analysis**

Wound samples were excised on days 3, 7, 11 and 15 (three rats in each group), fixed in 4% formaldehyde and stained with haematoxylin and eosin or blue trichrome. The thickness of the epithelium was measured in each histological section on both sides of the wound at four different sites.

**Hydroxyproline determination**

GT was dried at 60 °C and hydrolysed in 6 mol/L HCl at 105 °C for 16 h. Hydroxyproline content was determined by the Stegemann method as modified by Hurych and Chvapil. Citrate–acetate buffer pH 6.3 was added to the hydrolysate. Samples were oxidized with chloramin T (Riedel de Haen) dissolved in citrate–acetate buffer pH 6.0. The reaction was stopped by acidification with perchloric acid, then p-dimethylaminobenzaldehyde (Politechnika Slaska, Gliwice, Poland) dissolved in n-propanol was added and the reaction

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**Figure 1** Wounds on rat backs. (a) Rat with a contractible wound bandaged with gauze; (b) rat with a permanent wound with an inserted plexiglass ring; (c) contractible wound, day 0; (d) hyiodine-treated contractible wound, day 7; (e) permanent wound, day 0. The arrow indicates proliferating epithelium.
mixture was incubated in a water bath at 60 °C. The absorbance was measured at 540 nm.

**Protein and uronic acid determination**

The crust with jelly-like exudate was extracted with 0.5 mol/L NaOH at 60 °C for 2 h, as described by Simeon et al. The mixture was neutralized and ethanol was added to the final concentration of 80% (v/v). After centrifugation, the precipitate was redissolved in 0.5 mol/L NaOH and used for protein and uronic acid determinations. Protein was measured using a commercial protein assay (DC Protein Assay; Bio-Rad, Prague, Czech Republic) with bovine serum albumin (Sigma Chemical Co., Prague, Czech Republic) as a standard. The carbazole method of Bitter and Muir was used to determine uronic acid levels, and D-glucuronic acid (BDH Biochemicals, Poole, Dorset, UK) was used as a standard.

**RNA extraction and analysis**

RNA isolation and analysis was performed as described previously. Total cellular RNA was isolated from the GT and reverse transcribed. Purified cDNA, labelled with biotin dUTP, was hybridized with microarray chips containing specific oligonucleotides (50 bp in size) for 92 genes (Clondiag, Jena, Germany). The hybrids were incubated with streptavidin–horseradish peroxidase conjugate. The intensities of staining after peroxidase reaction were determined, and gene-expression intensities were normalized for all genes on the array.

For real-time reverse transcription PCR (RT-PCR), total cellular RNA was transcribed to cDNA and quantified (TaqMan Gene Expression Assays; Applied Biosystems, Prague, Czech Republic). The results were normalized to 18S RNA expression.

**Polyacrylamide gel electrophoresis**

Protein samples were boiled with dithiothreitol and SDS, applied to 8% acrylamide gel and electrophoresed. For the samples, 30 μg of rat serum or plasma, 30 μg of proteins extracted from the crust/exudate, or 15 μg of bovine serum albumin (Sigma, Prague, Czech Republic) were used. After resolution, the proteins were stained with Coomassie Blue.

**Statistical analysis**

One-way analysis of variance (ANOVA) with Fisher LSD multiple-comparison test or the nonparametric Kruskal–Wallis one-way ANOVA on ranks with Kruskal–Wallis multiple-comparison Z-value test (Bonferroni correction) were used. Significance was set at α = 0.05.

**Results**

**Determination of wound contraction**

Wound contraction in control rats was rapid in the first week of the experiment and it was almost complete by day 15, when < 5% of the wound area was not covered with epithelium. The contraction was significantly accelerated in the first days by the mixture of HA1200 and KI3 (hyiodine) treatment. The wound area in control rats was 60% of the original size on day 5. When the wounds were treated with hyiodine, this percentage was reached almost 2 days earlier (Fig. 2). The measurement of contracting wound size is illustrated in Fig. 1d.

**Histological analysis**

Figure 3 shows the thickening of epithelium in the wound on day 7 of hyiodine treatment. The thickness of the epithelial layer when measured immediately after wounding was 30 μm. It increased to 102 μm in both saline-treated and hyiodine-treated wounds on day 3. However, it was 109 μm in saline-treated and 146 μm in hyiodine-treated wounds on day 7, and the difference was significant (P < 0.05).

**Analysis of the granulation tissue**

Seven days of treatment of GT with the mixture of HA1200 and KI3 (hyiodine) resulted in a 18% increase
in the wet weight of the GT compared with saline treatment. The changes caused by HA1200 and by KI3 alone were smaller and were not significant. The concentration of hydroxyproline (the index of collagen), was decreased by hyiodine compared with saline or other solutions but little change was found in total hydroxyproline content (Table 1).

Analysis of the crust and exudate

The crust formed on the top of the GT. It could not be separated from the exudate gel, which was abundant especially after hyiodine treatment. HA1200 caused a 37% increase in the crust/exudate weight, whereas KI3 alone did not have any effect. When applied together, these substances increased the weight of the layer by 351% (Table 2).

Proteins and uronic acids were extracted from the crust/exudate with hot alkali. Protein concentration was similar in all groups but the total amount of protein was by far the highest in the hyiodine group (349%) compared with saline.

The source of the protein might be blood plasma. We therefore studied the composition of the protein mixture by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The protein pattern of four different exudate samples resembled that of plasma and serum. The band at 66 kDa corresponding to albumin was prominent (Fig. 4).

Compared with saline, total uronic acid content in dried crust/exudate was increased about 3-fold after treatment with HA1200 and 14-fold after hyiodine (Table 2).

![Figure 3](image)

**Figure 3** Histological sections of wounds treated with (a) saline and (b) hyiodine. B, blue trichrome; C, crust; D, dermis; epithelium; GT, granulation tissue.

<table>
<thead>
<tr>
<th>Granulation tissue</th>
<th>Saline</th>
<th>HA11</th>
<th>HA1200</th>
<th>Hyiodine</th>
<th>KI3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, mg</td>
<td>363 ± 18</td>
<td>410 ± 16</td>
<td>375 ± 14</td>
<td>428 ± 17*</td>
<td>409 ± 27</td>
</tr>
<tr>
<td>Dry weight, %</td>
<td>16.0 ± 0.9</td>
<td>16.1 ± 0.7</td>
<td>16.1 ± 0.6</td>
<td>15.9 ± 0.8</td>
<td>18.3 ± 1.2</td>
</tr>
<tr>
<td>Hyp concentration, mg/g</td>
<td>4.03 ± 0.22</td>
<td>3.99 ± 0.19</td>
<td>4.25 ± 0.24</td>
<td>3.66 ± 0.14†</td>
<td>3.88 ± 0.22</td>
</tr>
<tr>
<td>Hyp content, mg</td>
<td>1.45 ± 0.09</td>
<td>1.63 ± 0.09</td>
<td>1.58 ± 0.09</td>
<td>1.56 ± 0.08</td>
<td>1.56 ± 0.11</td>
</tr>
</tbody>
</table>

Hyp, hydroxyproline. Data are means ± SEM. Significance (P < 0.05): *hyiodine vs. saline; †hyiodine vs. HA11; ‡hyiodine vs. HA1200.

<table>
<thead>
<tr>
<th>Crust/exudate</th>
<th>Saline</th>
<th>HA11</th>
<th>HA1200</th>
<th>Hyiodine</th>
<th>KI3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, mg</td>
<td>95.0 ± 19.9</td>
<td>153.3 ± 22.3</td>
<td>130.0 ± 31.0</td>
<td>428.0 ± 81.2 *†‡</td>
<td>96.1 ± 25.1</td>
</tr>
<tr>
<td>Protein concentration, % dry weight</td>
<td>90.6 ± 9.2</td>
<td>91.4 ± 5.9</td>
<td>93.8 ± 6.8</td>
<td>92.5 ± 9.5</td>
<td>85.3 ± 8.9</td>
</tr>
<tr>
<td>Protein content, mg</td>
<td>36.8 ± 7.1</td>
<td>38.7 ± 5.6</td>
<td>36.6 ± 9.0</td>
<td>128.3 ± 27.0*†‡</td>
<td>48.2 ± 11.3</td>
</tr>
<tr>
<td>Uronic acid concentration, % dry weight</td>
<td>1.38 ± 0.22</td>
<td>1.25 ± 0.14</td>
<td>2.83 ± 0.52</td>
<td>4.07 ± 0.64*†‡</td>
<td>1.42 ± 0.20</td>
</tr>
<tr>
<td>Uronic acid content, mg</td>
<td>0.37 ± 0.06</td>
<td>0.49 ± 0.05</td>
<td>1.08 ± 0.34</td>
<td>5.10 ± 0.94*†‡</td>
<td>0.53 ± 0.10</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Significance (P < 0.05): *hyiodine vs. saline; †hyiodine vs. HA11; ‡hyiodine vs. HA1200; §hyiodine vs. KI3.
Gene-expression analysis

The mean expression of 92 genes studied was taken as being equal to 1. Only 54 genes for which the relative expression was > 0.3 were taken into account. Among these were genes coding for collagens I, III, V and XVIII, elastin, fibrillin-1, fibronectin, osteopontin, osteonectin (SPARC), vitronectin and thrombospondin 1 and 2, proteoglycans biglycan, decorin, lumican, perlecan and syndecans 1 and 4, and metalloproteinases 2, 7, 12, 13, 14 and their inhibitors TIMP-1 and TIMP-2. We also studied the cellular receptors intercellular adhesion molecule-1 and neural cell adhesion-1, integrins α5, α6, β1, β3, betaglycan and laminin receptor 1. Among the cytokines were fibroblast growth factor-2, insulin-like growth factor-1, the β chain of platelet-derived growth factor-1, the β chain of platelet-derived growth factor, transforming growth factor-β1 and vascular endothelia growth factor. Genes coding for hyaluronan synthases 1 and 2, hyaluronidases 1, 2 and 3, and hyaluronan receptors CD44 and RHAMM (receptor for hyaluronan-mediated motility) were also included. Cellular markers desmin, fibulin-2, protease P-100 and reelin were studied, as well as the housekeeping genes β-actin, glyceraldehyde-3-phosphate dehydrogenase and 18S RNA. No significant differences were found by DNA arrays when hyiodine-treated and saline-treated tissues were compared, and this result was confirmed by real-time RT-PCR on selected genes (not shown).

Discussion

HA is present in the skin in a free form as a component of extracellular matrix (ECM) and in a bound form in the pericellular matrix. In the ECM, it is found between collagen and elastin fibres. HA is highly hydrated and regulates cell migration and the movement of nutrients and other soluble compounds. Its concentration increases in wounded skin, especially in the skin of fetuses. High molecular weight HA has beneficial effects on wound healing.

Hyiodine is a novel product combining high molecular weight HA and iodine. It is highly viscous and when applied to rat skin wounds in our experiments, it made wound redressing easier because the hyiodine-soaked gauze did not stick to the wound. Hyiodine applied to the wounds immediately after skin excision accelerated wound contraction in the first days of healing. Later on, the course of wound closure was similar in the hyiodine-treated and saline-treated group, indicating that the influence of added HA may be greatest in the proliferative phase of healing. The antiseptic properties of iodine may be more important in humans than in rats.

Wounds treated with hyiodine showed thickened epithelium on day 7. HA is a component of GT but its synthesis is not limited to mesenchymal cells. HA is contained in normal epidermis and is synthesized by epidermal keratinocytes. Epidermal injury activates hyaluronan synthases in keratinocytes and causes an increase in epidermal HA. Keratinocyte migration is retarded when hyaluronan synthesis is blocked. Exogeneous HA may support epithelial hyperplasia.

The role of iodine in wound healing is not clear, but PVP–iodine hydrogel was reported to improve epithelization.

Hyiodine application did not change collagen accumulation in the GT. The expression of other high molecular weight ECM components, proteinases and cytokines was not changed when studied on mRNA level. However, iodine greatly potentiated the ability of HA1200 to stimulate exudate formation. The protein composition of the exudate was similar to that of rat plasma with a prominent albumin band, suggesting that a large part of the exudate came from plasma. The uronic acid content in the exudate was also increased. HA is a normal component of wound fluid but some HA applied to the wound may have been retained on its surface. The nature of the interaction between iodine and HA is not clear. Iodine may bind to the glycosaminoglycan or it may oxidize it. The action of HA may be more powerful or its absorption may be slowed down and the effect of HA may be protracted in the presence of iodine.

Hyiodine may positively influence wound healing by its effect on wound epithelium. Accentuated exudation keeps the wound moist and makes wound redressing
easier. The influence of HA is supported by iodine, which is not acting only as a disinfectant but is also potentiating some of the effects of HA. The formation of GT and its main component, collagen, is not changed.

We examined how the mixture affected the properties of GT and the wound epithelia. We found accelerated wound contraction in the first days of healing. We found hypertrophy of epithelia but little change in the GT. Hyiodine does not seem to affect gene expression in GT. However, great enhancement of exudate production was seen compared with wounds treated with HA or KI3 alone.

Acknowledgements
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References
Wound healing through synergy of hyaluronan and an iodine complex

Hyaluronan, a glycosaminoglycan (GAG), is a polysaccharide found in many locations in the human body, such as eye, skin and soft tissue. It is also found in other mammals and bacteria. As a component of the extracellular matrix, its role in wound repair, among others, is that of providing a temporary structure to support new tissue formation. Harnessing the therapeutic action of hyaluronan into a topical application of proven clinical benefit has proved challenging. A new development in hyaluronan technology, comprising sodium hyaluronate and iodine complex, offers a novel approach in exploiting the benefits of hyaluronan and delivering real clinical benefits for a wide range of wound types.

Hyaluronan; moist wound healing; antimicrobial; iodine: tri-iodide

Hyaluronan, also known as hyaluronic acid or hyaluronate, is a naturally occurring polysaccharide, found in all connective tissues, which is believed to play a significant role in tissue repair and wound healing. In recent years, manufacturers developed a small number of products containing hyaluronan that, when applied to wounds, were anticipated to support/accelerate healing. Despite initial positive trial results, hyaluronan-based wound care products have yet to make a significant impact in wound care. One possible reason for this may be the occurrence of wound infection (empirical observation), associated with application of some hyaluronan-based dressings. A newly developed topical wound preparation containing both hyaluronan and an antimicrobial compound (iodine complex), potentially offers a novel and innovative solution to providing vastly improved clinical outcomes. This paper will explore the role of hyaluronan in wound healing, looking at the potential for antimicrobial protection and report on the clinical outcomes of using this preparation in practice.

Hyaluronan

Hyaluronan is a synthesised polysaccharide polymer composed of chains of the monosaccharide glucuronic acid and N-acetylglucosamine-1. It is part of a group of substances known as glycosaminoglycans. It is synthesised on the cell membrane by the action of the enzyme hyaluronan synthase, and then secreted into the extracellular space. A large variety of cells are able to produce hyaluronan; however, the most important, in terms of the wound healing response, appears to be keratinocytes, fibroblasts and platelets. Hyaluronan is found in nearly all connective tissue. In the dermis it is mainly situated below the basement membrane and around skin appendages. The skin normally contains the highest concentration of hyaluronan within the body; however, the levels of free hyaluronan within the blood are relatively low due to its rapid clearance by the liver.

As well as long chain hyaluronan (known as high molecular weight hyaluronan), shorter chain fragments can also be found in the body (low molecular weight hyaluronan). During tissue trauma or following infection, hyaluronan accumulates and stimulates immune cells at the injury site to express inflammatory genes, leading to the release of enzymes and free radicals, which break the long chain molecules; these fragments will be of differing molecular weight. The bioactivity of the fragments strongly depends on their molecular weight. Lower molecular weight hyaluronan induces the release of pro-inflammatory cytokines from the cell, while high molecular weight hyaluronan is able to repress this activity.

Endogenous hyaluronan is synthesised on the cell membrane through the action of hyaluronan synthase, and discharged directly into the extracellular space. Immediately after wounding, there is a sharp rise in hyaluronan caused by increased production and decreased removal of the compound, reducing again by day seven, post-injury. Higher levels

Declaration of interest

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were also found in newly formed granulation tissue. In early healing, high mass hyaluronan is accumulated and interacts with fibrin to form clots, thus stopping the bleeding process. Fragments from endogenous hyaluronan are formed by enzymes, which are produced by different skin cells.

It is thought hyaluronan has a number of interdependent functions in wound healing and is an integral part of the extracellular matrix (ECM), providing it with stability and elasticity. Hyaluronan is a highly hygroscopic (moisture retaining) compound; it attracts large amounts of water into the extracellular space, with multiple effects in wound healing. By maintaining a moist wound environment, hyaluronan protects cells from the effects of desiccation, also assisting in cell movement by helping the dividing cell to disassociate itself from its substratum and providing a hydrated matrix, which facilitates easier cell movement. After the cell has undergone mitosis and dissociation, and the epithelial cells mature and migrate, hyaluronan levels gradually return to normal.

In addition to the physiochemical effects of hyaluronan, it also affects the migration of fibroblasts to the wound site. In vitro studies have demonstrated that, in the presence of specific growth factors, the higher the levels of hyaluronan, the greater the cell migration in cell cultures, suggesting hyaluronan is key in the mediation of cell migration. Another dose-dependent effect of hyaluronan on cell migration is its interaction with hyaluronan receptors (CD44, ICAM-1 and RHAMM) through which it activates intracellular signalling pathways.

While hyaluronan is important for fibroblast migration, there is currently no evidence that it acts directly on mitogenic activity. However, high levels of hyaluronan are present during cell mitosis, and inhibition of hyaluronan synthesis leads to the prevention of cell mitosis and proliferation. It is believed that this is the result of its ability to promote the hydrated environment in the extracellular space that aids cell detachment.

Hyaluronan does appear to have an active role in the modification of the inflammatory response. Inflammation initiates the healing process but the inflammatory response needs to be moderated, otherwise tissue repair cannot proceed normally and granulation tissue cannot be stabilised. Cell migration and proliferation are both essential in promoting the inflammatory response. Hyaluronan is capable of modulating this response by both up- and downregulation. Hyaluronan appears to have an effect against free radicals, although its specific action is unknown. In addition, hyaluronan is thought to moderate the inflammatory response through its specific interactions with constituents of the inflammatory response, stabilising cytokine activity and reducing protease-induced damage.

Hyaluronan can both stimulate and inhibit angiogenesis, depending on the molecular weight of the hyaluronan molecule. High molecular weight hyaluronan inhibits hyaluronan angiogenesis, while low molecular weight hyaluronan stimulates the process. However, this action only occurs in the presence of an injury response, indicating that it is the interaction of hyaluronan with a number of cytokines and receptors that initiate this response. It is thought that hyaluronan promotes endothelial cell proliferation by binding with cellular CD44 receptors, and receptors for HA-mediated motility (RHAMM, CD168). This, in turn, leads to an increase in production of another hyaluronan receptor, ICAM-1. The binding of hyaluronan to this receptor leads to the mediation of endothelial cell function and hence the stimulation of angiogenesis.

How can topical hyaluronan influence clinical practice?

Hyaluronan, discovered in 1934, is particularly suitable for wound healing as it is non-antigenic, pro-angiogenic, and helps to attract lymphocytes to inflammatory sites through hyaluronan-associated endothelial tissue, thus supporting integral steps in the progression to wound closure.

Commercially, hyaluronan is produced by extraction from animal tissues or by bacterial fermentation from group A Streptococcus, where it is synthesised as a byproduct from the extracellular capsule. The moist healing environment created following topical application of hyaluronan allows for the evaporation of water through the carrier dressing into the environment. As the water concentration reduces, the hyaluronan attracts more water and growth factors from the surrounding tissues (Fig 1). High molecular weight hyaluronan, therefore, acts as a pump, with growth factors becoming increasingly concentrated in the wound and optimising healing potential.

Despite the potential benefits of using hyaluronan, there are factors limiting its action in clinical practice. It is accepted that the wound is not a sterile environment. All wounds are colonised with bacteria (bioburden) that can influence healing outcomes. The host immune system is mobilised to control bacterial proliferation and maintain a balance, which ensures that healing can be achieved; however, there are times when the host’s defences are overrun and bacterial numbers rise to the extent that competition for nutrients and the production of bacterial toxins negatively impact on wound healing. Chronic, hard-to-heal wounds play host to multispecies communities of bacteria (biofilms) and this is thought to be an underlying cause of non-healing. Management of bacterial burden by the optimisation of host defences and the reduction of bacterial numbers is accepted as an important tenet in wound care.

References
The effects of bacterial burden are particularly relevant when considering the use of topical, exogenous hyaluronan. The promotion of a moist environment by the hygroscopic action of hyaluronan, not only provides an ideal environment for host cellular proliferation and mobilisation, but also induces an environment in which bacteria can multiply. In addition, a number of common wound pathogens produce hyaluronidase, the enzyme responsible for the splitting of high molecular weight hyaluronan, as a byproduct.

While fragmentation of high-molecular weight hyaluronan occurs naturally within the wound, the rapid breakdown of the molecule changes the action of the material, affecting the potential benefits of its topical application. One strategy to manage this is to esterify the hyaluronan. This gives the product a greater resistance to the action of hyaluronidase but does affect the product’s hygroscopic action; the more esterified the hyaluronan, the lower its hydrophilicity. Another approach is to combine the hyaluronan with an effective antimicrobial compound that provides protection from hyaluronidase degradation, such as iodine.

**Iodine**

Iodine has been used for the prevention and treatment of infection for over 150 years. It has a broad spectrum of antimicrobial activity, rapidly inhibiting bacteria, yeasts, moulds, protozoa and viruses. Enveloped viruses, those derived from components of host cell membranes that assist viral entry into host cells, such as influenza, are more susceptible to iodine than non-enveloped viruses, likely due to the binding of iodine to the lipid component of the envelope. Iodine is even effective against endospore-forming bacteria. Inhibition of mycobacteria has also been reported. Metillin-resistant staphylococci and metillin-sensitive staphylococci have been shown to be equally susceptible to iodine, with as little as 0.1×10^−3 mg (236 000 molecules) of iodine sufficient to destroy one bacterial cell.

It is believed that binding of iodine to proteins leads to denaturing by the oxidation of S–H bonds in amino acids, and the prevention of hydrogen bonding. These changes affect the structure and function of both the structural integrity of the bacteria and its enzyme activity, and therefore have extensive deleterious effects on microbial function. Furthermore, membrane structure is compromised by the reaction of iodine with C=C covalent bonds in unsaturated fatty acids, while hydrogen bonding in nucleic acids is prevented by iodine binding to nucleotides. Hence, changes in cell walls, membranes and cytoplasm result in rapid death following exposure to iodine.

One of the most remarkable features of iodine as an antiseptic is the lack of a selection of resistant strains. Over the years only one report of iodine resistance has been published; however, this study has subsequently been criticized for its methodology and doubts raised over its reliability. Other studies have failed to detect resistance in MRSA, and attempts to train bacteria to become resistant by repeated exposure in the laboratory have failed, as there are attempts to detect iodine resistance in bacteria isolated from nosocomial infections.

Some concern has been raised over the side effects (pain on application) and toxicity of iodine in wound care. There is some experimental evidence from an animal study that iodine might reduce wound strength, although this needs to be considered within the framework of infection management and the potential deleterious effects of wound infection on tensile strength. Some studies have shown that iodine can have negative effects on tissue cultures, namely granulocytes, monocytes, keratinocytes, fibroblasts; however, other reports have suggested that this topical toxicity is probably not of clinical relevance and is dose dependent.

There have been a small number of case study reports, which suggest that the topical use of iodine-containing products may affect thyroid function. In a large review, there was no clear risk to healthy patients. Leaper and Durani discovered in their review of iodine that only minor anomalies were found in multiple papers, such as a rise in protein-bound iodine, but there were no changes in thyroid function tests. Serious complications have only been found in extensive exposure to iodine in high concentration (risk of thyroid dysfunction, hyperthyroidism or metabolic acidosis), whereas the risk to normal patients is minimal. Caution should be used in applying iodine-based products to those with known thyroid dysfunction or extensive burns, children, pregnant mothers or lactating mothers.

A report of an international consensus meeting on the use of iodine in wound care, organised by the European Tissue Repair Society, was largely supportive of iodine. It was deduced that slow-release forms of iodine can have negative effects on wound healing, whereas non-iodine preparations provide a beneficial moist environment to promote wound healing. The side effects of iodine have also been minimised by slow-release systems, thus reducing the risk of thyroid function tests. Serious complications have only been found in extensive exposure to iodine in high concentration (risk of thyroid dysfunction, hyperthyroidism or metabolic acidosis), whereas the risk to normal patients is minimal. Caution should be used in applying iodine-based products to those with known thyroid dysfunction or extensive burns, children, pregnant mothers or lactating mothers.

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**Fig 1. Function of external Hyaluronan in wound healing process**

- **Sodium hyaluronate**
- **Exudate draining, water evaporation**
- **Attraction of water**
- **Growth factors attracted by sodium hyaluronate**
mulations that generate low concentrations of iodine in a wound were effective and non-toxic. This has not been the case with other forms of antimicrobials, where concerns exist that emerging bacterial resistance and cytotoxicity are still issues.49

Allergic reactions to iodine antiseptic preparations are rare.50 If they do occur, they appear to be related to compounds used in association with the iodine, such as povidone in povidone-iodine.

An early form of iodine used in clinical wound care was Lugol’s solution, although, this formulation is no longer in widespread use.51 Gottardi states that Lugol’s solution is a complex of iodine with potassium iodide, consisting of 5g iodine (I2) and 10g potassium iodide (KI),52 mixed with distilled water to make 100ml of brown solution; it thus contains 5% iodine. In its free form, iodine is relatively unstable, does not readily solubilise and can irritate tissues53 and cause skin staining.

Cadenxmer iodine comprises a starch-derived modified dextrin carrier coupled with iodine in the form of an ointment, powder or dressing. It contains 0.9% iodine that is slowly liberated from the starch carrier.53 Other iodinated liquid or gel preparations contain 4–10% polyvinylpyrolidone iodine, giving an approximate equivalent of 0.4–1% slowly liberated iodine.51

Hyiodine

Hyiodine (Contipro) is a patented complex of 1.5% sodium hyaluronate (sodium salt of hyaluronic acid, C14H22N4O11Na), 0.15% potassium iodide (KI) and 0.1% iodine, which has been developed for the treatment of wounds. According to the manufacturer, the concentration (0.1% iodine) is low in comparison to undiluted Lugol’s solution, or other antiseptic preparations, in order to minimise the occurrence of irritation, while maintaining the antiseptic (protective) properties of iodine against bacteria that could cause hyaluronan degradation. Hyiodine is indicated for the treatment of a variety of acute and chronic wound conditions and is presented as a sterile, non-toxic 50ml solution and is CE-marked.

Within the solution, the hyaluronan is intended to promote a moist wound environment and maximise the healing potential by supporting cell viability and migration,52 keeping the extracellular environment hydrated,53 drawing growth factors from surrounding dermis into the wound, and supporting the formation of the scaffold/matrix components of new tissue (Fig 1).54 The addition of iodine is intended primarily to act as antimicrobial protection to hyaluronan (minimising the impact of bacterial degradation of the hyaluronan chain), while at the same time providing antimicrobial protection to the wound. This prolongs the availability of hyaluronan in the wound, optimising its action on the wound healing cascade and increasing its beneficial effect.

In laboratory studies Hyiodine has been shown to be non-toxic to human cells.54 An investigation of the effects of Hyiodine on the functional properties of isolated human keratinocytes and leucocytes, and on those of U937 and HL60 cell lines, showed that while potassium iodide complex inhibited the viability and proliferation of the cells tested, Hyiodine did not have any significant effect. The expression of CD11b, CD62L and CD69 on PMNL, monocytes and lymphocytes, as well as the oxidative burst of blood neutrophils, were not changed. On the contrary, Hyiodine inhibited the PMA-activated oxidative burst and significantly increased the production of IL-6 and TNF-alpha by lymphocytes. It was concluded that hyaluronan content of Hyiodine reduces the toxic effect of KI3 complex on cells and speeds up the wound healing process by increasing the production of inflammatory cytokines.54

Efficacy of Hyiodine

Testing on an animal wound model was undertaken by Slavkovsky et al.55 Hyiodine was applied to full-thickness wounds made on the back of rats. The areas of the contracting wounds were calculated from digital photographs, with the moving edges of the wound studied by histological methods. The properties of granulation tissue were studied in wounds in which contraction was prevented by the insertion of plastic rings. The effects of the product Hyiodine were compared with those of high molecular weight (1200KDa) hyaluronic acid, low molecular weight (11KDa) hyaluronic acid and potassium iodide solution. It was found that wounds treated with Hyiodine demonstrated accelerated wound contraction in the first 5 days of healing. By day 3, Hyiodine-treated wounds had reduced to 63% of the original area, whereas the wound area in saline-treated animals was 75% of the original size. By day 7, it was found that the proliferating epidermis was thicker in Hyiodine-treated animals. In the wounds with inserted rings, Hyiodine caused little change in granulation tissue, but the weight of the crust/exudate formed on the top of the wound was increased by 351% compared with only minor changes caused by the Hyiodine components alone.

The authors concluded that Hyiodine supports wound healing by stimulating wound contraction and epidermal proliferation and by keeping the wound moist through increased exudation.55 The limitations are implicit here as this was an animal (rat) model, not a human study and, as such, care should be taken when generalising the results.

Human studies in the clinical setting have shown equally promising results. In a non-controlled, observational study by Sobotka et al.,56 18 patients (all peripheral neuropaths) with complicated diabetic foot ulcers were treated with Hyiodine. The distribution of Wagner grades for the patients’ ulcers were grade I: one patient, grade II: seven patients, grade III:
seven patients, grade IV: three patients. None of the patients were suitable for intra-arterial angioplasty or surgical revascularisation. The inpatients (n=18) were on bed rest and instructed not to weight bear. The product was either applied directly to the wound bed or soaked into gauze, which was placed in the wound. The wound was then dressed with several layers of dry gauze. Daily dressing changes were carried out. Complete healing was evident in 12 patients within 6–20 weeks after start of treatment, depending on the wound character, localisation and extent. At the time of reporting, two patients were still being treated with Hyiodine but it was reported that significant improvement in their wounds was noted. Treatment was not successful in two subjects, both of whom developed ischaemic defects as a result of simultaneous arterial occlusion (not product related). It is not clear what previous treatments the patients had received; however, all were challenging cases where the authors conclude that the hyaluronan-iodine complex Hyiodine was found to be an efficient method for treatment of difficult-to-heal diabetic defects, without complete arterial occlusion,56 and state that additional controlled studies need to be undertaken.

A 49-patient observational report of Hyiodine use on undetermined chronic, non-healing wounds with concurrent signs of infection was undertaken by Wild et al.,57 although the previous treatments were not specified. The study recorded the effect of Hyiodine on granulation tissue formation and reduction of wound size. Within 2 weeks of Hyiodine treatment, granulation tissue was increased by 46% (40% vs 51%) and wound size reduced by 21% (1128mm vs 895mm) on average. Pseudomonas infection was found to be successfully treated, avoiding inhibition of wound healing.57

The first US study on Hyiodine confirmed the European findings. Fourteen inpatients, with 19 difficult-to-heal wounds, were enrolled in a prospective study at St. Mary’s Hospital, Connecticut.48 Fourteen wounds progressed to complete healing, with a mean healing time of 18.1±15.1 weeks. Treatment was discontinued in three patients (four wounds), as a result of patient relocation or non-concordance with the treatment. Nonetheless, the wounds responded very well and were close to complete healing. Unusually, one patient discontinued the treatment due to pain related application of Hyiodine. Structure of wounds in the study was: lower extremity venous leg ulcers (n=4), postoperative wound infections (n=5), diabetic foot ulcers (n=5, one of them complicated with osteomyelitis and MRSA), three traumatic wounds and one perianal fistula with abscess. One patient in the study had multiple bilateral venous ulcers, which provided an interesting opportunity for a ‘controlled’ trial. Left leg wounds, treated with Hyiodine, healed within 33 weeks. The ‘control’ wounds, non-Hyiodine wounds on the contralateral leg, were treated with papain-urea and only managed a reduction of 38% within the same time frame. Control trial of acute wound showed similar result. Complete healing of a wound treated with hyaluronate-iodine was achieved in 33 weeks, whereas control wound had 45% reduction during the same time period. In the conclusion of the study, it was stated that the healing rate varied among the different types of wounds, depending on their complexity and presence of infection. Nonetheless, healing rates were generally better than those obtained with previous, unspecified treatment. The majority of wounds (12/15) healed in 21 weeks or less, with five wounds healing in less than 7 weeks. Two wounds, which required 49 and 62 weeks to heal, respectively, were both lower extremity wounds complicated by severe oedema, which was difficult to control despite elevation and compressive therapy.58

**Conclusion**

Hard-to-heal wounds are encountered on a daily basis by clinicians involved in wound care. They pose a significant problem in terms of progressive patient morbidity, with associated high treatment costs in terms of time and resources used. A newly developed hyaluronan and iodine complex offers a novel solution through this combination approach in delivering positive benefits to patient and health provider.■

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