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 KI_3 (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 KI_3 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.[1] 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.[2,3] 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.[4] The content of HA changes during skin repair, and in the wound fluid of adults is at its highest 2–4 days after injury.[5] 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

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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.[13] 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 evaluated 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 multiangle 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 shaven and disinfected. A full-thickness excision 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

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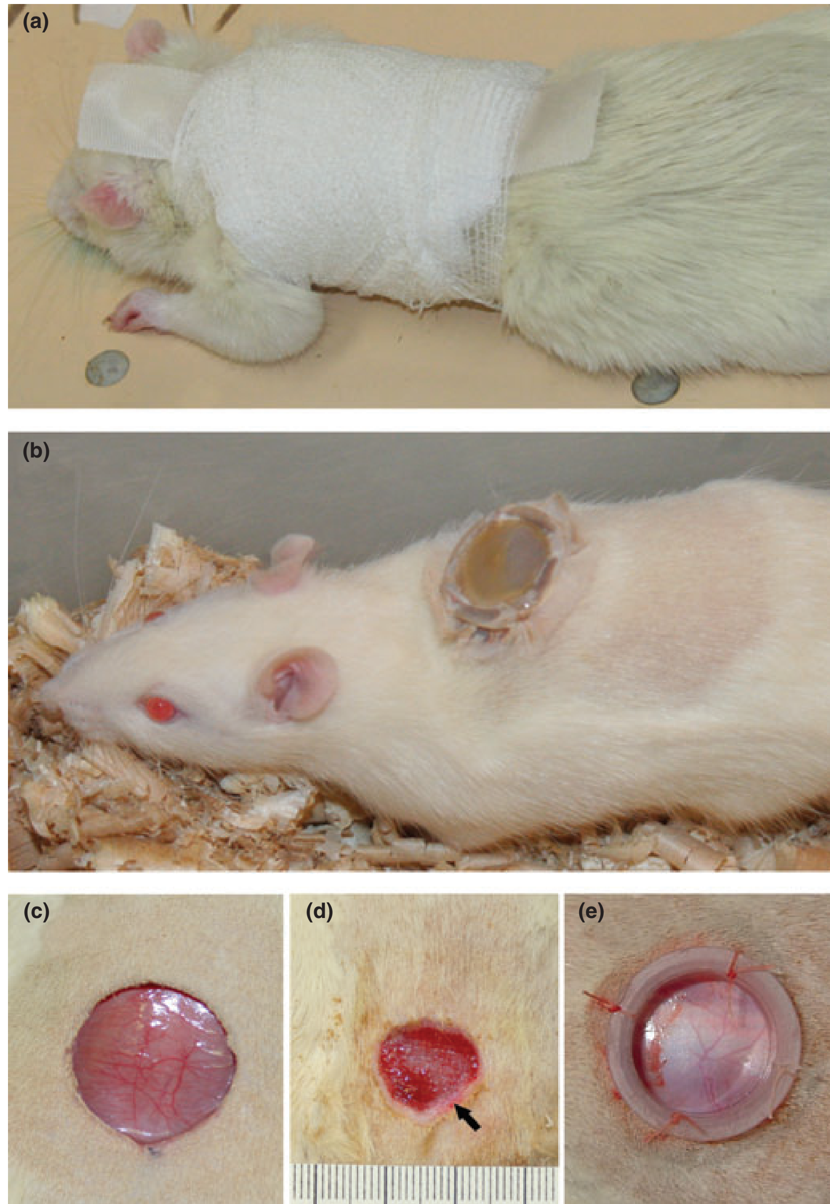

Figure 1 Wounds on rat backs. (a) Rat with a contractible wound bandaged with gauze; (b) rat with a permanent wound with an inserted plexiglass ring; (c) contractible wound, day 0; (d) hyiodine-treated contractible wound, day 7; (e) permanent wound, day 0. The arrow indicates proliferating epithelium.

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[19] as modified by Hurych and Chvapil.[20] 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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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.*[21] 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[22] 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.[23] 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 $\alpha = 0.05$.

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 KI_3 (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 KI_3 (hyiodine) resulted in a 18% increase

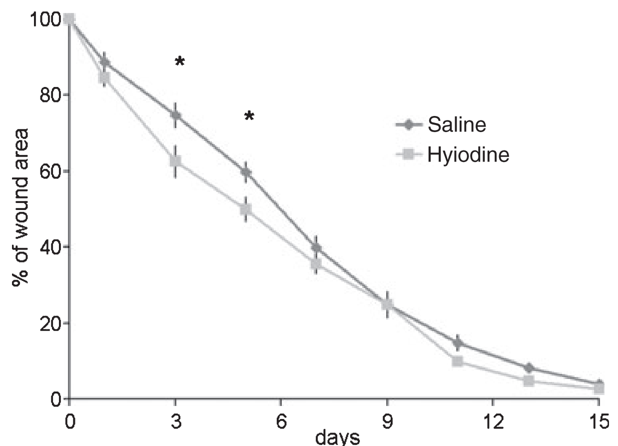

Figure 2 Wound area as a percentage of the original area measured immediately after wounding. Data are means ± SEM. *Significant results.

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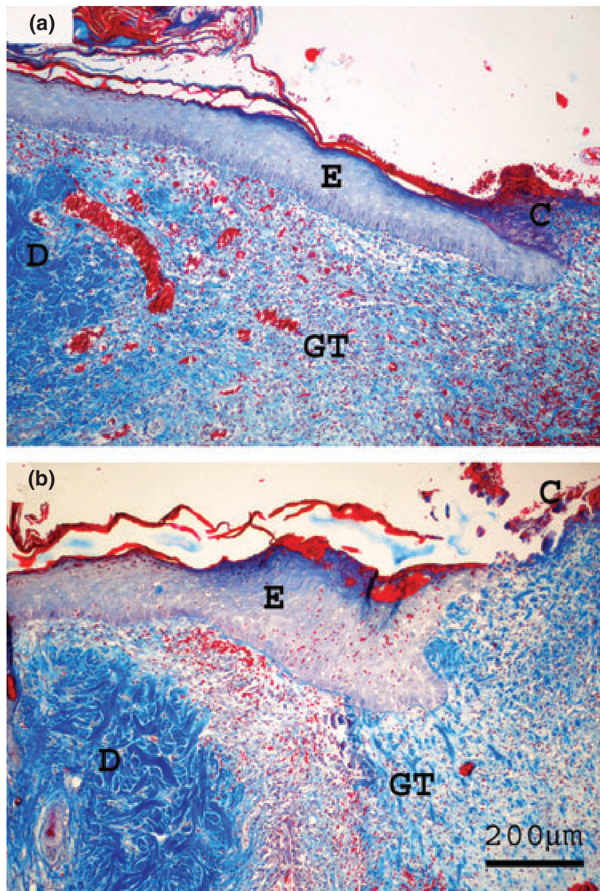

Figure 3 Histological sections of wounds treated with (a) saline and (b) hyiodine. B, blue trichrome; C, crust; D, dermis; epithelium; GT, granulation tissue.

in the wet weight of the GT compared with saline treatment. The changes caused by HA1200 and by KI_3 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 KI_3 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).

Table 1 Changes in granulation tissue weight, dry weight and hydroxyproline content after treatment of the wounds with saline, hyaluronan, iodine or hyiodine.

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

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

Table 2 Changes in the weight, protein and uronic acids content in the crust and exudate after treatment with saline, hyaluronan, iodine or hyiodine.

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

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

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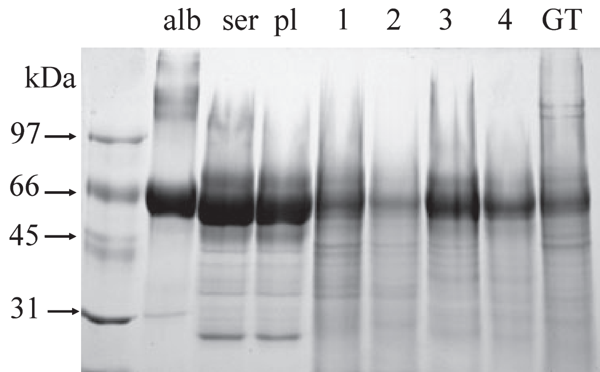

Figure 4 Gel electrophoresis of proteins contained in the crust/exudate. Alb, bovine serum albumin; ser, rat blood serum; pl, rat blood plasma; lanes 1–4, different samples of crust/exudate after treatment with hyiodine. GT, granulation tissue.

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, 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.[3] Its concentration increases in wounded skin, especially in the skin of fetuses. High molecular weight HA has beneficial effects on wound healing.[3]

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.[24] Exogeneous HA may support epithelial hyperplasia.[8] The role of iodine in wound healing is not clear,[12] but PVP–iodine hydrogel was reported to improve epithelialization.[25]

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

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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 KI_3 alone.

Acknowledgements

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