A novel formulation of metal ions and citric acid reduces reactive oxygen species *in vitro*

● **Objective:** Reactive oxygen species, including superoxide anions, are thought to play an important role in impairing wound healing. Additionally, superoxide anions react with nitric oxide produced by macrophages to form peroxynitrite, another strong oxidant with detrimental effects on surrounding tissue. This *in vitro* study investigated whether samples of metal ions and citric acid are able to reduce levels of reactive oxygen species.

● **Method:** Samples of materials were tested in assays for the following: inhibition of reactive oxygen species production by human polymorphonuclear neutrophils (PMNs); antioxidant activity (scavenging of superoxide anions in a cell-free system); inhibition of human complement (limiting the generation of complement factors that attract and stimulate PMNs, thereby reducing levels of reactive oxygen species).

● **Results:** Metal ions were shown to inhibit both PMN production of reactive oxygen species and the activation of complement via the classical pathway, whereas citric acid was found to be a scavenger of superoxide anions.

● **Conclusion:** The beneficial effects of using formulations containing metal ions and citric acid on chronic wounds may be explained in part by a reduction of reactive oxygen species in these wounds.

● **Declaration of interest:** The research was financed by Greystone Medical Group.

metal ions; citric acid; human polymorphonuclear neutrophils; antioxidant activity; human complement

A novel technology based on a composition of metal ions has been developed to improve wound healing. This *in vitro* study investigates whether samples of metal ions and citric acid can play a role in the healing of chronic wounds, specifically by inhibiting reactive oxygen species formation and due to their antioxidant activity.

Background

Unpublished data suggest that metal ions improve epithelialisation in partial-thickness excision wounds and contact burn wounds. A preliminary clinical study showed that a preparation containing metal ions and citric acid (DerMax, Dermagenics Europe BV, The Netherlands) was most effective on chronic wounds.[1] This pilot study involved four patients with non-healing wounds of more than 45 days' duration with a surface area greater than 1cm².
Application of DerMax resulted in loss of the fibro-necrotic cap covering the wound bed, re-activation of fibroblasts in the granulation tissue and a sharp decline in the expression of fibroblast MMP-2.[1]

For successful chronic wound management, modulation of matrix metalloproteinase (MMP) metabolism appears to be an important target.[2,3]

Free radicals

Free radicals are also implicated in impaired wound healing.[4-6] In both acute and chronic wounds, they cause cell damage and may inhibit healing. In chronic wounds, xanthine oxidase catalyses the conversion of oxygen into superoxide anions (free radicals toxic to tissue), although these are also produced by stimulated polymorphonuclear neutrophils (PMNs).[4,5] This results in formation of other reactive oxygen species, including hydroxyl radicals and the non-radical oxidant, hypochlorous acid.

Furthermore, superoxide anions react with the radical nitric oxide produced by macrophages — another inflammatory cell in the wound bed — to form peroxynitrite, which is also detrimental to surrounding tissue.[7]

Also, free radicals have been implicated in hypertrophic scar formation following thermal injury.[8]

It seems natural, therefore, to conclude that scavenging superoxide anions and inhibiting PMN production of reactive oxygen species will aid chronic wound management.

The complement system

The complement system is part of the non-adaptive humoral immune system and plays an important role in our defence mechanism.[9]

Activation of complement via either the classical, alternative or lectin pathway eventually leads to the formation of the high-molecular membrane attack complex that causes death of bacteria (or foreign red blood cells) through lysis.[10]

In addition, small split products are generated, which mediate many immunoregulatory effects. In this respect, complement factor C3b has a major bio-

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logical function as (pathogenic) micro-organisms and foreign particles (zymosan) are covered with C3b (opsonisation). This enables phagocytes with receptors for C3b (for example, PMNs) to recognise, ingest and destroy these invaders by producing reactive oxygen species. Fragment C5a is another activating agent for PMNs and is also a major chemotactic factor for these phagocytes.[11] Thus, inhibition of complement may promote wound healing by reducing levels of reactive oxygen species. This can be achieved by limiting the factors involved in PMN recruitment and activation.

PMN production of reactive oxygen species

Polymorphonuclear neutrophils, when recruited to the wound site and activated, consume an increased amount of oxygen, which is converted into reactive oxygen species. This process, known as respiratory burst, depends on NADPH oxidase, an enzyme that uses a reduced form of NADP or nicotinamide-adenine dinucleotide phosphate as a co-enzyme. This NADPH oxidase can be activated by both receptor-mediated and receptor-independent processes.[12,13]

Typical receptor-dependent stimuli include:
- Complement components C5a and C3b
- Bacterium-derived chemotactic tripeptide fMLP
- Opsonised zymosan.

Receptor-independent stimuli include long-chain unsaturated fatty acids.[14-17]

On activation of the PMNs, the multi-component NADPH oxidase is assembled in the cell membrane. The oxidase then transfers electrons from NADPH at the cytosolic side of the membrane to molecular oxygen at the other side of the membrane. This results in the generation of superoxide anions either in (intracellular) phagosomes containing ingested micro-organisms, or extracellularly. Most of the superoxide anions formed are converted into hydrogen peroxide.[18]

Some hydrogen peroxide is converted into extremely reactive hydroxyl radicals via the iron-catalysed Fenton reaction.[19] However, most is converted into hypochlorous acid, the most bactericidal oxidant known to be produced by PMNs. The latter conversion occurs in the presence of halide (chloride) ions and is catalysed by myeloperoxidase, an enzyme also released by activated PMNs.[20]

Although in the phagolysosome, intracellular reactive oxygen species — together with proteolytic and other cytotoxic enzymes released from lysosomes (granules) — kill ingested bacteria and prevent wound infection, excessive extracellular generation of these oxygen metabolites will have detrimental effects on surrounding tissue.

Production of nitric oxide by macrophages at the wound site is also significant. The radical nitric oxide may easily react with superoxide anions, resulting in the formation of peroxynitrite, an extremely potent, relatively stable oxidant with properties similar to those of the hydroxyl radical.[7,21]

Method

Samples of material (metal ions and citric acid) were tested in assays for:
- Inhibition of PMN production of reactive oxygen species (including superoxide anions)
- Scavenging of superoxide anions (antioxidant activity)
- Inhibition of complement activity.

Polymorphonuclear neutrophils

Inhibition of reactive oxygen species production by PMNs was determined using the chemiluminescence assay.

Human PMNs were activated with opsonised zymosan. Zymosan (cell walls of baker's yeast) was treated with human serum (opsonisation) before use, which results in C3b-covered particles. Polymorphonuclear neutrophils have membrane receptors for C3b, so opsonised zymosan is easily recognised and ingested by these phagocytes.

On activation, PMNs start producing reactive oxygen species, which can be measured as light in a luminometer, with luminol as the light enhancer.

To exclude cell death as the cause of inhibition, we investigated whether or not the test samples had any cytotoxic effects on the PMNs.

Superoxide anions

Scavenging activity was determined in an assay without cells by incubating hypoxanthine with xanthine oxidase, which results in generation of superoxide anions. The latter were measured as light in a luminometer, this time using lucigenin as the light enhancer. Lucigenin is more specific to superoxide anions, whereas luminol detects all reactive oxygen species.

Inhibition of complement

Complement was activated by incubation of antibody-coated sheep erythrocytes (classical pathway) and rabbit erythrocytes (alternative pathway) with human serum. Eventually, activation of complement results in lysis of these foreign red blood cells. The amount of haemoglobin released was measured spectrophotometrically and used to determine complement activity.

Test materials

The metal ions tested were derived from red oak bark burnt into an ash, boiled with water and concentrated to 5% solids after filtration. Since this metal ion solution is too alkaline for use in preparations for wound management (DerMax), its pH is lowered to 5.0 by adding citric acid. ▶

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The three test samples used in the study were:
● Metal ions acidified with citric acid to pH 5.0 (MI-CA5), representing the preparation clinically tested
● Metal ions neutralised with hydrochloric acid to pH 7.0 (MI-HA7) to exclude effects caused by citric acid or a low pH. In fact, MI-HA7 represents the metal ions only. The slight increase of chloride ions will not influence activity testing as buffers used in the assays also contain chloride.
● Citric acid — being an organic acid, citric acid was also tested on its own to investigate its contribution to the total activities of MI-CA5.

Assays
● **Chemiluminescence assay (inhibition of reactive oxygen species production by human PMNs)** Polymorphonuclear neutrophils were isolated from venous blood of healthy volunteers as described by Verbrugh et al.[22]

Experiments were performed in Hank's balanced salt solution (HBSS) buffered at pH 7.35 with $NaHCO_3$ and supplemented with 0.1% (w/v) gelatin to avoid cell aggregation (HBSS gel).

Opsonised zymosan A was obtained by incubating zymosan A with human pooled serum at 37°C for 30 minutes.

Test samples were serially diluted with HBSS gel to final volumes of 50µl (concentration range: 250–0.11µl/ml for metal ion preparations and 250–0.11µg/ml for citric acid) in white, 96-well, flat-bottom microtiter plates. To each well, we added 50µl of PMN suspension in HBSS gel (1x10^7 cells/ml) and 50µl of luminol (120µM in HBSS gel).

Polymorphonuclear neutrophils were triggered to produce reactive oxygen species by adding 50µl of optimised zymosan A (final concentration: 200µg/ml). Chemiluminescence was monitored for 0.5 seconds every two minutes over a 30-minute period using a Titertek Luminoskan luminometer (TechGen International, Zellik, Belgium). Peak levels of chemiluminescence of test samples and controls (identical incubations with 50µl of HBSS gel without sample) were used to calculate the inhibition of reactive oxygen species production.
● **Cytotoxicity assay** To exclude the possibility that inhibition in the chemiluminescence assay was due to the elimination of the PMNs, we investigated the cytotoxic effects of the test samples.

A stock solution of 5-carboxyfluorescein diacetate (10mg/ml) in acetone was prepared and stored at -20°C. Before use, this was diluted 1:1000 in HBSS gel. Propidium iodide (1.5mg) was dissolved in 10ml of phosphate-buffered saline (pH 7.4). Polymorphonuclear neutrophils were labelled with the vital stain 5-carboxyfluorescein diacetate (10µg/ml) at 20°C for 15 minutes, washed and resuspended in HBSS gel to a concentration of 10^7 cells/ml.

Samples (100µl) of this cell suspension were incubated with equal volumes of the test samples serially diluted in HBSS gel (concentration range: 250–0.11µl/ml) at 37°C for 15 minutes. Cells were then stained with 25µl of propidium iodide solution to discriminate between viable (green-fluorescent) and dead (red-fluorescent) cells. The percentage of dead cells was determined using a fluorescence microscope (Fluovert, Leitz, Wetzlar, Germany).
● **Superoxide anion scavenging assay** Test samples were serially diluted in phosphate-buffered saline (pH 7.4) to a final volume of 50µl (concentration range: 250–0.11µl or µg/ml) in white, 96-well, flat-bottom microtiter plates. Then hypoxanthine (50µl; final concentration 1mM), lucigenin (50µl; 0.4mM [0.4 millimolar or 0.4 milligrammolecule of lucigenin in one litre) and either buffer or superoxide dismutase (25µl; 80units/ml) were added.

Superoxide anion radical production was initiated by adding 25µl xanthine oxidase (80mU/ml). Chemiluminescence was monitored every minute for 0.5 seconds over 15 minutes, using a Fluoroskan Ascent FL luminometer (Labsystems, Breda, The Netherlands). Activity of the test compounds was calculated from the superoxide dismutase inhibitable part of the chemiluminescence signal.

In this assay, superoxide anions are generated by enzymatic conversion of hypoxanthine into uric acid. Inactivation of the enzyme xanthine oxidase will also result in inhibition of the chemiluminescence signal, but not due to scavenging effects. Thus, the samples' effects on xanthine oxidase were excluded by monitoring uric acid formation spectrophotometrically at 290nm.
● **Haemolytic assays for human complement activity (classical and alternative pathway)** Inhibitory activities of test samples on the classical and alternative pathways of human complement were determined using a modified version of the micro assay described by Klerx et al.[23]

Sheep or rabbit blood in Alsever solution served as sources of erythrocytes. Before use, erythrocytes were washed three times with saline.
● Sheep erythrocytes were sensitised by incubation with diluted amboceptor (1:800) for 10 minutes. After washing the sensitised erythrocytes were resuspended in veronal saline buffer/classical pathway (5mM veronal and 150mM saline; pH 7.35; supplemented with 0.15mM Ca^{2+} and 0.5 mM Mg^{2+}; 4×10^8 cells/ml)
● Rabbit erythrocytes were resuspended in veronal saline buffer/alternative pathway (buffer as above, this time supplemented with 0.5mM Mg^{2+} and 0.8mM EGTA; 3×10^8 cells/ml).

In U-well microtiter plates, the test samples were serially diluted in:
● Veronal saline buffer/classical pathway to final volumes of 50µl (concentration range: 333–0.15 µl/ml)

Fig 1. Formation of reactive oxygen species

PMN=polymorphonuclear neutrophil
Mθ=macrophage
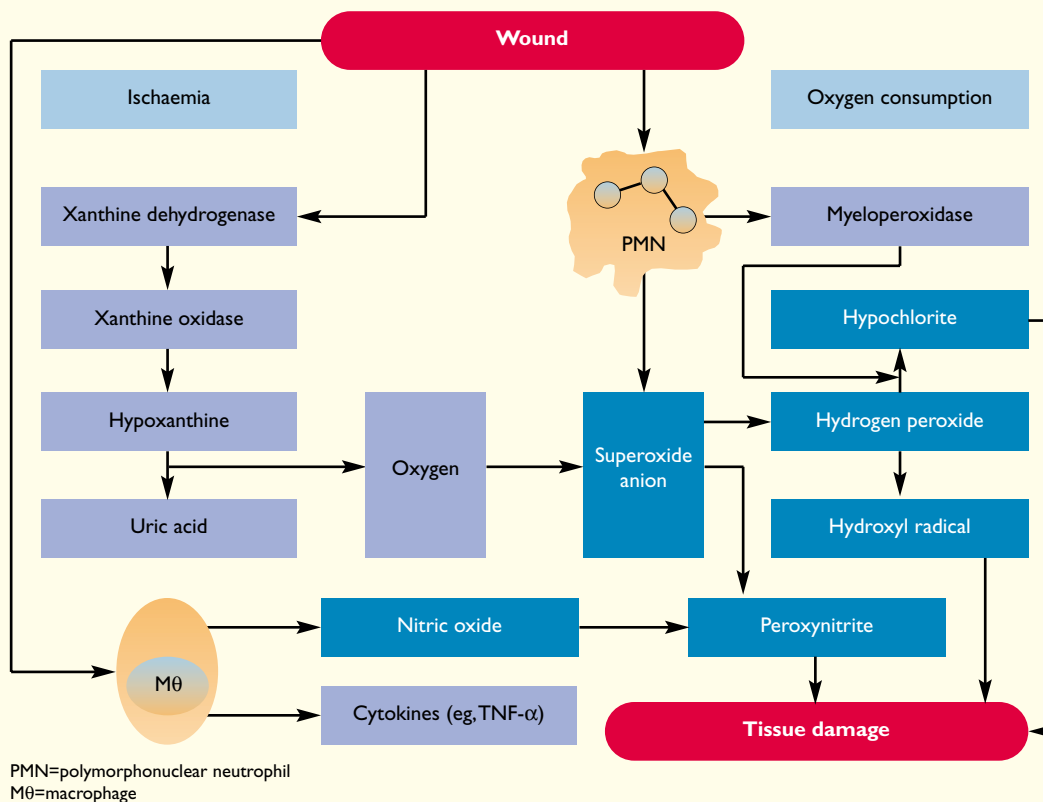
• Veronal saline buffer/alternative pathway to final volumes of 100μl (concentration range: 333–0.15 μl/ml).

Subsequently, 50μl (classical pathway) or 25μl (alternative pathway) of appropriate dilutions of human pooled serum were added to the microtiter plates, which were then incubated at 37°C for 30 minutes.

After adding 50μl of sensitised sheep erythrocytes (classical pathway) or 25μl of rabbit erythrocytes (alternative pathway), the plates were incubated again at 37°C (classical pathway: one hour; alternative pathway: 30 minutes).

Finally, the microtiter plates were centrifuged (900 x g, five minutes) to spin down intact cells and debris, and 50μl of the supernatants were transferred to 96-well flat-bottom microtiter plates containing 200μl of water per well.

In the latter plates, the amount of haemoglobin released by lysis of erythrocytes was measured spectrophotometrically using an automatic ELISA reader operated at 405nm.

Controls consisted of similarly treated supernatants of erythrocytes incubated with water (100% haemolysis) or buffer (veronal saline buffer/classical pathway or veronal saline buffer/alternative pathway; 0% haemolysis), and incubates in which human pooled serum was replaced with heat-inactivated HPS (56°C, 30 minutes; correction for the background colour of test samples).

Presentation of activities

Activities are given as IC50 values, representing the mean ± standard deviation (SD) of n determinations. The IC50 value is the concentration of the test sample required to achieve a 50% reduction in response. Therefore, low IC50 values represent strong activities.

Results and discussion

To summarise, the three test materials were:
• Metal ions neutralised with hydrochloric acid to pH 7.0 (MI-HA7)
• Metal ions acidified with citric acid to pH 5.0 (MI-CA5)
• Citric acid.

Inhibition of PMN production of reactive oxygen species

This study examined the inhibitory effects of the above three test materials on the production of reactive oxygen species by stimulated human PMNs. For MI-CA5 an IC50 value of 12 ±2μ/ml (n=6) was determined, for MI-HA7 it was 27 ±8μl/ml (n=5) and for citric acid it was >250μg/ml (n determinations were obtained with two batches of PMNs from two different donors).

Since inhibitory effects in the assay for reactive oxygen species production may be caused by cell death, potential cytotoxic effects of the test samples were investigated.

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We found that incubation with 100µl/ml MI-CA5 did not have any cytotoxic effects on PMNs, when compared with control cells similarly incubated with buffer (HBSS gel), whereas the presence of 100µl/ml MI-HA7 resulted in 20–30% cell death. The latter was also observed for metal ions neutralised to pH 7.0 with citric acid instead of hydrochloric acid.

We concluded that inhibition of reactive oxygen species production by MI-CA5 was not caused by cytotoxic effects on PMNs. The IC50 value determined for MI-HA7 (27µl/ml) concerned at least 80% viable cells, so most likely reflects real inhibition of production of reactive oxygen species.

Superoxide scavanging

Superoxide anions may also arise in chronic wounds, where ischaemic conditions may convert the enzyme xanthine dehydrogenase into xanthine oxidase, which catalyses the conversion of oxygen into superoxide anions.[4,5]

Therefore, antioxidant activity, including scavenging of superoxide anions, either produced by the PMNs or through xanthine oxidase, can be regarded as beneficial for the treatment of chronic wounds.[5,6]

MI-CA5 was also shown to be a significant scavenger of superoxide anions (IC50 value 7 ±3µl/ml, n=9). With citric acid, an IC50 value of 49 ±6µg/ml (n=3) was found. In contrast, the activity of MI-HA7 was poor or absent (IC50 >250µl/ml). We conclude that scavenging of superoxide anions by MI-CA5 is mainly due to the presence of citric acid.

Inhibition, as found in the assay for reactive oxygen species production, may also be caused by unspecific scavenging of superoxide anions. The fact that MI-HA7 did not scavenge superoxide anions but inhibited reactive oxygen species production by stimulated PMNs (IC50 27µl/ml) indicates that the metal ions themselves have a direct effect on PMN functioning. The increase in activity observed in the latter assay for MI-CA5 (IC50 12µl/ml) was most probably due to additional scavenging of superoxide anions by citric acid.

Although the scavenging and inhibitory activities of MI-CA5 on reactive oxygen species production may seem moderate when compared with other compounds tested in our laboratory (such as apocynin), the amounts of metal ions and citric acid in clinically applied formulations are quite high, and therefore considered to be effective in this respect.[21]

Both the superoxide anion-scavenging properties of citric acid and the metal ions' inhibition of reactive oxygen species production by the PMNs provide a scientific basis for the use of MI-CA5 in chronic wound management.

Inhibition of complement

Finally, MI-HA7 was tested in the haemolytic assays for modulation of complement activity. MI-HA7 showed significant inhibition of classical pathway complement activity (IC50 value 10 ±5 µl/ml, n=3), whereas activation of complement via the alternative pathway was much less affected (IC50 value 107 ±4µl/ml, n=3).

MI-CA5 resulted in brown colouration of target cells (erythrocytes) in the test system. Activities determined for MI-CA5 were therefore regarded as invalid, and are not reported here.

Inhibition of complement activation will limit the generation of complement split products such as C5a. As outlined above, this may result in less influx and decreased stimulation of PMNs in the wound bed, and therefore reduced extracellular formation of reactive oxygen species as well as peroxynitrite. The end result may be reduced tissue damage.

In general, decreased PMN recruitment will reduce the number of bacteria killed, which may lead to infection. In chronic wounds, however, limitation of PMN influx is favourable as too many of these inflammatory cells are already present.

Several compounds are known to inhibit complement activation — for example, certain polysaccharides or triterpenoids.[24] The activity of metal ions encountered here may point to a new class of complement inhibitors.

Summary

Although other factors governing wound healing may also be important, such as MMPs, these results show that metal ions inhibited human complement activation via the classical pathway and inhibited production of reactive oxygen species by activated PMNs, and that citric acid scavenged superoxide anions.

In addition, incubation of PMNs in up to 100µl/ml MI-CA5 did not have any cytotoxic effects. Unpublished data suggesting the beneficial effects of formulations containing metal ions and citric acid (DerMax) on chronic ulcers may be in part explained by a reduction of reactive oxygen species in these wounds.[1]

Future research

DerMax is currently being tested in clinical studies. In vitro effects of this preparation on nitric oxide and cytokine production by macrophages, which we consider other relevant targets for wound healing, are being investigated. Future research should also include animal experiments concerning the impact on PMN recruitment. The synthetic version of metal ions has already been developed and is being tested in vitro and in animal models. We expect that formulations based on this special metal ion composition and citric acid will offer an effective alternative for patients with wounds that are not responding to conventional treatment. ■

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