Title: TREATMENT AND PREVENTION OF LIPODERMATOSCLEROSIS

Abstract: The invention provides the use of an iron chelating agent for the preparation of a composition for use in the prevention or treatment of lipodermatosclerosis by topical application to the lower leg.
TREATMENT AND PREVENTION OF LIPODermatosclerosis

The present invention relates to the use of iron scavenging compounds for the preparation of compositions for use in the treatment and prevention of lipodermatosclerosis.

In the United Kingdom, 10-20% of the adult population is affected by the complications of venous disease, including deep vein thrombosis, varicose veins, lipodermatosclerosis and venous ulceration. Approximately 50% of all venous ulcers are present for periods greater than nine months and two-thirds are recurrent. Venous problems are not only limited to the elderly, since greater than 40% of patients form venous ulcers before the age of 50. Venous disease consumes approximately 2% of the total annual healthcare budget in the UK.

In patients with venous disease, the valves in the veins of the lower limb become incompetent, generally due to deep vein thrombosis and/or superficial vein incompetence, resulting in venous hypertension on exercise and the retrograde flow of blood through the superficial venous system (venous reflux) to the blood vessels in the skin. Chronic venous disease often progresses from defective valves and the appearance of varicose veins, to the development of skin changes in the lower leg and finally to the formation of venous ulcers. Despite the frequency of venous disease, surprisingly little is understood of the mechanisms by which chronic venous hypertension leads to venous ulceration.

Many patients with venous insufficiency develop skin changes in the lower leg known as lipodermatosclerosis (LDS). LDS is also known as hypodermitis sclerodermaformis, liposclerosis, hypodermatosclerosis sclerodermiformis, lipomembranous change in chronic panniculitis and sclerosing panniculitis. LDS skin is characterised by skin induration or thickening and brown pigmentation caused by haemosiderin deposition. These features often occur in skin before ulceration as well as in skin surrounding an ulcer. The degree of skin thickening (induration or fibrosis) is thought to be directly related to venous ulcer formation and subsequently delayed healing. Removal of the fibrotic LDS skin surrounding
venous ulcers by debridement often leads to increased ulcer healing. Treatment of this problem has for many years relied on established principles of compression and limb elevation and drug treatment has been of little benefit. Yet there is clearly a role for drugs in the management of venous disease either alone or in combination with compression therapy. It is likely to be beneficial to intervene in the initial processes that cause the lipodermatosclerotic skin changes leading to ulceration rather than to modify the repair processes when an ulcer has formed. The same processes that caused the ulcer are likely to contribute to its perpetuation and delayed healing.

The present invention relates to the inhibition of the effects of excess iron deposition in the skin following chronic venous insufficiency. The present inventors have found that excess iron may have a much larger influence on the early stages of lipodermatosclerosis than previously thought.

During venous insufficiency, repeated damage of cutaneous blood vessels due to venous hypertension, causes the leakage of blood proteins and erythrocytes. The damaged erythrocytes deposited in the interstitial tissue are thought to be cleared by dermal macrophages and the iron derived from haemoglobin stored within the cells as ferritin and in haemosiderin granules.

Ackerman et al., in 1988 performed x-ray spectrometry, a method based on x-ray fluorescence analysis, for non-invasive determination of iron levels in the skin of sixteen patients with venous ulceration. The mean (± SEM) iron concentration in the skin around the venous ulcer was elevated, compared with control values of nonulcerated skin (250±54 vs 128±39µg) and compared with normal skin from the forearm (250±54 vs 14±2.5µg). This group suggested that dermal iron deposition may not be an incidental by-product of increased venous pressure, but may actively perpetuate tissue damage in venous ulcerations. However, attempts to treat venous ulcers with inhibitors of the effects of excess iron have not been overly effective. The present inventors have found that there is little evidence of haemosiderin deposition in ulcer base tissue. It is thought that elevated levels of
iron in ulcer wound fluid may be due to iron leaching into the wound fluid, rather than to iron accumulation in the ulcer tissue itself.

It is known that dissolved iron in high concentrations is cytotoxic because the iron catalyses the formation of oxygen free radicals. The present inventors have found that even quite low concentrations of iron below the threshold for cytotoxicity can cause increased collagen production which may explain the fibrotic skin changes seen in LDS. It therefore seems that low levels of dissolved iron play an important part in the early stages of venous disease, in particular the fibrotic stages of lipodermatosclerosis. It further appears that removal or sequestering of dissolved iron could be an effective method to prevent or treat the fibrotic symptoms of lipodermatosclerosis.

In a first aspect the present invention provides the use of an iron chelating agent for the preparation of a composition for use in the prevention of lipodermatosclerosis by topical application to the lower leg.

In a second aspect the present invention provides the use of an iron chelating agent for the preparation of a composition for use in the treatment of lipodermatosclerosis by topical application to the lower leg.

The term “chelating agent” refers to a chemical moiety that functions as a bidentate or multidentate ligand for dissolved iron. Chelating agents which may be used herein should be safe for topical application and should exhibit a sufficiently high binding coefficient with dissolved iron to lower the concentration of dissolved iron in vivo and should neutralise the fibrotic effect of the iron when the iron is bound thereto. The chelating agent should also be capable of penetrating into the skin following topical application. By "safe" is meant chelating agents which may be used topically, at typical usage levels for extended periods of time, without causing any significant adverse skin reactions or other side effects.

The chelating agents useful in the present invention may be classified according to their donor groups; see The Design and Synthesis of Chelating Agents". 
Development of Iron Chelators for Clinical Use, Martell, Anderson and Badman, eds., Elsevier North Holland, Inc., New York, N.Y. (1981), pp. 67-104, which is hereby incorporated by reference. Because some chelating agents have more than one type of donor group, they may fall into more than one of the classes defined in this reference.

Preferably, the chelating agent is selected from the group consisting of aromatic amines, carbonyls, oximates, amines, carboxylates, alkoxides, enolates, phenoxides, catecholates, hydroxy acids, hydroxamates, ketoenolates, mercaptides, hydroxy aromatic amines, aromatic hydroxy acids, and mixtures thereof.

Preferably, the chelating agent is selected from the group consisting of aromatic amines, carbonyls, oximates, enolates, phenoxides, catecholates and hydroxylates, and mixtures thereof.

Aromatic amine chelating agents preferably are selected from the group consisting of: 2,2'-dipyridylamine; 1,10-phenanthroline (o-phenanthroline); di-2-pyridyl ketone; 2,3-bis(2-pyridyl) pyrazine; 2,3-bis(2-pyridyl)-5,6-dihydropyrazine; 1,1'-carboxyldiimidazole; 2,4-bis(5,6-diphenyl-1,2,4-triazine-3-yl)pyridine; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 4,4'-dimethyl-2,2'-dipyridyl; 2,2'-biquinolone; di-2-pyridyl glyoxal (2,2'-pyridiil); 2-(2-pyridyl)benzimidazole; 2,2'-bipyrazine; 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine; 3-(4-phenyl-2-pyridyl)-5-phenyl-1,2,4-triazine; 3-(4-phenyl-2-pyridyl)-5,6-diphenyl-1,2,4-triazine; 2,3,5,6-tetrakis(2'-pyridyl)-pyrazine; 2,6-pyridinedicarboxylic acid; 2,4,5-trihydroxyprymidine; phenyl 2-pyridyl ketoxime; 3-amino-5,6-dimethyl-1,2,4-triazine; 6-hydroxy-2-phenyl-3(2H)-pyridazinone; 2,4-pteridinediol (lumazine); 2,2'-dipyridyl; and 2,3-dihydroxypyridine.

More preferably the aromatic amine chelating agent is selected from the group consisting of: di-2-pyridyl ketone; 1,1'-carboxyldiimidazole and 2,2'-pyridiil.

Oximate chelating agents preferably are selected from the group consisting of: 2-furilidioxime; phenyl 2-pyridyl ketoxime; and 1,2-cyclohexanedione dioxime.
In other preferred embodiments the chelating agent is selected from the group consisting of: ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester; diethylidithiocarbamic acid; 1-pyrrolidinocarbodithioic acid; and 3-amino-5,6-dimethyl-1,2,4-triazine.

Carboxy chelating agents preferably are selected from the group consisting of: 2,3-dihydroxybenzoic acid; 3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinecarboxylic acid (pyridoxic acid); ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester; and 2,6-pyridinedicarboxylic acid.

In other preferred embodiments the chelating agent is selected from the group consisting of: 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (kojic acid) and pyridoxic acid.

In other preferred embodiments the chelating agent is selected from the group consisting of: 1,2-dimethyl-3-hydroxypyrid-4-one; 3-hydroxy-2-methyl-4-pyrone; kojic acid; 1-hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)-2(1H)-pyridone (piroctone olamine--Octopirox); and 6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridinone (Ciclopirox).

In other preferred embodiments the chelating agent is selected from the group consisting of: Octopirox; 6-hydroxy-2-phenyl-3(2H)-pyridazinone; Ciclopirox; 2,3-dihydroxybenzoic acid; 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron); ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester; pyridoxic acid; 2,3-dihydroxypyridine; 2,4,5-trihydroxypyrimidine; and 2,3-dihydroxynaphthalene.

In other preferred embodiments the chelating agent is selected from the group consisting of: 2,3-dihydroxynaphthalene; 2,4,5-trihydroxypyrimidine; kojic acid; 2,3-dihydroxypyridine; 3-hydroxy-2-methyl-4-pyrone; Tiron; 2,3-dihydroxybenzoic acid; 4-(2-amino-1-hydroxyethyl)-1,2-benzenediol; Ciclopirox; and Octopirox.
In other preferred embodiments the chelating agent is selected from the group consisting of: 2,3-dihydroxybenzoic acid; and pyridoxic acid.

In other preferred embodiments the chelating agent is selected from the group consisting of: N-benzoyl-N-phenyl-hydroxylamine; desferrioxamine B (Desferal); Ciclopirox; and Octopirox.

In other preferred embodiments the chelating agent is selected from the group consisting of: kojic acid.

In other preferred embodiments the chelating agent is selected from the group consisting of: diethylthiocarbamic acid; and 1-pyrrolidinecarbodithioic acid.

Preferred chelating agents useful in the present invention which fall within the class of hydroxy aromatic amines include the following: 5,7-dichloro-8-hydroxyquinoline.

In other preferred embodiments the chelating agent is selected from the group consisting of: 2,3-dihydroxybenzoic acid; pyridoxic acid; and 2,6-pyridinedicarboxylic acid.

In other preferred embodiments the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline; di-2-pyridyl ketone; 2-furildioxide; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid); dimethyl ester; 1,1'-carbonyldiimidazole; 1,2-dimethyl-3-hydroxypyrid-4-one; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 1-pyrrolidinecarbodithioic acid; diethyldithiocarbamic acid; Ciclopirox; 2,2'-dipyridyl; 1,2-cyclohexanedione dioxide; 3-hydroxy-2-methyl-4-pyrone; 2,3-bis(2-pyridyl)-5,6-dihydropyrazine; 3-(4-phenyl-2-pyridyl)-5-phenyl-1,2,4-triazine; kojic acid; 2,3-dihydroxyypyridine; 2,2'-biquinoline; 2,2'-bipyrazine; 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine; 4-(2-amino-1-hydroxyethyl)-1,2-benzenediol; and 4,4'-dimethyl-2,2'-dipyridyl.
In other preferred embodiments the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline, di-2-pyridyl ketone; 2-furildioxime; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid), dimethyl ester; 1,1'-carbonyldiimidazole; 1,2-dimethyl-3-hydroxypyrid-4-one; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 1-pyrrolidinecarbodithioic acid; diethylidithiocarbamic acid; and Ciclopirox. More preferred still chelating agents for use in the compositions and methods of the present invention including the following: 2,2'-dipyridylamine; o-phenanthroline, di-2-pyridyl ketone; 2-furildioxime; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; and ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid), dimethyl ester.

In other preferred embodiments the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline; di-2-pyridyl ketone; and 2-furildioxime.

Preferably, the chelating agent has a binding coefficient with aqueous Fe$^{3+}$ of at least $10^6$, preferably at least $10^9$ and more preferably at least $10^{12}$. The binding coefficient is defined as the equilibrium constant for the reaction

$$Fe^{3+} + L^{n-} \leftrightarrow FeL^{(3-n)+}$$

Preferably, the chelating agent is present in the composition at a concentration by weight of from about 1% to about 10%, preferably from about 2% to about 5%, of the composition.

The preferred chelating agents listed hereinabove are generally commercially available from one or more of the following suppliers: Aldrich Chemical Company, Milwaukee, Wis.; G.F.S. Chemicals, Columbus, Ohio; Dojindo Laboratories, Kumamoto, Japan; Sigma Chemical Company, St. Louis, Mo.; Ciba-Geigy, Summit, N.J.; Strem Chemicals, Newburyport, Mass.; and American Hoechst Corp., Summerville, N.J. A method for synthesizing 1,2-dimethyl-3-hydroxypyrid-4-one is disclosed in Kontogiorghes, G. J., "L1--1,2-dimethyl-3-hydroxypyrid-4-

In addition to the active agent, the compositions used in the present invention contain a safe and effective amount of an acceptable carrier. The term "acceptable topical carrier" encompasses both pharmaceutically-acceptable carriers and cosmetically-acceptable carriers, and it encompasses substantially nonirritating compatible components (either taken alone or in mixtures) which are suitable for delivering the active component to the skin. The term "compatible", as used herein, means that the components of the carrier must be capable of being commingled with the chelating agent, and with each other, in a manner such that there is no interaction which would substantially reduce the efficacy of the composition during use for protecting the skin from the effects of UV radiation. These carriers must, of course, be of sufficiently high purity and sufficiently low toxicity to render them suitable for chronic topical administration to the skin of humans or lower animals. The term "safe and effective amount" of carrier means an amount sufficient to deliver the chelating agent to the skin but not so much as to cause any side effects or skin reactions, generally from about 50% to about 99%, preferably from about 90% to about 98%, of the composition.

The composition made in the present invention may be of any conventional topical application type. These include, for example, lotions, creams, gels, sticks, sprays, ointments, pastes, mousses and cosmetics. These product types may comprise either of two basic types of carrier systems, i.e., solutions and emulsions.

The compositions formulated as solutions typically include a pharmaceutically- or cosmetically-acceptable organic solvent. The most typical example of such a solvent is water. Examples of other suitable organic solvents include: propylene glycol, polyethylene glycol (200-600), polypropylene glycol (425-2025), glycerol, 1,2,4-butanetriol, sorbitol esters, 1,2,6-hexanetriol, ethanol, isopropanol, butanediol, and mixtures thereof. These solutions contain from about 1% to about 20%, preferably from about 2% to about 10%, of the chelating agent, and from about 80% to about 99%, preferably from about 90% to about 98%, of an acceptable organic solvent.
Emollients may be included in the carrier system of the present invention formulated as a solution. Such compositions contain from about 1% to about 20% of the chelating agent and from about 2% to about 50% of a pharmaceutically/cosmetically-acceptable emollient. As used herein, "emollients" refer to materials used for the prevention or relief of dryness, as well as for the protection of the skin. A wide variety of suitable emollients are known and may be used herein. Sagatin, Cosmetics, Science and Technology, 2nd Edition, Vol. 1, pp. 32-43 (1972), incorporated herein by reference, contains numerous examples of suitable materials.

Lotions typically comprise from about 1% to about 20%, preferably from about 2% to about 10%, of the chelating agent; from about 1% to about 20%, preferably from about 5% to about 10%, of an emollient; and from about 50% to about 90%, preferably from about 60% to about 80%, water. Another type of product that may be formulated from a solution carrier system is a cream. A cream of the present invention would comprise from about 1% to about 20%, preferably from about 2% to about 10%, of the chelating agent; from about 5% to about 50%, preferably from about 10% to about 20%, of an emollient, and from about 45% to about 85%, preferably from about 50% to about 75%, water. Yet another type of product that may be formulated from a solution carrier system is an ointment. An ointment may comprise a simple base of animal or vegetable oils or semi-solid hydrocarbons (oleaginous). Ointments may also comprise absorption ointment bases which absorb water to form emulsions. Examples of such ointment bases include, anhydrous lanolin and hydrophilic petrolatum. Emulsion ointment bases may be oil-in-water or water-in-oil emulsions. Ointment carriers may also be water soluble. Examples of such ointment carriers include glycol ethers, propylene glycols, polyoxyl stearates, and polysorbates. An ointment may also comprise from about 2% to about 10% of an emollient plus from about 0.1% to about 2% of a thickening agent. Examples of suitable thickening agents include: cellulose derivatives (e.g., methyl cellulose and hydroxy propylmethyl cellulose), synthetic high molecular weight polymers (e.g., carboxyvinyl polymer and polyvinyl alcohol), plant hydrocolloids (e.g., karaya gum and tragacanth gum), clay thickeners (e.g.,
colloidal magnesium aluminum silicate and bentonite), and carboxyvinyl polymers (Carbopol®)

If the carrier is formulated as an emulsion, from about 1% to about 10%, preferably from about 2% to about 5%, of the carrier system comprises an emulsifier. Emulsifiers may be nonionic, anionic or cationic. Preferred emulsifiers are anionic or nonionic, although the other types may also be used. Single emulsion skin care preparations, such as lotions and creams, of the oil-in-water type and water-in-oil type are well-known in the cosmetic art and are useful in the present invention.

Lotions and creams can be formulated as emulsions as well as solutions. Typically such lotions comprise from about 1% to about 20%, preferably from about 2% to about 10%, of the chelating agent; from about 1% to about 20%, preferably from about 5% to about 10%, of an emollient; from about 25% to about 75%, preferably from about 45% to about 95%, water; and from about 1% to about 10%, preferably from about 2% to about 5%, of an emulsifier. Such creams would typically comprise from about 1% to about 20%, preferably from about 2% to about 10%, of the chelating agent; from about 1% to about 20%, preferably from about 5% to about 10%, of an emollient; from about 20% to about 80%, preferably from about 30% to about 70%, water; and from about 1% to about 10%, preferably from about 2% to about 5%, of an emulsifier. If the compositions are formulated as a gel or a cosmetic stick, a suitable amount of a thickening agent, as disclosed supra, is added to a cream or lotion formulation.

The compositions may also be formulated as makeup products such as foundations, thereby enabling the compositions also to cover up the discoloration symptoms of lipodermatosclerosis. Foundations are solution or lotion-based with appropriate amounts of thickeners, pigments and fragrance.

The compositions may also include a safe and effective amount of a penetration enhancing agent. By "safe and effective amount" is meant an amount sufficient to enhance penetration of the chelating agent into the skin but not so much as to cause any side effects or skin reactions, generally from about 1% to about 5% of

The compositions may comprise, in addition to the chelating agent, a safe and effective amount of a radical scavenging compound. By "safe and effective amount" is meant an amount sufficient to inhibit the deleterious effects of iron catalysed free radical formation when the composition is properly applied, but not so much as to cause any side effects or adverse skin reactions. The use of the radical scavenger tocopherol sorbate in the present invention in combination with the chelating agent is preferred. From about 1% to about 5% of these radical scavenging compounds may generally be used in the present invention in combination with the levels of chelating agent taught herein. Exact amounts will vary depending on which particular compound is used as these compounds vary somewhat in potency.

Preferably, the treatment or prevention comprises daily topical application for a period of at least three weeks.

Preferably, the patient is suffering from a venous disease or venous insufficiency. Preferably this is still at an early stage, for example the patient may have varicose veins but still little or no LDS.

Typically a safe and effective amount is from about 0.001 mg to about 1.0 mg, preferably from about 0.01 mg to about 0.5 mg, more preferably from about 0.05 mg to about 0.1 mg of the chelating agent per cm² skin. The chelating agent may be simply spread or sprayed onto the skin or may preferably be rubbed into the skin to enhance penetration. For protection against chronic damage, application of the chelating agent several times daily; generally from about 2 times to about 5 times, preferably 2 times daily is preferred. Preferably, application of the chelating agent is continued daily for as long as the underlying venous insufficiency persists.
EXPERIMENTAL

The current study addressed the hypothesis that excess iron deposited in skin subjected to chronic venous insufficiency is phagocytosed by dermal cells and at low levels causes enhanced collagen deposition leading to fibrotic skin changes but when in excess it mediates cell death and subsequent ulceration. The initial aims of the study were to assess the level and distribution of iron in the lower leg skin of patients with progressive venous disease. Histological and ultrastructural analysis of LDS skin biopsies showed that dermal fibroblasts as well as inflammatory cells contained iron in the form of ferritin and haemosiderin. The effects of increasing levels of iron on dermal fibroblast collagen production and cell viability were determined in vitro. Results suggest that iron at low concentrations causes increased collagen production but higher concentrations induces a significant decrease in cell viability which is likely to be due to iron induced apoptosis.

1. Experimental Study of the Concentration and Distribution of Iron in LDS Skin

Patient selection and sample collection

Patients undergoing varicose vein stripping were initially assessed by colour duplex ultrasonography and photoplethysmography to determine the cause and extent of venous disease, and then separated into two groups according to the International Society for Cardiovascular Surgery/Society for Vascular Surgery CEAP (clinical, etiologic, anatomic and pathologic) classification. Patients in class 3 CEAP (n=10) demonstrated varicose veins and oedema with no detectable skin changes on physical examination whereas CEAP class 4 patients (n=12) presented with trophic skin changes ascribed to lipodermatosclerosis, including skin thickening and hyperpigmentation. Control patients, CEAP class 0 (n=12), undergoing coronary artery by-pass surgery, demonstrated no clinical signs of venous disease when assessed. Patients with a history or clinical evidence of co-existent arterial disease, diabetes mellitus, connective tissue disease or
medication known to affect collagen metabolism, were excluded from the study. Furthermore patients that had a previous healed ulcer were excluded. The average age (in years) and sex distribution (male :female) of the patients in each class was as follows; Class 0 CEAP 59± 6, 11:1; Class 3 CEAP 39± 6, 1:9; Class 4 CEAP 41± 14, 6:6 respectively.

Full-thickness excisional skin biopsies (4mm) were removed from above the medial malleolus of distal leg in the three patient groups. The Royal Free and University College London Medical School Ethics committee gave ethical approval for this study and all subjects provided informed written consent. Skin samples were immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4, overnight before being processed for wax histology. Tissue sections (5μm) were obtained from wax embedded samples and stained with Haemotoxylin and eosin and Perls’ Prussian blue to assess tissue architecture and the distribution of haemosiderin respectively.

**Immunocytochemistry**

Inflammatory cells, including as macrophages, mast cells, neutrophils and lymphocytes, were identified by indirect immunocytochemistry. Sections were dewaxed, rehydrated, and treated with 0.3% H₂O₂ in PBS for 20min to block endogenous peroxidase activity followed by blocking serum corresponding the host of the secondary antibody. Sections were incubated with well-characterised primary antibodies for 1hr at room temperature in a humidified chamber. Monoclonal primary antibodies were raised against human macrophage, CD68 (1:100 dilution; Dako, Cambridge, UK), neutrophil, CD15s (1:50 dilution; Serotec Ltd, Oxford, UK), mast cell tryptase (1: 100 dilution; Serotec Ltd, Oxford, UK) and T-lymphocyte, CD3 (1:50 dilution, Serotec Ltd, Oxford, UK). Following three washes with PBS, the sections were incubated with biotinylated-conjugated secondary antisera against mouse or rat IgG (1:300 dilution; Dako, Cambridge, UK), for 1hr at room temperature. Sections were washed three times with PBS prior to incubation with streptavidin-conjugated horseradish peroxidase (1:100 dilution; Dako, Cambridge, UK) for 30min. Peroxidase activity was visualised with chromogen diaminobenzidine substrate (FAST DAB, Sigma, Poole, UK) and
sections were counterstained with 1% eosin and mounted. Control sections were incubated with either normal blocking serum or mouse/rat IgG1 instead of primary antisera. For dual localisation, sections were initially assessed for inflammatory cells by immunoperoxidation and counterstained with Perls' Prussian blue for haemosiderin.

*Ultrastructural analysis*

Three additional LDS and normal skin samples were fixed in a solution of 1% paraformaldehyde and 5% glutaraldehyde in 100mM sodium cacodylate buffer, pH 7.4 at room temperature overnight, and post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.4 for 1hr. Specimens were washed, stained with an aqueous saturated solution of uranyl acetate for 1hr, dehydrated and embedded in araldite epoxy resin. Semi-thin sections (0.5-1µm) were cut on a Reichert microtome, stained with 1% toluidine blue and examined under a Zeiss Axiophot light microscope equipped with phase contrast optics. Ultra-thin sections (70-100nm) were collected on copper grids, stained with uranyl acetate and lead citrate and viewed in a Philips 400 transmission electron microscope (Eindhoven, Holland).

*Results*

The presence and distribution of iron was assessed in skin biopsies from patients with progressive stages of venous disease by Perls' Prussian blue staining. Red blood cell extravasation and iron in the form of haemosiderin was observed in all LDS samples examined whereas it was absent in skin samples from patients with venous insufficiency but without clinical evidence of LDS and normal control skin. In LDS, haemosiderin was deposited throughout the dermis, in both a cellular and extracellular distribution. In addition, it was clearly found in the few remaining septal cells of the fibrotic subcutaneous layer. Dual localisation of inflammatory cells and haemosiderin demonstrated that macrophages, mast cells and neutrophils contained iron deposits. However, there was also an abundance of Perls staining in elongated cells aligned along collagen fibres which was not associated with brown DAB localisation of inflammatory cells. This suggests that a non-inflammatory cell type may also take up excess iron in LDS.
Ultrastructural analysis of LDS samples confirmed that macrophages and neutrophils contained clusters of electron dense iron particles recognised as ferritin and haemosiderin laden siderosomes. Furthermore, cells within the collagen fibres were identified as dermal fibroblasts and found to contain similar deposits of iron.

2. Experimental Study of the effect of iron on dermal fibroblast collagen production

Experimental Approach
Adult human dermal fibroblasts were isolated from normal dermal skin by explant culture and were grown in DMEM medium supplemented with penicillin (100U/ml), streptomycin (100μg/ml) and L-glutamine (2mM), containing 10% FCS at 37°C, in a humidified, 5% CO₂ atmosphere. Cells were characterised using well-characterised antibodies against specific markers and were used at passage 7 to 9.

Primary dermal fibroblasts were plated at 10⁶ cells/ml in DMEM with 10% FCS in a 12 well plate. At confluence, cells were washed twice with PBS and loaded with iron according to a modified method of Ruiz et al. (2000). Briefly, serum-free DMEM incubation media containing 50μM ascorbate as a reducing agent, 200μM proline, 250μM citric acid as a carrier with and without 10-100μM iron chloride was added to the cells. After 24hr, ascorbate at 200μM final was re-added to the media and the cultures incubated for a further 24hr before being processed for collagen analysis (n=6) and cell proliferation (n=4). Positive controls contained TGF-β1 at 1ng/ml instead of iron chloride. Cell layer and media proteins were precipitated with 67% ethanol at 4°C and samples filtered to separate proteins from free amino acids and small peptides prior to drying. Protein samples were hydrolysed with 6M HCl. Hydroxyproline levels were measured as an index of procollagen synthesis using a reverse-phase HPLC method developed in our laboratory. Cell
proliferation was assessed by counting viable cells following trypsinisation. Collagen production was expressed as the amount of hydroxyproline per $10^5$ cells.

Preliminary data suggest that iron causes an increase in collagen production after 48 hours.

**Experiment 3: The effect of iron on dermal fibroblast viability in culture.**

**Fibroblast viability assay**

Primary dermal fibroblasts were plated in 96-well plates at $10^5$ cells/ml in 100μl media supplemented with 0.4% FCS. Intact cells are relatively impermeable to iron therefore iron was loaded using the iron-chelating fungistat 8-hydroxyquinoline (8HQ). 8HQ chelates with iron and rapidly transfers the metal across the intact plasma membrane of cells. After 24 hrs, cells were washed twice with PBS and loaded with 100μl serum-free DMEM containing stoichiometric quantities of ferric chloride and ferric ammonium citrate (to prevent precipitation of insoluble iron hydroxides) with 8-hydroxyquinonine (8-HQ) used as a carrier at concentrations of 1-100μM in a method modified from Jacob AK, Hotchkiss RS, DeMeester SL, Hiramatsu M, Karl IE, Swanson PE, Cobb JP, Buchman TG. Surgery 122:243-53 (1997). Control experiments added iron without 8-HQ carrier and 8-HQ without iron. In addition, the membrane-impermeable iron chelator, deferoxamine (DFX; Sigma) at 200μM was added to prevent the uptake of iron by the cells. Previous studies have shown that iron loading is complete after 30min (Jacob et al., 1997), therefore 30 min incubation with iron was used in all experiments. Viable cells accumulate the vital dye, neutral red, in their lysosomes and so neutral red (0.003%) in 100μl serum-free media was applied to washed cells and incubated for a further 2 hr. Excess dye was aspirated, the cells were washed with
phosphate buffered saline, pH 7.4 (PBS), and lysed with 100μl 50% ethanol containing 1% acetic acid. Absorbency was measured at 560nm and viability calculated as a fraction of control cell absorbency after blank absorbency was deducted.

5

*Apoptosis assays*

To determine the cause of cell death, TUNEL assay for apoptosis was used. For TUNEL staining, cells were grown on glass Labtek slides and when semi-confluent rinsed with PBS and fixed with 10% formalin overnight. After several PBS washes, cells were treated with 20μg/ml proteinase K in 20mM Tris/HCl, pH 8 for 30min at 37°C, rinsed four times with PBS and air-dried. The TUNEL reaction was performed using the apoptosis kit according to the manufacturer’s instructions (Boehringer Mannheim, UK). Stained cells were illuminated at 488nm and fluoresced at 520nm.

15

To assess the effect of iron on dermal fibroblast viability, the uptake of neutral red dye following incubation with iron was analysed. Dermal fibroblasts did not absorb ferric iron from the media unless iron was complexed to 8-HQ. Iron with the carrier (8HQ) significantly decreased dermal fibroblast viability from 1μM which reached a plateau at higher concentrations (>25μM). Concentrations of iron below 1μM did not produce a significant decrease in cell viability over serum free controls. Iron added in combination with the iron chelator (DFX) completely abrogated this effect at all concentrations of iron tested. However, iron or carrier alone had no significant effect on dermal fibroblast viability.

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The TUNEL stain, which enzymatically adds tagged deoxyribonucleotides to blunt-ended DNA fragments, identified nucleic acid fragmentation characteristic of apoptosis. Cells loaded with iron and carrier, 8-HQ, were TUNEL positive compared with untreated control cells.

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**Discussion**
In the current study, the amount and distribution of iron in LDS was investigated. It was demonstrated for the first time that dermal fibroblasts contain iron in the form of ferritin and haemosiderin in addition to inflammatory cells. It is likely that inflammatory cells are unable to accommodate the continuous deposition of iron from extravasated erythrocytes and redistribution into mesenchymal cells takes place. The effect of iron on dermal fibroblast function is thought to be to stimulate collagen deposition. Iron is known to act in the stabilisation of procollagen by acting as a cofactor in the hydroxylation of lysine and proline residues. The current study showed that a low concentration of iron caused an increase in collagen production by dermal fibroblasts.

Iron accumulation in dermal fibroblasts in LDS may induce the production of collagen leading to the fibrotic skin changes observed and this may be through lipid peroxidation products. One of the hallmarks of LDS is the progressive replacement of the subcutaneous adipose layer by fibrotic tissue. In the current study, septal cells in the subcutaneous fat layer showed haemosiderin accumulation by Perl's Prussian blue staining and we have previously demonstrated that these cells are actively expressing procollagen mRNA. This suggests that iron may also play a role in collagen production by subcutaneous adipose cells as in haemochromatosis.

Iron at higher concentrations than those used to induce collagen caused a decrease in dermal fibroblast cell viability, possibly due to the stimulation of apoptosis.

Ischaemia/reperfusion, which has been suggested to occur in legs subjected to venous insufficiency, may exaggerate the effects of iron by acting as an inducer of stress. Ferrous iron release from ferritin occurs in the presence of superoxide generated by xanthine oxidase system on reperfusion or partial ischaemia. Free iron generated in this manner would enhance the formation of toxic hydroxyl radicals leading to the perpetuation of tissue damage.
Based on these findings a new hypothesis of venous ulceration is postulated. Chronic venous insufficiency in the lower leg causes leakage of erythrocytes from cutaneous blood vessels into the interstitial tissue. Inflammatory cells initially phagocytose these, however with continued extravasation these cells become overloaded and excess iron is then taken up by dermal fibroblasts. Accumulation of low levels of iron in dermal fibroblasts in skin subjected to chronic venous insufficiency leads to the production of lipid peroxidation by-products and the development of fibrotic skin changes. However, the build-up of iron to high levels in dermal fibroblasts results in cell death and eventual ulcer formation. These events are likely to be through the production of free radicals and enhanced by ischaemia/reperfusion. The presence of iron-loaded LDS skin around a venous ulcer will perpetuate the fibrotic process so preventing ulcer healing and may lead to more extensive tissue damage and an increase ulcer size.

Treatment of this problem has for many years relied on established principles of compression and limb elevation and no drug treatment has been shown to be more effective. Yet there is clearly a role for drugs in the management of venous disease either alone or in combination with compression therapy. It is likely to be beneficial to intervene in the initial processes that cause the lipodermatosclerotic skin changes leading to ulceration rather than to modify the repair processes when an ulcer has formed. The same processes that caused the ulcer are likely to contribute to its perpetuation and delayed healing. The free radical scavengers, topical allopurinol or dimethyl sulfoxide, were given to 88 ulcer patients, on a 3 months, double blind controlled study and showed a significant increase in healing (Salim A.S. The role of oxygen derived free radicals in the management of venous (varicose) ulceration: a new approach. World J. Surg. 15, 264-269 (1991)).

We have found that there is little evidence of haemosiderin in ulcer base tissue, although others have found raised iron levels in chronic wound fluid suggesting
that much of the iron leaks out of the wound. Therefore, it is logical to address the problem of iron overload by applying iron chelators topically to pre-ulcerated skin.

EXAMPLES

A gel ointment suitable for application to intact skin for the prevention or treatment of LDS is prepared according to the following formulation (percentages are by weight of the composition):-

<table>
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<tr>
<th>Ingredient</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Carboxymethylcellulose</td>
<td>2.4%</td>
</tr>
<tr>
<td>Hydroxyethylcellulose</td>
<td>0.3%</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.24%</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>20.2%</td>
</tr>
<tr>
<td>o-phenanthroline</td>
<td>2.0%</td>
</tr>
<tr>
<td>Water</td>
<td>balance</td>
</tr>
</tbody>
</table>

The ointment may be applied to intact skin of a patient exhibiting symptoms of venous insufficiency or LDS, or of an asymptomatic patient as a preventative treatment. The ointment may be applied at least once a day in an effective amount.

Many other embodiments falling within the scope of the accompanying claims will be apparent to the skilled reader.
CLAIMS

1. Use of an iron chelator for the preparation of a composition for use in the prevention of lipodermatosclerosis by topical application to the lower leg.

2. Use of an iron chelator for the preparation of a composition for use in the treatment of lipodermatosclerosis by topical application to the lower leg.

3. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of aromatic amines, carbonyls, oximes, amines, carboxylates, alkoxides, enolates, phenoxides, catecholates, hydroxy acids, hydroxamates, ketoenolates, mercaptides, hydroxy aromatic amines, aromatic hydroxy acids, and mixtures thereof.

4. Use according to claim 3, wherein the chelating agent is selected from the group consisting of aromatic amines, carbonyls, oximes, enolates, phenoxides, catecholates and hydroxylates, and mixtures thereof.

5. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; 1,10-phenanthroline [o-phenanthroline]; di-2-pyridyl ketone; 2,3-bis(2-pyridyl) pyrazine; 2,3-bis(2-pyridyl)-5,6-dihydropyrazine; 1,1'-carbonyldimidazole; 2,4-bis(5,6-diphenyl-1,2,4-triazine-3-yl)pyridine; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 4,4'-dimethyl-2,2'-dipyridyl; 2,2'-biquinoline; di-2-pyridyl glyoxal [2,2'-pyridil]; 2-(2-pyridyl)benzimidazole; 2,2'-bipyrazine; 3-(2-pyridyl)5,6-diphenyl-1,2,4-triazine; 3-(4-phenyl-2-pyridyl)-5-phenyl-1,2,4-triazine; 3-(4-phenyl-2-pyridyl)-1,2,4,5-diphenyl-1,2,4-triazine; 2,3,5,6-tetrakis(2'-pyridyl)-pyrazine; 2,4,5-trihydroxypyrimidine; phenyl 2-pyridyl ketoxime; 3-amino-5,6-dimethyl-1,2,4-triazine; 6-hydroxy-2-phenyl-3(2H)-pyridazinone; 2,4-pteridinediol [lumazine]; 2,2'-dipyridyl; and 2,3-dihydroxypyridine.
6. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: di-2-pyrindyl ketone; 1,1'-carbonyldiimidazole and 2,2'-pyrididil.

7. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2-furildioxime; phenyl 2-pyridyl ketoxime; and 1,2-cyclohexanedione dioxime.

8. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester; diethyldithiocarbamic acid; 1-pyrrolidinecarbodithioic acid; and 3-amino-5,6-dimethyl-1,2,4-triazine.

9. Use according to claim 3, wherein the chelating agent is selected from the group consisting of: 2,3-dihydroxybenzoic acid; 3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridine-carboxylic acid [pyridoxic acid]; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester; and 2,6-pyridinedicarboxylic acid.

10. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one {kojic acid} and pyridoxic acid.

11. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 1,2-dimethyl-3-hydroxypyrid-4-one; 3-hydroxy-2-methyl-4-pyrone; kojic acid; 1-hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)-2(1H)-pyridone {piroctone olamine--Octopirox}; and 6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridinone {Ciclopirox}.

12. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: Octopirox; 6-hydroxy-2-phenyl-3(2H)-pyridazinone; Ciclopirox; 2,3-dihydroxybenzoic acid; 4,5-dihydroxy-1,3-benzene-disulfonic acid {Tiron}; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid) dimethyl ester;
pyridoxic acid; 2,3-dihydroxypyridine; 2,4,5-trihydroxypyrimidine; and 2,3-dihydroxynaphthalene.

13. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,3-dihydroxynaphthalene; 2,4,5-trihydroxypyrimidine; kojic acid; 2,3-dihydroxypyridine; 3-hydroxy-2-methyl-4-pyrone; Tiron; 2,3-dihydroxybenzoic acid; 4-(2-amino-1-hydroxyethyl)-1,2-benzenediol; Ciclopirox; and Octopirox.

14. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,3-dihydroxybenzoic acid; and pyridoxic acid.

15. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: N-benzoyl-N-phenyl-hydroxylamine; desferrioxamine B {Desferal}; Ciclopirox; and Octopirox.

16. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: kojic acid.

17. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: diethylthiocarbamic acid; and 1-pyrrolidinecarbodithioic acid. Other preferred chelating agents are analogs, homologs and isomers of the above mercaptides which exhibit at least about 50% inhibition of iron-catalyzed hydroxyl radical formation in the in vitro solution radical assay or at least about a 20% reduction in skin wrinkle grade in the in vivo mouse skin wrinkling test. Preferred chelating agents useful in the present invention which fall within the class of hydroxy aromatic amines include the following: 5,7-dichloro-8-hydroxyquinoline.

18. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,3-dihydroxybenzoic acid; pyridoxic acid; and 2,6-pyridinedicarboxylic acid.
19. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline; di-2-pyridyl ketone; 2-furildioxime; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid), dimethyl ester; 1,1'-carbonyldiimidazole; 1,2-dimethyl-3-hydroxy pyrid-4-one; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 1-pyrrolidine carbodithioic acid; diethylldithiocarbamic acid; Ciclopirox; 2,2'-dipyridyl; 1,2-cyclohexanediol; 3-hydroxy-2-methyl-4-pyrene; 2,3-bis(2-pyridyl)-5,6-dihydropyrazine; 3-(4-phenyl-2-pyridyl)-5-phenyl-1,2,4-triazine; kojic acid; 2,3-dihydroxy pyridine; 2,2'-biquinoline; 2,2'-bipyrazine; 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine; 4-(2-amino-1-hydroxyethyl)-1,2-benzenediol; and 4,4'-dimethyl-2,2'-dipyridyl.

20. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline, di-2-pyridyl ketone; 2-furildioxime; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid), dimethyl ester; 1,1'-carbonyldiimidazole; 1,2-dimethyl-3-hydroxy pyrid-4-one; 2,4,6-tri(2-pyridyl)-1,3,5-triazine; 1-pyrrolidines carbodithioic acid; diethylldithiocarbamic acid; and Ciclopirox. More preferred still chelating agents for use in the compositions and methods of the present invention including the following: 2,2'-dipyridylamine; o-phenanthroline, di-2-pyridyl ketone; 2-furildioxime; 2,3-bis(2-pyridyl) pyrazine; Octopirox; 2,3-dihydroxybenzoic acid; and ethylenediamine-N,N-bis-(2-hydroxyphenylacetic acid), dimethyl ester.

21. Use according to claim 1 or 2, wherein the chelating agent is selected from the group consisting of: 2,2'-dipyridylamine; o-phenanthroline; di-2-pyridyl ketone; and 2-furildioxime.

22. Use according to claim 1 or 2, wherein the chelating agent has a binding coefficient with aqueous Fe$^{3+}$ of at least 10$^6$. 
23. Use according to any preceding claim, wherein the chelating agent is present in the composition at a concentration by weight of from about 1% to about 10%, preferably from about 2% to about 5%, of the composition.

24. Use according to any preceding claim, wherein the treatment or prevention comprises daily topical application for a period of at least three weeks.

25. Use according to any preceding claim, wherein the patient is suffering from a venous disease.

26. Use according to claim 25, wherein the patient has varicose veins.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7  A61K31/47  A61K31/4745  A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 7  A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, CHEM ABS Data, EMBASE, WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y        | FR 7 302 M (ABBOTT LABORATORIES)  
12 November 1969 (1969-11-12)  
page 1  
page 3  |

Y

REINHAREZ D: "Pigmentation following sclerosis! Les pigmentations apres sclerose."  
PHLEBOLOGIE, (1983 OCT-DEC) 36 (4) 337-44,  
XX001095988  
page 337  
page 341  
page 343  |

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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:
*A* document defining the general state of the art which is not considered to be of particular relevance
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*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
*C* document referring to an oral disclosure, use, exhibition or other means
*P* document published prior to the international filing date but later than the priority date claimed

**I** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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27 September 2002

Date of mailing of the international search report
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Authorized officer
Brück, M

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