ORIGINAL RESEARCH – CLINICAL SCIENCE

New dressing materials derived from transgenic flax products to treat long-standing venous ulcers—a pilot study

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ABSTRACT

A new flax dressing product was developed based on three components (fibers, oil emulsion, and seedcake extract) from genetically engineered flax plants that were obtained by plant transformation using three genes controlling the synthesis of antioxidative compounds from the phenylpropanoid pathway. Simultaneous flax explant transformation with three genes coding for chalcone synthase, chalcone isomerase, and dihydroflavonol reductase resulted in an accumulation of phenolic acids in the fibers, polyunsaturated fatty acids in the oil, and lignans in the seedcake. The fibers, oil, and seedcake from transgenic flax contained a broad spectrum of antioxidative compounds. They were tested for cytotoxicity, and none were found to have a negative effect on the growth and morphology of Balb/3T3 cells. In this preliminary report, we present pilot data on the effects of using linen dressing treatment on its own or in combination with oil emulsion and/or seedcake extract on chronic wound healing. After a 12-week study, we concluded that an application of a modified flax-dressing (linen) bandage might yield a more rapid rate of healing and reduce the wound exudes and wound size. In several cases, wound healing was completed during the period of investigation. Interestingly and importantly, the patients reported that the new bandage made from modified flax diminished the pain accompanying chronic venous ulceration. Further study is required to determine any definitive effects of flax bandage on wound healing. This is the first pilot study report suggesting the benefits of a flax-based dressing on wound healing.

The most common types of chronic, nonhealing wounds are venous, pressure, diabetic, and ischemic ulcers. Chronic venous ulcers are considered to be the most common disorder of vascular origin.[1] The frequency of occurrence of venous ulcers is reaching epidemic proportions, with elderly or disabled people most at risk.[2–4] These ulcers significantly impair the quality of life and increase healthcare expenditures for millions of people around the world.[5–7] Successful treatment that stimulates healing is an essential step toward eliminating morbidity, improving quality of life for patients, and decreasing healthcare costs. Although scientists and clinicians are developing novel therapeutic approaches to promote healing, we are still far from success.[8]

Recent reports indicated oxidative stress as an important mechanism in the aggravation of chronic wound progression.[9] It is suggested that reactive oxygen species are responsible for chronic wound pathogenesis and antihealing processes, because they reduce the proliferation capacity. Oxidative stress causes damage to cellular macromolecules, deregulation of key proteins involved in DNA replication, cell cycle, cellular resistance to such stress, and promotion of wound fibroblast apoptosis.[10,11] Via multidirectional analysis, it was found that the fibroblasts in chronic wounds have a decreased ability to withstand oxidative stress.[12]

In this study, we assessed a new material (which we named FlaxAid) and method for the treatment of chronic lesions, based on products from transgenic flax plants overproducing various antioxidative compounds. The increase in the levels of phenolics in the fibers and unsaturated fatty acids in the seeds and the strong increase in the lignan content of the seedcakes were the characteristic features of these plants.[13] We decided to test the coordinative use of fibers, oil emulsion, and seedcake extract from these plants for the healing of chronic skin ulceration.

The primary goal of this study was to investigate the degree of clinical improvement in chronic lesions by assessing the change in wound exudation, the proportion of fibrin, and the granulation tissue level. The effect of the new flax bandage on the wound size and on the pain usually accompanying chronic ulceration was also assessed.

MATERIALS AND METHODS

Patients

The study group consisted of 30 subjects (16 female and 14 male, mean age 68 ± 10 years), patients of the Dermatology Department of the Wroclaw Military Hospital.

All were suffering from chronic nonhealing venous ulcers located on the leg. The treatment with new bandage was proposed to those patients whose local and general treatment (compression therapy, antibiotics, and analgesic and anticoagulant therapy) thus far had not resulted in healing of the ulcer or even in stopping its progression. Selected information on the subjects is presented in Table 1.

The wound pathophysiology was diagnosed using the patients' medical history, physical examination, Doppler sonography of the leg vessels, and the ankle–brachial index (ABI) measurement.

Only patients with wounds that, had lasted at least 2 years were included in the study; the mean duration of ulceration was 9 ± 7 years, and in no case was bacterial infection detected.

The study was approved by the independent bioethics committee. All the patients were provided with written information on the purpose and design of the study and accepted it with a signature.

Table 1. Classification of patients chosen for treatment

						Prior treatment (local therapy)		
No.	Gender	Age	Wound duration (years)	ABI R; L	Coexisting diseases	Surgery	Compression therapy (class)	Pharmacotherapy
1	M	74	2	0.9; 1.0	HT, IHD, MI	None	II	0.9% NaCl, hydrocolloid dressings, hydrofiber dressings
2	M	72	2.5	1.0; 1.0	IHD, HT	None	II	0.9% NaCl, silver dressings, dressings including alginians
3	M	58	5	1.1; 1.0	Nephrolithiasis	None	II	Ethacridine, hydrofiber dressings, hydrocolloid dressings
4	M	60	5	0.9; 1.1	HT	None	II	0.9% NaCl, ethacridine, hydrocolloid dressings
5	M	56	5	1.0; 0.9	None	None	II	TenderWet dressings, hydrofiber dressings, hydrocolloid dressings, dextranomer dressings
6	F	70	18	0.9; 1.1	HT, stroke, cholelithiasis	Subcutaneous extirpation of a saphenous vein	III	0.9% NaCl, povidone iodine, silver dressings
7	F	72	18	1.0; 1.0	HT, hypothyreosis	Subcutaneous extirpation of a saphenous vein	III	Hydrocolloid dressings, chlorhexidine, povidone iodine
8	F	80	2	0.9; 0.9	DVT, coxartrosis	None	III/IV	Hydrocolloid dressings, 0.9% NaCl, antiseptic agents
9	M	66	2.5	1.1; 1.2	HT, IHD, DVT, hyperlipidemia, obesity	Thrombectomy	II	Blood extract, silver sulfathiazole, prednisolone, neutral ointment, 0.9% NaCl
10	M	68	2.5	1.1; 1.0	HT, IHD	None	II	Silver sulfathiazole, prednisolone, hydrocolloid dressing, neutral ointment
11	F	73	23	0.9; 0.8	HT	None	III	0.9% NaCl, blood extract, silver dressings, antiseptic agents
12	M	56	3	1.0; 1.1	HT, gastric ulcer	None	II	0.9% NaCl hydrocolloid, dressings, hydrogel dressings
13	F	62	3	1.0; 1.1	IHD, HT; cholelithiasis	Dermal graft	III	Hydrocolloid dressings silver sulfathiazole neutral ointment
14	F	91	2	1.0; 0.9	HT, IHD, asthma	None	II	Bacitracine, antiseptic agents, 0.9% NaCl
15	F	88	2.5	0.9; 0.8	HT, IHD, DVT	None	II	Bacitracine hydrocolloid, dressings
16	F	61	15	1.2; 0.9	Nephrectomy, HT	Dermal graft	III/IV	Silver sulfathiazole 0.9% NaCl
17	F	63	15	1.1; 1.0	HT, hypothyreosis	None	II	Povidone iodine, 0.9% NaCl
18	M	71	6	1.0; 0.8	HT, AF, DVT	None	III	Hydrocortisone, silver sulfathiazole, octanisept, 0.9% NaCl

Table 1. Continued.

No.	Gender	Age	Wound duration (years)	ABI R; L	Coexisting diseases	Prior treatment (local therapy)		
						Surgery	Compression therapy (class)	Pharmacotherapy
19	F	55	22	0.9; 0.9	Coxarthrosis, DVT	Perforating-vein ligation	III	0.9% NaCl, dressings including alginians
20	F	57	5	1.0; 1.0	Obesity, gonarthrosis	None	II	Hydrocolloid dressings, antiseptic agents
21	F	53	5	0.9; 1.1	None	None	II	Antiseptic agents, 0.9% NaCl
22	F	83	20	1.0; 1.1	Renal cysts	None	II	Neutral ointment, 0.9% NaCl mometasone
23	F	59	2	1.2; 1.1	IHD, SAS, obesity	None	III	Antiseptic agents, hydrocolloid dressings, including alginians
24	M	83	10	1.0; 1.0	Pulmonary embolism, DVT	Thrombectomy	II	0.9% NaCl, antiseptic agents, prednisolone
25	M	81	10	1.0; 1.1	HT, nephrolithiasis, gonarthrosis	None	II	0.9% NaCl, antiseptic agents, prednisolone, neutral ointment
26	M	61	18	1.2; 1.0	HT, stroke	Subcutaneous extirpation of a saphenous vein	III	Chlorhexidine, povidone iodine, hydrocolloid dressings
27	M	64	18	1.1; 1.1	HT, renal cysts	Subcutaneous extirpation of a saphenous vein	I/II	Prednisolone, dressings including alginians, povidone iodine
28	M	59	18	1.0; 1.0	HT, stroke	Subcutaneous extirpation of a saphenous vein	II	Chlorhexidine, hydrogel dressings
29	F	74	6	0.8; 0.9	IHD, HT coxarthrosis, cholelithiasis	None	II	Antiseptic agents, 0.9% NaCl
30	F	72	6	1.0; 1.1	IHD, HT	None	II	Hydrocolloid dressings, 0.9% NaCl

ABI, ankle/brachial index; R, right lower extremity; L, left lower extremity; AF, atrial fibrillation; HT, hypertension; IHD, ischemic heart disease; MI, myocardial infarction; SAS, sleep apnea syndrome; DVT, deep vein thrombosis. Compression therapy was realized using widely available compression articles (class of compression refers to approximate pressure in ankle region: class I, < 25 mmHg; class II, 25-35 mmHg; class III, 35-45 mmHg; class IV, > 45 mmHg).

Transgenic flax generation and selection

Plant material

Flax seeds (cv. *Linola*) were obtained from the Flax and Hemp Collection of the Institute of Natural Fibres, Poland. For the analysis, the control and selected transgenic plants were grown in a field, and seeds were harvested 3 months after the transfer of the tissue-cultured plants to the soil.

Transgenic plant construction and selection

Two-week-old cotyledon and hypocotyl explants were transformed using *Agrobacterium tumefaciens* strain C58C1:pGV2260 carrying a binary vector containing three cDNAs from *Petunia hybrida*, encoding chalcone synthase (CHS, EMBL/GenBank database accession no. X04080), chalcone isomerase (CHI, EMBL/GenBank database accession no. X14589), and dihydroflavonol reductase (DFR, EMBL/GenBank database accession no. X15537) in the sense orientation under the control of the 35S promoter and OCS terminator.[14,15] The transgenic plants were preselected via PCR using primers specific for the kanamycin-resistance gene (*npt II*), and then selected by means of Northern blot analysis.[16] The details on plant transformation, selection, and transgenic plant analysis were reported previously.[13]

Preparation of the linen dressing

Flax fabric was prepared from raw yarn using the standard weaving method. The linear mass of the warp and weft was 140TEX. The warp density was 65/dm and the linear density of the weft was 85/dm. The density of the final flax

fabric was 220 g/m^2. An appropriate quantity of linen dressing was sterilized by autoclaving at 120 °C for 20 min. The size of the dressing for wound treatment was 10×10 cm. Where indicated, the linen dressing for the wound treatment was covered with 2 mL of sterile seedcake extract or with 2 mL of oil emulsion.

Preparation of the oil emulsion

A flax oil emulsion was prepared according to the published protocol.[17] Briefly, soybean lecithin (Lipoid S75 from Lipoid, Ludwigshafen, Germany) and Tween 80 (Sigma-Aldrich, St Louis, MO, USA) were mixed with flax oil, and then an aqueous phase containing glycerol (Sigma-Aldrich) was added. The oil and aqueous phases were mixed vigorously. The sample was further sonicated using a Microson ultrasonic cell disruptor (Misonix INC, farmingdale, NY, USA) for 10 min at 4 W. The sonicated preparations were filtered through sterile Acrodisc (Gelman Sciences, Ann Arbor, MI) 0.22 μm filters. All the emulsion samples were prepared at room temperature. The final concentrations of the chemicals in emulsion were 1% lecithin, 2.5% flax oil, 2.5% Tween 80, and 2.5% glycerol.

Preparation of the seedcake extract

One hundred grams of hexane defatted flax seeds were extracted three times with 400 mL of 80% methanol (v/v) for 15 min at 70 °C. The extract was centrifuged, and the methanol was evaporated at 40 °C. The aqueous fraction of the extract was subjected to alkaline hydrolysis in a final concentration of 0.3 M sodium hydroxide for 2 days at room temperature, followed by neutralization using 2 M hydrochloric acid. After centrifugation the supernatant was sterilized by filtration through an Acrodisc (Gelman Sciences) 0.22μm filter or by autoclaving at 120 °C for 20 min. The final concentration of lignans was 8.4 μg/mL and that of phenolic acids was 191.5 μg/mL.

Cytotoxicity assay

The cytotoxicity of the flax fibers, oil emulsion, and seedcake extract was determined in vitro using a culture of Balb/3T3 mouse fibroblasts, which are widely used to measure acute toxicity when screening various chemicals and biomaterials. The Balb/3T3 cells were seeded in a six-well plate. 1×10^5 cells/1 mL of the culture medium (Dulbecco's- modified Eagle's [DMEM] enriched with 10% calf serum, penicillin, and streptomycin) was deposited into each well. Samples of the material for testing were added to each well, and the whole well was incubated at 37 °C in an air atmosphere containing 5% CO_2. The control cells were incubated without any of the test materials. The number of cells and their morphology were assessed after 24 and 72 hours using reversed phase-contrast microscopy.

Additionally, the morphology of the adhered cells was evaluated using scanning electron microscopy. Before this analysis, the cells were fixed in 2.5% glutaraldehyde, dehydrated in a graded ethanol series, dried in CO_2 at the critical point, and finally sputter coated with a thin silver layer. The examination of the specimens was performed in a Tesla BS 300 at an accelerating voltage of 15 kV.

Patient treatment

Routine laboratory tests were performed on all the patients during their first visit in order to assess their general state of health and to confirm a pure venous origin for the ulcers, i.e., to exclude the presence of any internal disease that could have contributed. Peripheral blood was collected in the morning after an overnight fast.

Arterial pathology was excluded by anamnesis, physical examination, ABI measurement (Sonodop 4000 DSM 2P Doppler Segmental Sphygmometer, Sonotechnic GmbH), and Doppler ultrasonography (Vivid 7) of the leg vessels.

The lab tests performed included a complete blood count with a differential chemistry profile including blood urea nitrogen, creatinine, uric acid, serum protein, CRP, fibrinogen, alanine aminotransferase, and aspartate aminotransferase; a lipid profile with total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides; a coagulation profile with PT, APTT, and INR; the levels of fasting glucose and glycosylated hemoglobin; and a urinalysis. All the laboratory tests were in a normal range. In all subjects, a wound swab was taken to detect bacterial infection; no pathogenic organisms detected in any of the samples obtained. Although some patients had a history of antibiotic treatment within the 6 months preceding the study none of them received antibiotic therapy for at least 2 weeks before the start of the study.

The treatment with the newly developed flax dressings was part of a complex ulcer therapy regime that included education, analgesic (if necessary), and compression therapy.

The study was divided into four stages (zero, first, second, and third), each lasting 4 weeks. After each week, during a consultation, a physician performed an evaluation of the ulcers, measuring the ulcers using sterile dressings with a millimeter scale, and reading a questionnaire filled in by the patients the day before each visit. The physicians also prepared photographic and descriptive documentation. In the zero stage, each patient's wound was treated with popular, widely available cotton dressings wetted with an isotonic salt solution. This part of the study was treated as the control stage. In the first stage, the wounds were treated with linen dressings wetted with an isotonic salt solution. The second stage involved ulcer treatment with linen dressings wetted with oil emulsion. During the third stage, the therapy was based on wound treatment with linen dressing wetted with seed cake extract.

The dressings were changed every 24 hours; qualified hospital personnel applied the first in each stage. The patients themselves or someone from their family changed the dressings thereafter, having been thoroughly instructed by a qualified nurse during the weekly visits.

The amount of exudates was measured as a number of pads wetted in the last 12 hours before each patient's visit. Twelve hours before each visit, six gauze pads were placed on top of a flax dressing, and during a visit, the number of wet pads was recorded.

The changes in fibrin and granulation tissue levels within the ulcer were measured as the area of red tissue or fibrin as a yellow surface by using ulcers photos and the respective computer program (GIMP). The same program was also used for measuring the size of the ulcer. The change in the level of pain was expressed in the Numerical Rating Scale (1–10) and the

patients reported this during each visit. The last was confirmed by reduction of analgesics admitted to patients.

Statistical analysis

The data obtained were statistically analyzed using the *t*-test for dependent samples. Additionally, the repeated measures ANOVA were performed to test equality of means. Repeated measures variance analysis has been performed using Wilks's test.

RESULTS

Transgenic plant generation and selection

The hypocotyl and cotyledon explants of flax plants were transformed with a multigene vector containing three cDNAs encoding the key enzymes of flavonoid biosynthesis using the Agrobacterium method.[15] The construct, consisting of the *CHS*, *CHI*, and *DFR* cDNAs from *Petunia hybrida* under the control of the CaMV 35S promoter and OCS terminator, was inserted into the genome of the flax plants.[14] *Petunia DFR* was used because it preferentially converts dihydromyricetin to leuco-delphinidin. Delphinidin 3-*O*-3-xylosylrutinoside was determined as the main pigment in *Linum grandiflorum*.[18] The regenerants obtained were prescreened using the PCR method with specific primers for the neomycin phosphotransferase gene and further selected by means of Northern blot analysis. W 92.40 was the transgenic line that showed the highest level of mRNA for the three introduced cDNAs, and it was used for further analysis. The levels of the products of the everexpressed enzymes were assessed in the leaves and seeds. The transgenics were characterized by a significant increase in the levels of flavonoids in the seeds and in the green parts of the plant. The major differences in the antioxidants levels were observed in the phenolic acid content (particularly protocatechuic, caffeic, and ferulic acid). Transgenic flax also produced a high quanties of SDG.[19] The quantity of antioxidants in the seedcakes, fibers, and linseed oil are presented in Table 2.

Investigation of the cytotoxic effect of the fibers, oil emulsion, and seedcake extract

The cytotoxicity of the flax fibers, oil emulsion, and seedcake extract was determined in a culture of Balb/3T3 mouse fibroblasts of the permanent cell line, widely used for acute toxicity in vitro screening of various chemicals and biomaterials.[20–22] The cells were grown in Dulbecco's modified Eagle's (DMEM) medium enriched with 10% calf serum, penicillin, and streptomycin. The test material and cells were incubated together in six-well plates at 37 °C in an air atmosphere containing 5% CO_2. For the control, the cells were grown in the absence of plant materials. The number of cells was counted after 24 and 72 hours of growth, and as an example, the data from fiber treatment are presented in Figure 1. The results from other treatments were remarkably similar and are presented in supporting information Table S1. There were no significant changes in the total number of fibroblasts grown in the presence of each type of material, but a slight consistent increase in cell numbers was detected in the medium containing plant material. Trypan blue cell staining revealed comparable numbers of dead cells in the control fibroblast culture and in the fibroblast cultures with the test material (supporting information Table S1). The morphology of cells grown for 24 and 72 hours in the control and test media was also investigated, and the data from reversed-phase contrast microscopy are presented in Figure 2 and from scanning electron microscopy in Figure 3. The microscopy revealed no difference in the shape and size of the cells in cell morphology culturing in the presence of plant material when compared with the control.

Patient treatment

It has been reported that phenylpropanoid compounds, in particular flavonoids, exhibit health-protecting activities because of their strong antioxidant properties.[23] Their antioxidant activity might have a great significance for chronic ulceration, because they inhibit enzymatic and nonenzymatic peroxidation.[24] In addition, flavonoids have antiallergic, antiviral, antiinflammatory, and vasodilatory activities.[23,25] All these features make them attractive targets for genetic engineering strategies aimed at producing transgenic plants with increased antioxidant properties. It is believed that applying a high level of phenylpropanoid compounds strengthens wound tissue defenses against biotic and abiotic stresses.[26–28] Because the transgenic plant material had no negative impact on the fibroblast culture, the next step was to investigate all three plant elements as wound dressing. The flax bandage consists of three elements (Figure 4): the linen layer, the oil emulsion, and the seedcake extract. These three elements of new flax dressing were sequentially used for chronic ulceration treatment.

Table 2. The content of antioxidants in three products originated from transgenic plants

	Fiber	Seedcake	Seed oil
Ferulate (μg/gFW)	0.432 ± 0.002	23.862 ± 0.01	u/d
Coumarate (μg/gFW)	u/d	91.025 ± 0.002	u/d
Lignan (μg/gFW)	1.056 ± 0.05	878.160 ± 0.1	u/d
γ-tocopherol (μg/gFW)	u/d	u/d	782.7 ± 1.3
β-carotene (μg/gFW)	u/d	u/d	1.5 ± 0.1
Linolenic acid (μg/g of fat)	u/d	u/d	861.9 ± 49.7
Linolic acid (μg/g of fat)	u/d	u/d	591.93 ± 15.5

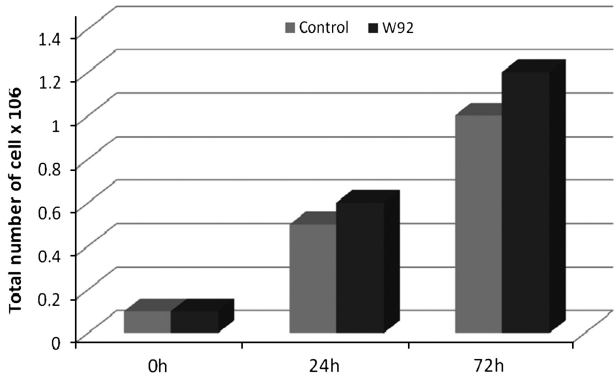

Figure 1. The total number of Balb/3T3 cells after 24- and 72-hour incubation in the presence of flax fibers (60 mg) isolated from nontransformed (control) and transgenic (W92) plants.

To evaluate changes in the ulcers yielded by flax dressing therapy, several parameters were considered: the change in wound exudates (measured in semiquantitative analysis as a number of gauze pads wetted with exudates in the last 12 hours before each patient's visit (scale: one to six gauze pads), and the changes in fibrin and granulation tissue levels within the ulcer (measured as the area of red tissue or fibrin as a yellow surface) using ulcers' photos and graphic computer program (GIMP). The same program was also used for measuring the size of the ulcer. The change in the level of pain was expressed in the Numerical Rating Scale (1–10) and the patients reported this during each visit. The last was confirmed by reduction of analgesics admitted to patients.

After the control, zero stage, slight but negative changes were observed in all the considered parameters (Figure 5), suggesting that the common treatment (cotton gauze wetted with an isotonic salt solution) has no positive impact on ulcer healing within 4 weeks.

The next three stages covered a 12-week period of time in which FlaxAid was used. In the first 4 weeks, the wounds were treated with a linen dressing wetted with an isotonic salt solution. In the next 4 weeks, a linen dressing wetted with oil emulsion was applied, and during the last 4 weeks, a linen dressing wetted with seedcake extract was used. The results of these stages were evaluated together and divided into two parts: objective evaluation and subjective evaluation.

Objective evaluation:

(1) *Ulcer size* (Table 3): this is the most objective parameter describing the healing of wounds. During the three stages of flax fabric dressing treatment, the majority of the subjects (80%) showed a statistically significant reduction in wound size. The rate of reduction did not depend on the initial size of the ulcer. However, it should be pointed out that the most effective treatment to reduce the ulcer size was that with seedcake extract containing lignans as the major antioxidant. An increase in the size of the wounds was detected in 20% of the patients, with an increase from 10% (patient no. 18) to 53% (patient no. 21) of the initial size.

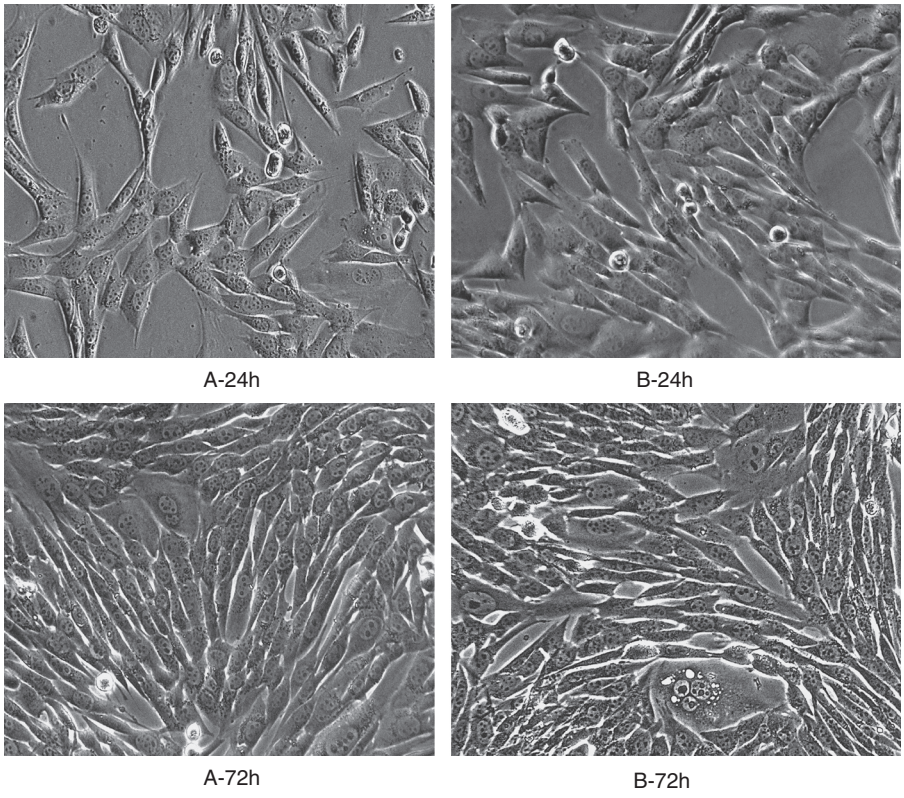

Figure 2. The morphology of Balb/3T3 cells after 24- and 72-hour culture in medium only (A, control) and in medium supplemented with flax fibers isolated from transgenic plants (B, W92).

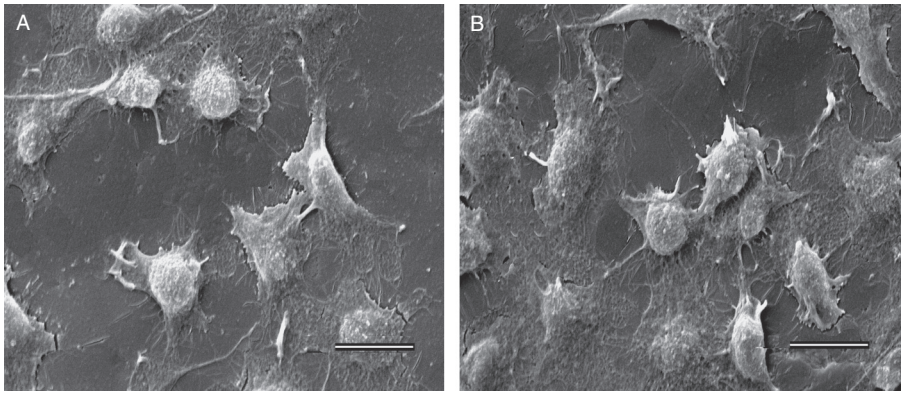

Figure 3. SEM of fibroblasts cultured for 24 hours in medium only (A, control) and in medium supplemented with flax fibers isolated from transgenic plants (B). Scale bar=30 μm.

(2) *Ulcers healed*: in seven subjects (23%), the wounds were cured after the 12-week period (Table 3). An example of a completely cured wound is shown in Figure 6.

Subjective evaluation (Figure 5):

(1) *Exudate*: there was an observed systematic decrease in the level of exudates over the three stages of treatment with the new dressing. At the end of the final stage, over 66% of the patients showed a reduction in the level of exudates, with the onset in reduction having appeared in the second or the third stages. The 33% of subjects who did not show changes in the level of exudates already had dry wounds before treatment. There was no observed worsening of this parameter in any case during the investigation.

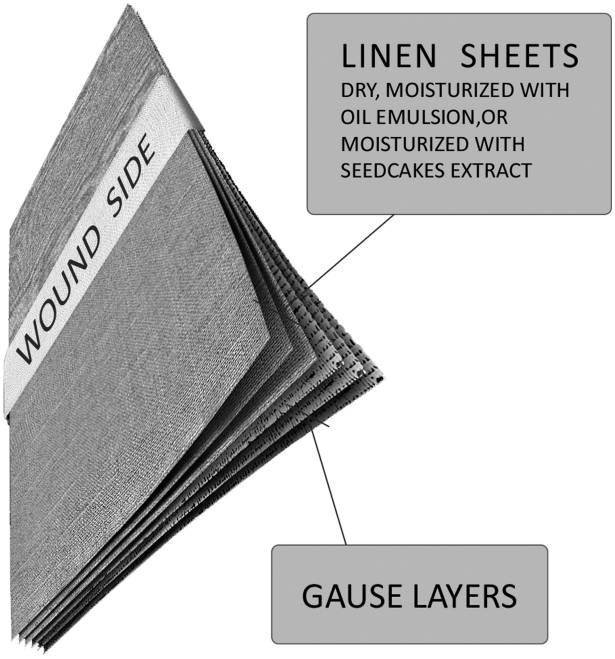

Figure 4. A schematic view of the FlaxAid bandage. The layers of linen were supplemented with an isotonic salt solution or a flax oil emulsion or seedcake extract, and covered with two to three layers of wetted (isotonic salt solution) cotton gauze.

(2) *Fibrin and granulation tissue levels*: both ulcer parameters were highly changed upon FlaxAid treatment. Over 93% of the subjects showed a decrease or even complete disappearance of fibrin. New granulation tissue was observed in all the patients examined (100%). There was no worsening observed in the evaluated parameters.
(3) *Reported pain*: it is known that chronic ulceration is frequently accompanied by pain. It was noticed that over 96% of subjects reported a decrease in the level of pain at the end of the FlaxAid therapy. It should be pointed out that pain reduction was already reported in the first stage of the study, suggesting the healing feature of linen dressing. Only 3.3% of patients reported no changes in the level of pain, and these were mostly patients who reported having painless wounds at the beginning of the study. None reported a worsening in their pain.

It is interesting to note that in the majority of cases, the subjective evaluation of the wound morphology (exudates, fibrin, and granulation tissue) concurred with the objective parameter (the size of wounds; Table 3).

We performed the *T*-test for dependent samples, which is the most commonly used method to evaluate the differences in means between two groups. We used this test, because the within-group variation (normally contributing to the error of the measurement) can be easily identified and excluded from the analysis. We treated each treatment stage as a group, and compared each pair of groups, which allowed us to check for detailed differences between them (with the statistical significance $p < 0.05$). We additionally performed the repeated measures ANOVA to test the equality of means. In this way, we were able to show more generally the tendency of repeated measures results in the whole designed experiment. Repeated measures variance analysis has been performed using the Wilks test, and with $F=3,16$ the statistical significance p was 0.030316.

DISCUSSION

The phenylpropanoid pathway is the source of a large number of compounds that are derivatives of phenylalanine such as flavonoids, lignin monomers, lignans, phenolic acids, and their esters.[29] All these compounds act as antioxidants, chelators of divalent cations,

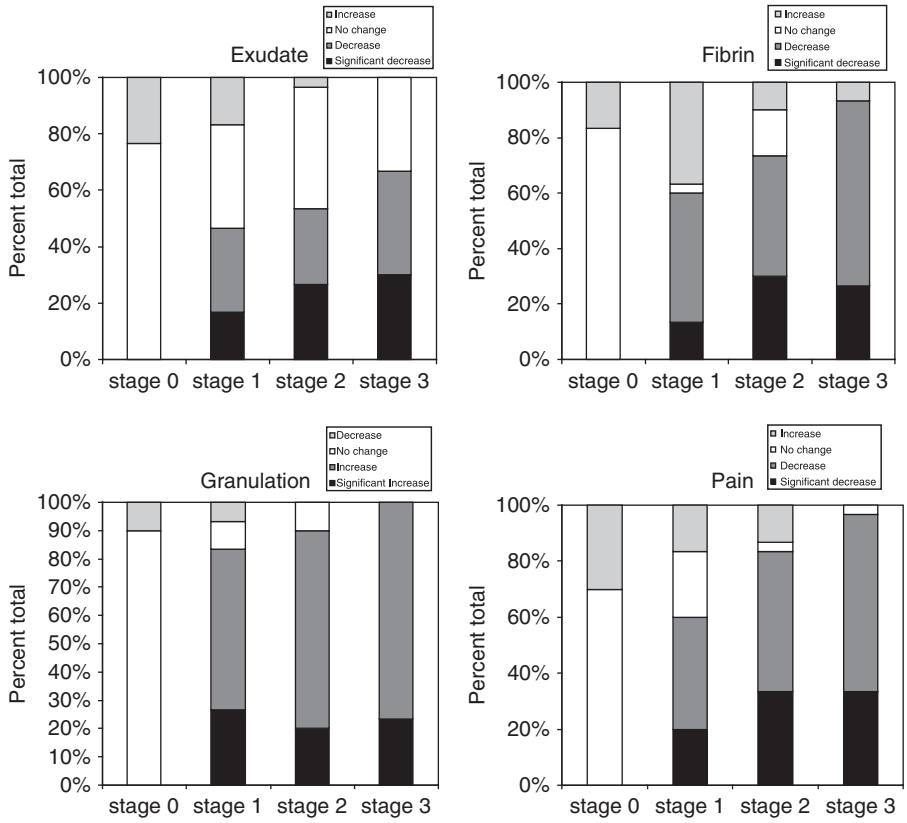

Figure 5. Patients (30 subjects) suffering from chronic ulceration were investigated. The wounds were treated either with a cotton gauze wetted with an isotonic salt solution (zero stage) or with a linen dressing wetted with an isotonic solution (first stage) or an oil emulsion (second stage) or seedcake extract (third stage) as described in ''Materials and methods'' section. The subjective parameters, including the exudates, fibrin, and granulation tissue and pain levels, were noted at the end of each stage and expressed in arbitrary units. The change in wound exudates was measured in a semi-quantitative analysis of number of gauze pads wetted with exudates for 12 hours before last in each period patient's visit, scale: one to six gauze pads. If the number of gauze pads decreased by more than 3, the change was considered significant. The changes in fibrin levels within the ulcer were measured by surface of tissue or fibrin using ulcers' photos and computer program. If the decrease in the area measured was $< 20\%$, it was considered significant. The changes in granulation tissue levels within the ulcer were measured by surface of tissue or fibrin using ulcers' photos and computer program. If the increase in the area measured was more than 50%, it was considered significant. The change in the level of pain was reported by the patients (in questionnaires given to patients during each visit with the pain scale 1–10). If the pain decrease was more than 50%, it was considered significant.

photoreceptors, and visual attractors[23] and are also beneficial for elevating oxidative damage to the skin.[30,31]

Chronic venous ulcers are considered to be the most common disorder of vascular origin.[1] It was recently pointed out that oxidative stress is an important factor in chronic wound progression and pathogenesis.[9] Oxidative stress promotes damage to many cellular mechanisms and causes fibroblast apoptosis.[12] It is also reported that low-molecular-weight antioxidative compounds originating from the phenylpropanoid pathway can promote the healing of chronic ulcers.[32] Furthermore, flax oil is a rich source of omega-3 and omega-6 fatty acids that has been reported as having a beneficial influence on wound healing.[33] Thus, it was hypothesized that wound treatment with a product based on flax might promote wound tissue healing. In this study, chronic wounds were treated with a new bandage made from genetically modified flax, a bandage that contains strong antioxidants (phenolic acids, lignans) from transgenic flax plants.

There are several known parameters that can describe the features of wounds. For this study, we chose changes in exudates and in the levels of fibrin and new granulation tissue, changes in the level of pain reported by the patient, and the most objective parameter, changes in wound size (using the methods described in ''Results'').

There were slight but negative changes in the measured parameters of the wounds during the first 4 weeks (the zero stage), which involved treatment with the conventional method (cotton gauze wetted with an isotonic salt solution), and this stage was taken as the control stage. After this stage, the patients were treated with the transgenic flax bandage, and the wound-healing parameters were assessed.

The major points suggesting a possible positive effect of a flax dressing was hygroscopicity of a flax fibers, the high

Table 3. The size of the wounds at the end of the initial stage (zero stage) and at the end of FlaxAid treatment (first through third stages)

Patient no.	Duration of ulcer (years)	Wound size (cm^2)				Wound morphology		
		Zero stage	First stage	Second stage	Third stage	First stage	Second stage	Third stage
1	2	1.87	1.70	1.45	0.0	+	+	+++
2	2.5	2.38	2.25	1.70	0.0	+	++	+++
3	5	5.24	4.05	2.75	0.0	++	++	+++
4	5	8.62	6.30	3.95	0.0	++	++	+++
5	5	5.75	4.30	3.10	0.0	++	++	+++
6	18	2.87	1.85	0.75	0.0	+	–	+++
7	18	20.12	28.30	30.50	28.75	+	–	+
8	2	233.70	230.10	225.30	196.50	–	–	0
9	2.5	8.40	7.50	6.25	4.00	+	++	++
10	2.5	6.25	5.50	4.90	3.80	+	++	++
11	23	38.50	38.50	37.45	36.60	+	+	+
12	3	19.60	18.00	16.45	12.25	0	+	+
13	3	135.30	135.30	135.00	133.87	+	+	+
14	2	4.50	3.75	2.45	0.0	+	++	+++
15	2.5	3.63	3.60	2.75	1.20	+	++	+
16	15	26.75	25.00	23.30	20.55	+	+	++
17	15	17.75	18.00	17.50	16.70	+	+	++
18	6	336.50	384.30	380.00	376.25	0	0	0
19	22	14.12	19.20	20.30	18.37	–	–	+
20	5	3.15	5.50	5.10	5.12	–	–	+
21	5	5.90	13.20	13.20	11.12	–	–	+
22	20	13.87	13.95	12.65	10.12	+	+	+
23	2	41.25	42.50	40.30	36.62	+	+	+
24	10	18.75	19.50	18.60	16.00	+	–	+
25	10	22.37	17.80	16.00	12.37	0	–	+
26	18	119.70	130.10	125.00	123.25	0	0	+
27	18	1.37	1.40	1.10	0.75	0	+	+
28	18	10.37	9.60	8.30	6.50	0	0	+
29	6	2.50	2.10	1.30	0.50	+	–	+
30	6	4.50	4.00	2.80	1.20	+	–	+

The changes in the size of the ulcers at the end of each treatment stage are expressed in cm^2. The data were analyzed statistically using Student's *t*-test. Changes in the ulcer size measured at the end of the first and second stages were not statistically significant compared with the measurements for the end of the zero stage ($p < 0.05$). Statistically significant changes were found at the end of the third stage compared with all the other stages (p=0.000964, 0.000001, and 0.000007, for third vs 0, first, and second, respectively). Significant changes in the size of ulcers were also observed at the second stage compared with the first (p=0.000016). To test the general tendency of repeated measures results, repeated measures variance analysis has been performed using the Wilks test, and with F=3,16 the statistical significance of p was 0.030316.

Changes in the morphology of the wounds (exudates, fibrin, and granulation tissue) reported by the patients are also presented and expressed in arbitrary units (+++, wound cured; ++, significant decrease; +, decrease; 0, no change; –, increase).

The respective meanings of first, second, and third stages are ulcer treatment with linen dressing wetted with isotonic salt solution, with emulsion of flax oil and with seedcake extract.

content of polyunsaturated fatty acids and hydrophobic antioxidants (tocopherol, carotene) in oil emulsion, and a high antioxidant potential of a seedcake extract derived mostly from ferulic acid and SDG content.[34]

The antioxidant capacity of seedcake extract as determined by a luminometric method is very high, in fact 10 times higher than that reported for Vitamin C at the same concentration (supporting information Table S2). Thus, it

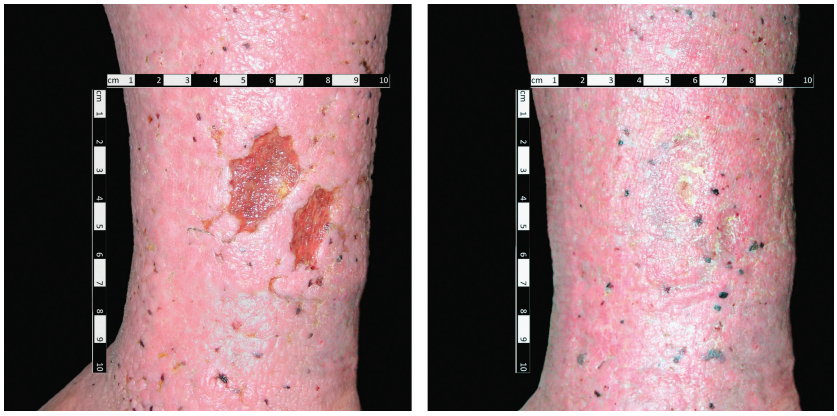

Figure 6. An example of a completely healed wound after three stages of treatment with FlaxAid.

was possible that supplying the cells with essential fatty acids and antioxidants can help strengthen the plasma membranes and combat oxidative stress in damaged tissues and thus facilitate the healing process based on how a topically applied phenolic compound can help combat oxidative damage to a cell.[27,35] However, the rate of transport of the extracts used through the tissues and how they interact with the cell need to be studied.

The reduction of exudates was found in almost 67% of the subjects. An excess of exudates usually promotes bacterial infection and inflammation processes, which are also responsible for the pain associated with chronic nonhealing wounds. An optimal level of exudates promotes wound healing because it contains vital proteins and cytokines.[36] The remaining 33% of the patients had an optimal level of wound exudates from the beginning of the study. In no case was there an allergic reaction, which is a highly positive feature of the bandage.[37]

One of the steps of healing a wound is decreasing the fibrin level. A decrease was observed in 93% of the patients, while the rest (7%) were those patients who had shown clean ulceration and no fibrin before treatment. It is thought that the linen dressings with their special texture (thick loosely bound fibers) and rich antioxidant levels acted as a barrier to all unnecessary tissues, such as fibrin and necrotic tissue, and also to microbes, excess water, and irritants.

We believe that the decrease in exudates and fibrin contents, concomitant with the presence of antioxidants, promoted new granulation tissue. All the participants were found to have new tissue in the last stage of transgenic flax application.

It should be pointed out that decreases in the levels of pain were reported by more than 96% of the subjects, as measured in the reduction of analgesic therapy and by a 1- to 10-point pain scale. No one reported any aggravation of the pain. This observation is very important, because long-lasting pain associated with ulcers is known to be a negative factor in the healing prognosis. The mechanism of easing of pain at the molecular level is as yet unknown, but we suggest that the decrease in pain might derive from the reduction in the exudates level, the decrease in the level of fibrin, and the antiinflammatory effect of the antioxidants.

The size of the wound was the most objective parameter assessed. We observed that in almost 80% of the patients, the ulcer size had reduced after the 12-week flax dressing treatment, and even more importantly, 23% of patients were completely cured after this short duration of treatment. It should be noted that $< 20\%$ of the patients showed an increase in ulcer size. Although the reason for this is unknown, the age of the patients, the length of time the wound had existed before treatment, the presence of coexisting diseases, and the effects of previous treatments cannot be excluded at this time. Further study is needed. It can be speculated that for those patients, the 12-week treatment was far too short. This speculation is reasoned from the observation that in all cases, the size of the ulcer decreased in the third stage, and in all cases, an improvement in the wound morphology was reported in the third stage.

This study could indicate that a flax-based dressing may have beneficial effects on a chronic wound but further investigation is required. To the best of our knowledge, this is the first report on transgenic flax product application for chronic wound treatment.

In conclusion, treatment with a new flax dressing effectively reduced the wound exudates, fibrin levels, and ulcer size, and increased the level of new granulation tissue. It is believed that the beneficial nature of flax bandage is derived from its high levels of a broad spectrum of antioxidants. The observation of an easing of pain symptoms is also important, although the reason for this is as yet unknown. However, these are suggestions derived from the pilot study data, which obviously need further investigation in a randomized group of treated patients and containing a representative control group of patients. Such a study is now in progress.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Table S1. The measurements of cell numbers grown for 24 h and 72 h in medium only (control) and in the presence of flax fiber, oil emulsion, and seed cake extract made from transgenic plant (W92) after trypan blue staining.

Table S2. The antioxidant capacity of seedcake extract measured by luminometric method and presented as a uMol necessary to quench the luminol fluorescence by 50%.

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