Comparison of the Effects of Contractubex® Gel in an Experimental Model of Scar Formation in Rats: An Immunohistochemical and Ultrastructural Study

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ABSTRACT

Background: Contractubex® gel, a commercial treatment for scars, consists of a mixture of onion extract (cepea extract), heparin sodium, and allantoin. It exerts a softening and smoothing effect on indurated, hypertrophic, painful, and cosmetically-disfiguring scar tissue.
Aim: To compare and discuss the immunohistochemical and ultrastructural effects of treatment of an experimental scar in a rat model with Contractubex gel.
Methods: Thirty-two Sprague-Dawley rats were divided into four groups. Skin biopsies were taken to develop full thickness wounds. After 10 days, Contractubex gel, heparin, and allantoin were topically applied daily to groups 2, 3, and 4, respectively. Group 1 was the control group. On the 30th day, scar tissues were excised to investigate the immunohistochemical and ultrastructural effects of these agents. For this purpose we used TGF-beta, laminin, and fibronectin primary antibodies.
Results: Increased immunoreactivities of laminin, fibronectin, and TGF-beta in control group, moderate immunoreactivities in heparin and allantoin groups, and mild immunoreactivities in the Contractubex gel group were observed. In semi-thin sections, Group 2 showed the thinnest epidermis of the four groups. In electron micrographs of Group 2, completely keratinized and normally appearing cells could be seen.
Conclusions: Immunohistochemical and ultrastructural observations demonstrated that the Contractubex gel significantly improved the quality of wound healing and reduction of scar formation. Also, it was a more appropriate treatment choice than heparin monotherapy and allantoin monotherapy in keloidal and hypertrophic scars.

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INTRODUCTION

There are two kinds of abnormal scar formation in the skin: keloid and hypertrophic scars. The causative mechanism of such scars is not fully understood, but local histological factors in connection with a hereditary component are thought to be involved in most cases. In genetically predisposed individuals, traumatic factors including burns, incisional wounds, infections, acne, and other inflammatory disorders may trigger keloid formation. Keloid and hypertrophic scars, which result from excessive collagen deposition may lead to significant morbidity as well as pruritus, pain, restriction of motion, or cosmetic disfigurement.[1,2]

There is no universally accepted treatment protocol in the management of keloid and hypertrophic scars. Hypertrophic scars, which are often resistant to treatment and have a higher rate of recurrence, may be more responsive to treatment than keloids.[1,3] Laser surgery, surgical removal, radiotherapy, silicone gel sheeting and other dressings, cryotherapy, interferon, bleomicin, 5-fluorouracil, and intralesional corticosteroids have all been used alone or in various combinations, with variable but largely transient success.[3,4]

Clinical studies demonstrated that the gel-based mixture of extractum cepae-heparin sodium-allantoin (Contractubex® gel, Merz Pharma, Frankfurt, Germany) [components: 10% aqueous onion extract (extractum cepae), 50 U heparin per gram of gel, 1% allantoin] was effective in the treatment, reduction, and prevention of hypertrophic scars and keloids.[5-8] These agents exert a softening and smoothing effect that visually and functionally improves scar tissue.[5,9] However, there has been little research on how these agents influence scar formation processes and key wound-healing components such as transforming growth factor-beta (TGF-β)

TABLE 1.

Glogau Photodamage Classification Scale

Groups n=8 (each)	Control Group (Group 1)	Contractubex Group (Group 2)	Heparin Sodium Group (Group 3)	Allantoin Group (Group 4)
Fibronectin	2.63±0.74*** *	1.25±0.46	1.75±0.45	1.75±0.46
Laminin	2.75±0.46 *** **	1.00±0.53	1.65±0.53	1.63±0.52
TGF-β	2.88±0.35 *** **	1.13±0.35	1.75±0.47	1.75±0.46

CON-FIB, CON-LAM, CON-TGF-β vs CTB-FIB, CTB-LAM, CTB-TGF-β	***	$P<0.001$
CON-FIB vs HEP-FIB, HEP-TGF-β, HEP-LAM, ALL-FIB, ALL-TGF-β, ALL-LAM	*	$P<0.05$
CON-LAM, CON-TGF-β vs HEP-FIB, HEP-TGF-β, HEP-LAM ALL-FIB, ALL-TGF-β, ALL-LAM	**	$P<0.01$

Control > Heparin sodium Group = Allantoin Group
Contractubex Group < Heparin = Allantoin < Control Group

(CON: Control, FIB: Fibronectin, CTB: Contractubex, LAM: Laminin, TGF-β: Transforming Growth Factor -β, HEP: Heparin, ALL: Allantoin)

and extracellular matrix proteins (e.g., laminin and fibronectin).[10-12] The anti-microbial, anti-inflammatory, anti-neoplastic, and anti-oxidant effects of onion extract have been well accepted for many years.[13] It is also popular in treating postoperative scars.

In this study, we aimed to compare and discuss the immunohistochemical and ultrastructural effects of Contractubex gel, heparin sodium, and allantoin in the rat model.

METHODS

Study Design

All of the protocols used in the present study were approved by the Animal Research Ethics Committee of Celal Bayar University. Thirty-two female Sprague-Dawley rats, weighing 200 to 230 g were purchased from Experimental Animals Research Laboratory of Aegean University and used in the study. Guidelines for using of laboratory animals were strictly adhered throughout the study. The rats were fed with standard rodent feed and kept in a 20 ºC room. After shaving the hair of the left dorsal side of each rat, an incisional skin biopsy was taken to develop a full-thickness wound. The dimensions of the samples were 1 x 1 x 0.3 cm. The animals were randomly divided into four experimental groups, i.e., a control group (Group 1) and three treatment groups (Groups 2, 3, and 4). After the animals' wounds had completely healed over a period of 10 days, the wounds were treated topically and daily as follows: Group 2 (n=8), extractum cepae-heparin sodium-allantoin mixture (Contractubex gel, Merz Pharma, Frankfurt, Germany [components: 10% aqueous onion extract, 50 U heparin per gram of gel, 1% allantoin]); Group 3 (n=8), heparin sodium 5000 IU (Liquemine® flac, Roche Pharmaceuticals, Istanbul, Turkey); and Group 4 (n=8), allantoin 1 g (Allantoin, H.G.u.C. Blau GmbH, Hamburg, Germany). No treatment was applied to control Group 1 (n=8). At the beginning the scars were not keloidal or hypertrophic in our study. On the 30th day of treatment, scar tissues of the animals in all groups were excised and examined microscopically.

Histochemical and Immunohistochemical Analysis

Specimens were fixed in 10% buffered formaline solution for 24–48 hours and then performed routine paraffin procedure. Sections (5 µm thick) were cut and prepared for both histochemical and indirect immunohistochemical stainings. For immunohistochemical evaluation, avidin-biotin peroxidase system (Santa Cruz Staining System, immunocruz sc-2051, Santa Cruz, CA) was used. Anti-transforming growth factor beta (anti-TGF-β) (Neomarkers, mouse Mab, MS-1106, Lab Vision Corp, Fremont, CA), anti-laminin (Neomarkers, rabbit Pab, RB-082, Lab Vision Corp, Fremont, CA), and anti-fibronectin (Neomarkers, mouse Mab, MS-1351, Lab Vision Corp, Fremont, CA), primary antibodies were used. After 18 hr of incubating with primary antibodies at 4 °C, tissue sections were washed with phosphate buffered saline (PBS), then incubated for one hour with streptavidin-peroxidate conjugate (Histostain-DS Kit-95-9999, Zymed Laboratories, San Francisco, CA) in a 1:100 dilution. After a second PBS wash, chromogen diaminobenzidine tetrahydrochloride (DAB) was applied to the sections for five minutes for color development, which was followed by counterstaining with Mayer's hematoxylin. A semiquantitative grading system was used to compare the staining intensities using light microscopy. Immunohistochemical staining on each section was graded as mild (+), moderate (++), or strong (+++). ANOVA non-parametric test was used to compare the staining intensities, $P<0.05$ was accepted as significant (Table 1).

Electron Microscopic Analysis

For electron microscopic examination, scar tissue samples from control and experimental groups, were fixed in 2.5% glutaraldehyde solution in phosphate buffer, pH 7.4 for four hours, and post-fixed for one hour in 1% osmium tetroxide solution, dehydrated in ethanol, treated with propylene oxide and embedded in Araldite. After heat polymerization, sections were cut using an ultramicrotome. Semi-thin sections were stained with methylene blue-azure II and examined using an Olympus BX-40 microscope. Ultra-thin sections were double-stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 900 transmission electron microscope.

RESULTS

Immunoreactivity

We observed immunoreactivity to TGF-β in the fibroblasts, adipose cells, and muscle cells, to fibronectin on the extracellular compartments of dermal layer of the skin, and to laminin in the basal lamina component of epithelium, adipose cells, and muscle cells. In the dermal layer of the scar samples, we also observed increased immunoreactivities of laminin, fibronectin, and TGF-β in the control group; moderate immunoreactivities in the heparin group and allantoin group; mild immunoreactivity in the Contractubex gel group (Figure 1). The intensities of immunohistochemical scores were shown in Table 1. It was observed that the immunoreactivities of TGF-β, fibronectin, and laminin had increased in control group. On the other hand, the results were statistically significant in Contractubex group (P<.001), heparin group (P<0.05), and allantoin group (P<0.05).

Ultrastructural Effects

In Group 1, the semi-thin tissue sections revealed a thickened epidermis, active fibroblasts, and granulation-like connective tissue in the dermis. There were intercellular separations in the granular layer and significant separation between the cells of the stratum corneum, however, normal dermal structures had not yet formed. In Group 2, appearance of keratinocytes forming the epidermis was close to normal, and papillary layer of dermis occupies a large area in semi-thin sections. In Group 3, normal structure in stratum granulosum and stratum corneum could be seen. There was granulation-like connective tissue, consisting of loosely-structured collagen fibers and vessels in the papillary dermis. In Group 4, epidermal keratinocytes were rather normal in shape; separations between stratum granulosum and stratum corneum were not as large as in the control group; and dermal papillary layer consists of collagen bundles rich in vessels (Figure 2).

Electron microscopy examination of the scar tissue samples of Group 1 revealed cells that had not completed keratinization, lost their intercellular bindings, and been separated from the epidermis. In the wound-healing area, the modified cells of stratum corneum had not completed their keratinization and degenerating cells were observed. The scar tissues of Group 2 exhibited the thinnest epidermis of the four groups, with normal-appearing epidermal cells that had completed keratinization, normal-appearing keratinocytes forming the epidermis, papillary wavy structures in the epidermo-dermal junction, and a dermal papillary layer occupying a large area (Figure 3).

The scar tissues of Group 3 revealed intercellular bindings with a firm appearance among cells of stratum corneum and stratum granulosum; separations only in the cells of the outer layer of stratum corneum; continuing keratinization in lower cells of the stratum corneum; desmosomes still connected to the intact epidermis; epidermal keratinocytes that showed keratinization; and normal-appearing cells of the granular and corneal layers. In the dermal area close to epidermis, granular tissue has transformed into papillary dermis. Beneath the epidermis, formation of connective tissue consisting of loosely-structured collagen fibers and blood vessels could be seen, but this area was not as wide as in the applied mixture group (Group 2) (Figures 2, 3).

The scar tissues of Group 4 demonstrated normal-appearing keratinocytes forming the epidermis; separations between the cells of stratum corneum and stratum granulosum that were not as large as in the control group (Group 1). A dermal papillar layer that consisted of well-vascularized collagen bundles; and granulation tissue that had almost transformed into dermal connective tissue. Throughout the stratum corneum, cells were tightly spaced and intercellular binding units were evident, but some cells had not completed keratinization (Figures 2, 3).

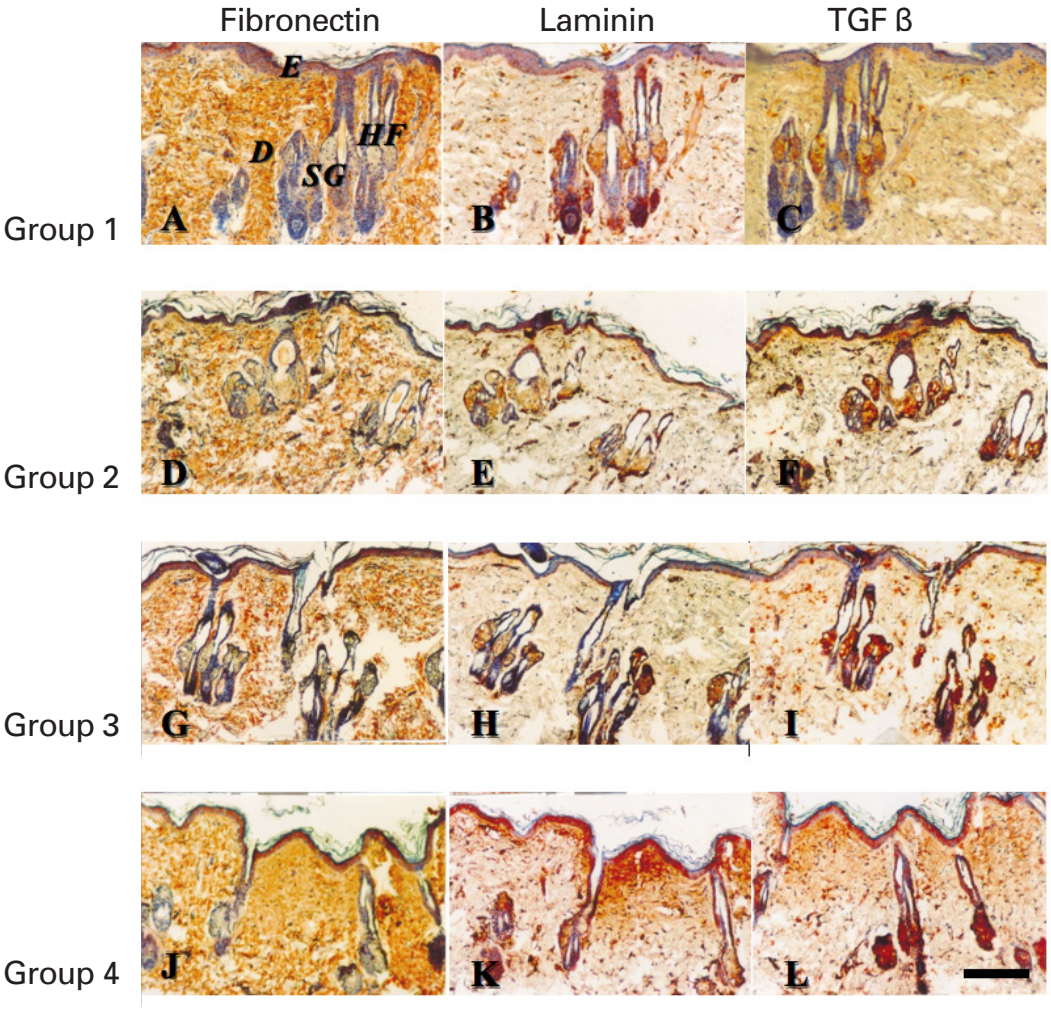
DISCUSSION

Wound healing is a complex pathophysiological process involving interplay of several cellular and biochemical processes. It includes the interaction of inflammation, re-epithelialization, angiogenesis, granulation tissue formation and collagen deposition.[14] This process is driven in part by a complex mixture of growth factors and cytokines, which are released coordinately into the wounds.[15] Cytokines play important roles in the evolution of granulation tissue through recruitment of inflammatory leukocytes and stimulation of fibroblasts and epithelial cells. In case of any impairment in the normal reparative process, delayed healing or excessive fibrosis may occur. Overhealing or excessive fibrosis of wounds is observed in fibroproliferative disorders such as keloids and hypertrophic scars. Keloids and hypertrophic scars are considered to be atypical manifestations of the wound healing process following trauma to the skin. These scars consist of excessive dense fibrous tissue growing in all directions, resulting in a prominent elevation above the skin.[16]

Transforming growth factor-β (TGF-β) is known to be the most potent growth factor involved in wound healing throughout the body.[17] It is synthesized by several types of cells in vivo and in

FIGURE 1. Immunohistochemical staining of scar samples from control and topically treated groups. While increased immunoreactivities of laminin, fibronectin, and TGF-β were observed in the dermis (D) of the skin samples from the control group (Group 1), moderate immunoreactivities observed in heparin (Group 3) and allantoin (Group 4) groups, and mild immunoreactivity in extractum cepea mixture group (Group 2), respectively. Group 1 = control group; Group 2 = extractum cepae, heparin sodium, and allantoin mixture in a gel base group; Group 3 = heparin sodium group; Group 4 = allantoin group.

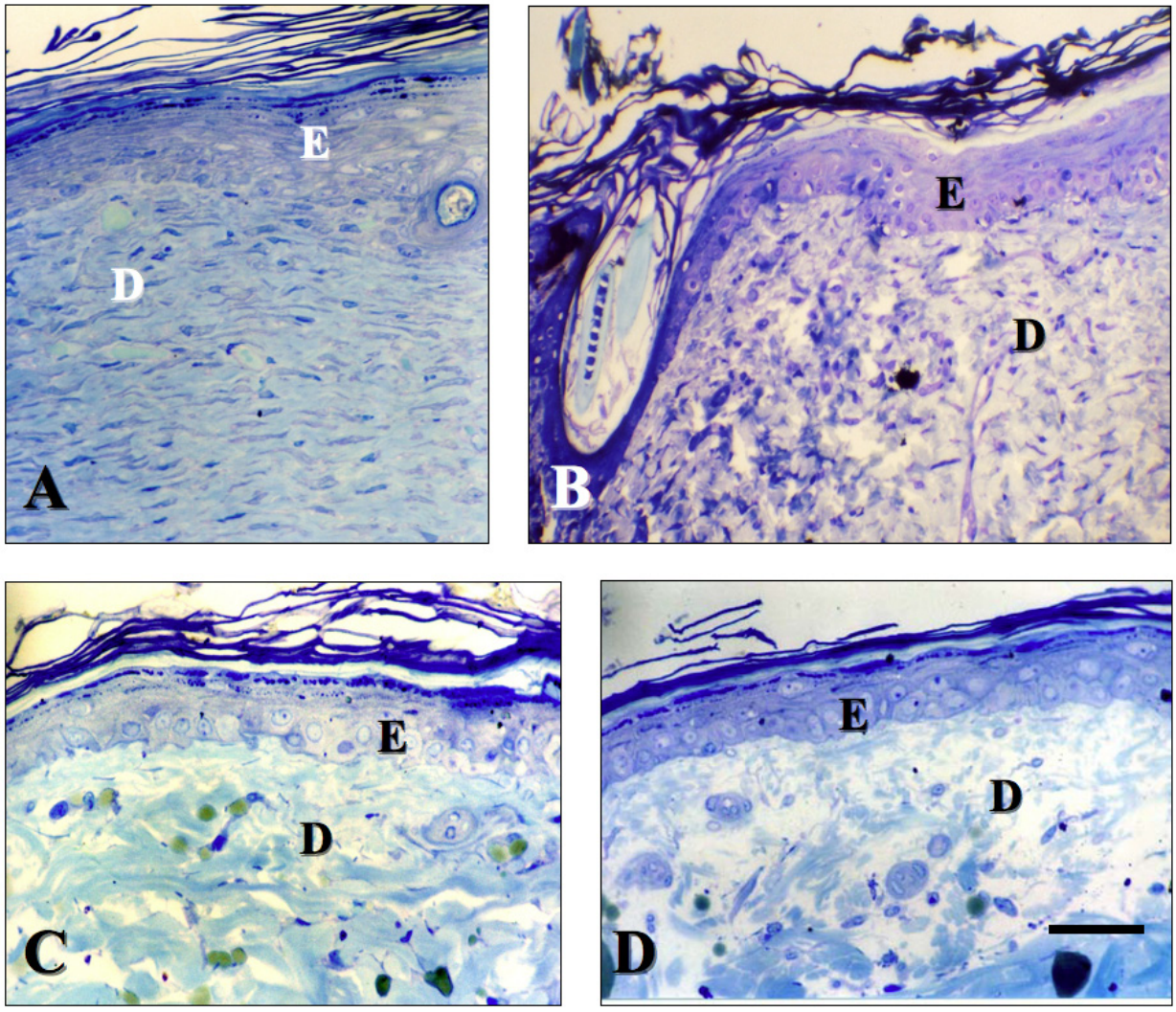
Primary antiantibodies: anti-fibronectin, anti-laminin, and anti-TGF-β
Cromogen: AEC, Counter stained: Mayer's Hematoxylin. HF: Hair Follicle, SG: Sebase gland. Scale bar: 25 μm.



vitro, particularly platelets, placenta and spleen, and by human skin and cultured keratinocytes. It has multiple activities, some of which are synergistic for or inhibitory to the activities of other growth factors. It inhibits proliferation of epithelial cells.[18] TGF-βactivates extracellular matrix production and seems to be the most responsible for excessive scar tissue formation. Fibroblasts on hypertrophic scars and keloids proved to be more sensitive to and respond to a lower concentration of TGF-β.[19] In vitro, TGF-β reduces the collagenase-mediated degradation of wound matrix.[20] Inhibitors of TGF-β reduce scarring.[21] Recent studies have indicated that targeting of TGF-β might result in accelerated wound healing and reduced scarring.[17,22]

A group of receptors called integrins regulate cell attachment in tissue growth, wound healing, and leucocyte extravascular emigration. They are the expression of a family of supergenes. Fibronectin, laminin, and collagen type I are some of the integrin receptor ligands.[18] Fibronectin functions to promote cell adhesion, cell shape and spreading, organization of the cytoskeleton, hemostasis and thrombosis, cell migration, and phagocytosis. In normal conditions skin fibronectin receptors in basal cells were few, but following experimental wounds, when granulation tissue was abundant, greater amounts of fibronectin receptors were evident in epidermis and fibroblasts.[18] Laminin is a multifunctional protein with diverse biological activities. Like

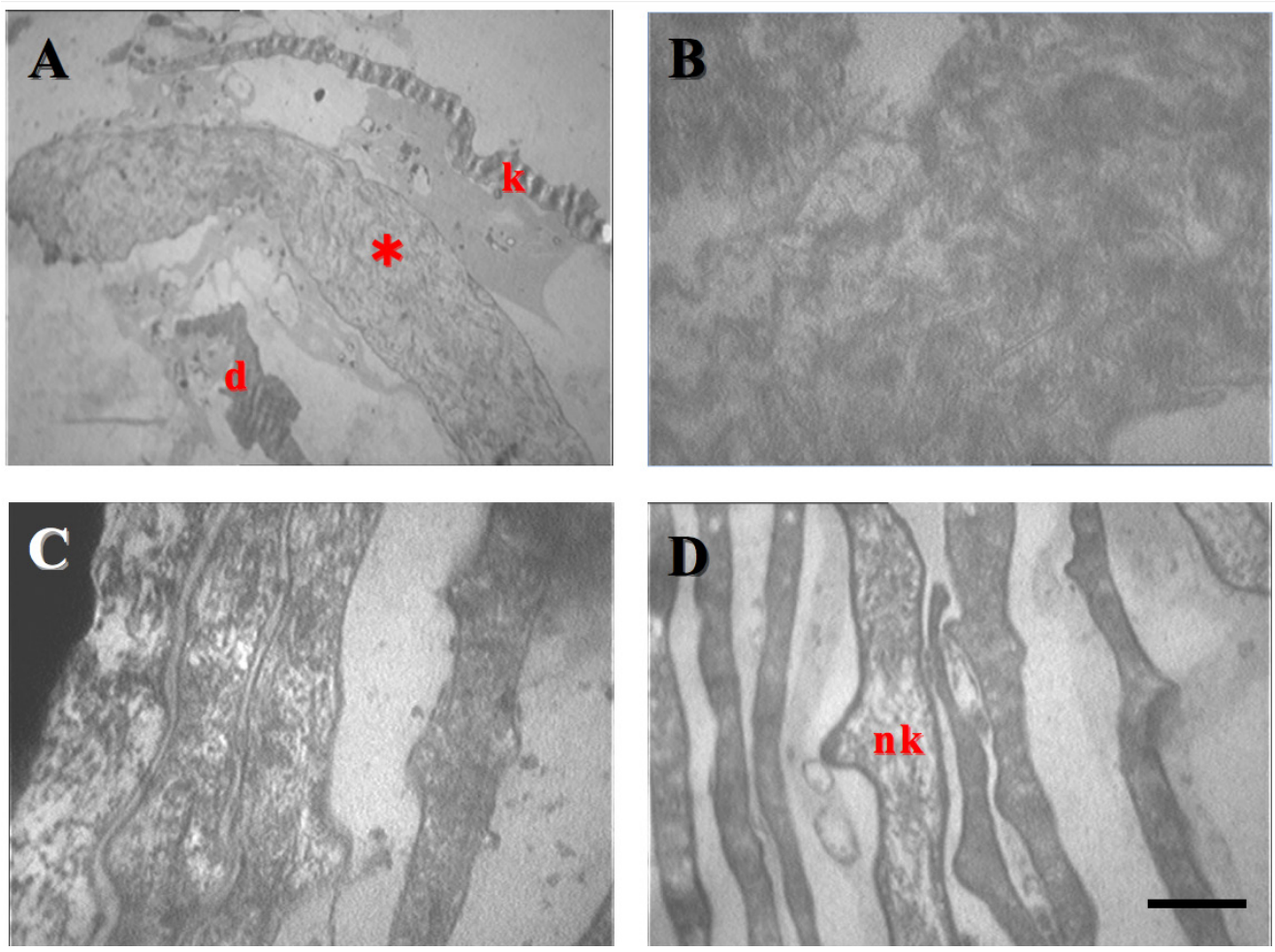
FIGURE 2. Ultrastructural effects of topical scar treatments: results of semi-thin section assessments. Group 1 = control (A); Group 2 = extractum cepae, heparin sodium, and allantoin mixture in a gel base (B); Group 3 = heparin sodium (C); Group 4 = allantoin. E = Epidermis, D = Dermis (D). Scale bar: 100 µm.



fibronectin, it can influence cell adhesion, growth, morphology, differentiation, migration, and agglutination as well as the assembly of the extracellular matrix. Because most differentiated cells are difficult to maintain in culture, laminin may be an important supplement in studies on cell differentiation in vitro.[23]

There are numerous clinical studies, in which the efficacy and the tolerability of extractum cepae-heparin sodium-allantoin mixture have been conducted.[5,8,24] A reduction in both inflammation, fibroblast proliferation, and connective-tissue components such as collagen, can explain the effects of extractum cepae-heparin sodium-allantoin mixture, not only have anti-inflammatory effects, but they also exert similar antiproliferative effects that, in the case of excessive scar formation in hypertrophic and keloidal scars, depress fibroblast proliferation and reduce scar size. Its depressing effect on collagen structure may promote physiologic scar development.[5] In in-vitro studies to show the exact mechanism of action of extractum cepae-heparin sodium-allantoin mixture, onion extract reduced the proliferative activity and the production of substances in the extracellular matrix. Heparin can interact strongly with collagen molecules, and induces the formation of thicker fibrils, typical for a mature tissue, and promotes intermolecular bonding in collagen.[24] These two active ingredients of extractum cepae-heparin sodium-allantoin mixture, therefore, affect scar development by the inhibitory effects on inflammatory processes, fibroblast proliferation, and the synthesizing capacity of fibroblasts.[4,24] In a later stage, which is further characterized by connective tissue fibers, a loosening effect on collagen structure is achieved by the combination of onion extract, heparin, and allantoin. The polyelectrolytes heparin and allantoin have a hydrating effect, which, in addition, promotes the softening of the indurated scar structures.[8]

FIGURE 3. Ultrastructural effects of topical scar treatments: results of electron micrograph assessments. Group 1 = control (A); Group 2 = extractum cepae, heparin sodium, and allantoin mixture in a gel base (B); Group 3 = heparin sodium (C); Group 4 = allantoin (D). (*) Cells that have not completed keratinization. (d) degenerated cells, (k) modified cells of corneum, (nk) stratum corneum cells that have not completed keratinization. Scale bar: 1μm.



Ho et al. showed that extractum cepae-heparin sodium-allantoin mixture reduced the risk of scarring significantly in Chinese patients having laser removal of tattoos.[24] In a study which was carried out by Maragakis et al., it was shown that early treatment with Contractubex gel is suitable for reducing the risk of developing pathological scars after thoracic surgery.[8] In addition, it is well tolerated and permanently improves the appearance of scars. Sigrist treated facial acne scars with microdermabrasion, after which interval and follow-up treatment with Contractubex gel was performed.[25] Subjectively, Contractubex gel resulted in a more rapid disappearance of the residual erythema in all cases and in satisfactory skin care.[25] Willital and Heine concluded that topical Contractubex gel treatment is efficacious both in minimizing the severity of excessive scar formation and in reducing the scar size after surgical incisions, as well as in promoting the paling of the scar erythema.[5] However, Chung et al. treated 24 patients with onion extract and petrolatum emollient on new surgical scars and found no statistically significant difference between the two treatment groups.[26]

In their study, Hosnuter et al. evaluated the efficacy of onion extract, silicon sheet gel, and the combination of these two agents on hypertrophic and keloid scars in 60 patients. They found that onion extract should be used in combination with an occlusive silicon dressing to achieve a satisfying decrease in scar height.[27] In an open, randomized, controlled, comparative study, Koc et al. compared intralesional triamcinolone acetonide alone and combined with onion extract in keloidal and hypertrophic scars of 27 patients. They concluded that combined with onion extract gel, intralesional triamcinolone acetonide appeared to be superior to triamcinolone acetonide alone in the treatment of keloids and hypertrophic scars.[4]

CONCLUSIONS

In our study, we compared and discussed immunohistochemical and ultrastructural effects of extractum cepae-heparin sodium-allantoin mixture, heparin sodium and allantoin in the rat model. Since the effects of the onion extract-based compound have not been deeply characterized so far, we could not find a study comparing immunohistochemical and ultrastructural effects of Contractubex gel, heparin sodium, and allantoin in any animal model in the literature. The reduced dermal immunoreactivity for laminin, fibronectin, and TGF-β was associated with heparin-, allantoin-, and Contractubex gel treatment in our study. The gel-based extractum cepae-heparin sodium-allantoin mixture was found to have apparent keratinolytic and collagenase effects. The thinnest epidermis of the four groups and normal-appearing epidermal cells that had completed keratinization showed that Contractubex gel had apparent keratinolytic effects. The following findings show the effect of Contractubex gel on collagenase: [1] dermal papillary formation occurred earlier in the second group comparing to the other groups; [2] connective tissue formed, consisting of loosely-structured collagen fibers and vessels in the papillary dermis (Fibrosis did not develop, and scar formation completed close to normal cutaneous morphology.), and [3] scar formation reduced more effectively than no treatment, heparin monotherapy, or allantoin monotherapy groups.

We can emphasize that Contractubex gel results in better reduction of scar formation, since TGF-β, laminin, and fibronectin immunoreactivities were mild or moderate. We conclude that wound healing with extractum cepae-heparin sodium-allantoin mixture is better than no treatment, heparin monotherapy, and allantoin monotherapy groups according to immunohistochemical and ultrastructural measurements.

DISCLOSURES

The authors have no relevant conflicts of interest to disclose.

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