Angiotensin-Converting Enzyme Inhibitor Enalapril Reduces Formation of Hypertrophic Scars in a Rabbit Ear Wounding Model

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Background: Angiotensin-converting enzyme inhibitors are widely used in medicine because of their antihypertensive and antifibrogenic effects. Angiotensin-converting enzyme activates angiotensin I to angiotensin II, which plays an important regulatory role in wound healing and collagen production. The authors investigated whether systemic administration of angiotensin-converting enzyme inhibitors has any effect on formation of hypertrophic scars using the rabbit ear wound model.
Methods: Sixteen New Zealand albino rabbits were divided into four groups, and four punch defects were created on each ear. The first group received oral enalapril immediately after the creation of punch defects. The second group received oral enalapril on day 28 after the formation of scars. The third group received intralesional steroid injections on days 28 and 35. The fourth group was the control group. The rabbits were killed on day 40. The harvested specimens were analyzed histomorphometrically and immunohistochemically.
Results: Early enalapril application decreased the scar elevation index and fibroblast and capillary counts significantly, compared with the values in the control group. Late enalapril application decreased fibroblast counts significantly; however, there was no difference in scar elevation index compared with the control group. There was no difference between early enalapril application and steroid therapy in terms of scar elevation index and capillary and fibroblast counts. However, early and late enalapril groups displayed lower collagen type III immunoreactivity compared with the steroid and control groups.
Conclusion: Early application of enalapril following dermal injury reduces formation of hypertrophic scars, probably because of its down-regulatory effects on type III collagen production. (*Plast. Reconstr. Surg.* 132: 361e, 2013.)

Hypertrophic scarring is the clinical manifestation of a dysfunctional wound healing response of skin to injury. It is characterized by excessive collagen deposition, which results in a stiff, elevated scar.[1] Associated contractures, burning, itching, and prolonged tenderness can mask the success of surgery and are a major concern for patients and surgeons.

There is no definitive treatment for hypertrophic scarring. Some widely accepted and routinely used options for suboptimal treatment of this condition include steroid injections, radiotherapy, and topical silicone gel application.[2] Intralesional steroid therapy can be considered the criterion standard against which any prospective therapeutic option would be judged. However, even steroid injections have significant shortcomings, such as repetitive painful injections and unpredictable efficacy. Other treatment modalities such as irradiation and silicone-sheet applications are not easy to use, nor do they perform better. Treatment that is easy to apply, painless, and effective would fill a very large gap in plastic surgery practice and be welcomed by patients and surgeons.[3]

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Received for publication October 23, 2012; accepted February 20, 2013.
Presented at the 32nd Annual Meeting of the Turkish Society of Plastic, Reconstructive, and Aesthetic Surgery, in Trabzon, Turkey, September 15 through 19, 2010.

DOI: 10.1097/PRS.0b013e31829acf0a

Disclosure: *The authors have no financial interest in any of the products, devices, or drugs mentioned in this article.*

Angiotensin-converting enzyme inhibitors act by inhibiting the converting enzyme that hydrolyzes angiotensin I to its active form, angiotensin II.[4] Angiotensin II promotes collagen production[5] and plays an important role in wound healing and fibrosis. Angiotensin II overactivity is associated with pathologic fibrosis in the heart,[6] aorta,[7] kidney,[8] and lungs.[9] Angiotensin-converting enzyme inhibitors decrease left ventricular collagen content in heart failure.[10]

Angiotensin-converting enzyme and angiotensin II levels are higher in hypertrophic scars than in normal skin and normal scars.[11] Case reports have claimed that angiotensin-converting enzyme inhibitors may prevent (or even treat) hypertrophic scars clinically.[12] Between this hypothetical biochemical background and the case reports, there exists a gap of basic science research. No experimental study has investigated the relationship between hypertrophic scarring and angiotensin-converting enzyme inhibitors. We investigated the preventive potential of angiotensin-converting enzyme inhibitors in scar hypertrophy using an established model of ear wounding in rabbits.

MATERIALS AND METHODS

This study protocol was approved by the Review Board of Experimental Ethics, Faculty of Medicine, Hacettepe University (Ankara, Turkey). The animals were kept, fed, and killed under standardized conditions. A model of ear wounding was used in rabbits that has been described and widely used.[13] Sixteen female New Zealand albino rabbits (2500 to 3500 g) were used. Animals were divided into four groups of four.

Surgical Procedure

Rabbits were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg). Four punch defects were created on each ear using a 5-mm biopsy punch (Fig. 1). The defects involved the epidermis, dermis, and perichondrium. A dissecting microscope was used to ensure removal of the perichondrium. Hemostasis was achieved by applying manual pressure, and each ear covered with Tegaderm (3M Co., St. Paul, Minn.). After this procedure, each group consisted of 32 standardized punch defects (Fig. 2).

Groups

After the creation of wounds, rabbits were divided into four groups. The first group was the early enalapril group; administration of enalapril (0.75 mg/kg/day) was initiated immediately after wound creation. The second group was the late enalapril group; administration of enalapril (0.75 mg/kg/day) was initiated on day 28, when hypertrophic scars had already formed. This dosage regimen was adapted from studies using enalapril in rabbits.[14,15] Enalapril formulations were prepared by diluting commercial 10-mg enalapril tablets with water. Suspensions were administered through transoral gavage. The third group was the steroid group; a suspension of triamcinolone acetonide, 40 mg/ml (Kenacort A; Bristol-Myers Squibb, New York, N.Y.), was administered intralesionally on days 28 and 35. At two points, 180 degrees apart and peripheral to the original wound, a needle was introduced and advanced intradermally so that its tip was positioned under the center of the scar, and 10 μl of material was injected using 28-gauge needles.[13] The fourth group was the control group, and no additional procedure was applied. Histologic properties of experimental hypertrophic scars diminish after day 40, so the rabbits were killed on this day.

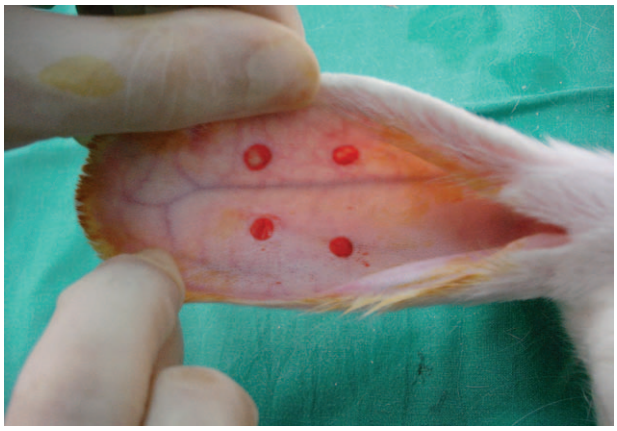


Fig. 1. Formation of the punch defects.

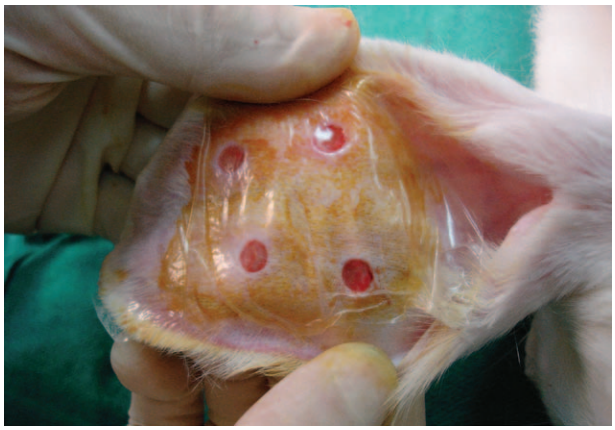


Fig. 2. Coverage of the punch defects with Tegaderm dressing.

Histomorphometric and Immunohistochemical Analysis

Half of the specimens were fixed in 10% neutral-buffered formalin and processed into paraffin blocks. Slides were prepared from 5-μm sections and stained with hematoxylin and eosin and Masson trichrome. Each stained slide was examined using a Leica DM-6000 image analysis system (Leica Microsystems, Wetzlar, Germany) and images were recorded digitally. Indices of scar elevation were calculated using the Leica Q-win V3 program. Scar elevation index is an established measurement tool that correlates well with other methods of measurements of wound healing and is used to calculate the ratio of total wound area (scar and normal tissue) to the area of normal tissue below the hypertrophic scar. The scar elevation index integrates cellular proliferation, matrix deposition, and epithelial thickness as a whole (Fig. 3). Fibroblasts were counted using an ocular micrometer under 400× magnification in three random fields of 1 mm^2 by two histologists blinded to the groups. Capillaries were also counted under 40× magnification. The other half of the specimens were processed for immunohistochemical analyses. The density of type I and type III collagen was measured qualitatively. Immunostaining was performed by using the streptavidin–biotin horseradish peroxidase technique (LSAB2 System, HRP Universal; Dako, Glostrup, Denmark), which involves anti-collagen type I (GeneTex, Irvine, Calif.) and anti-collagen type III (Acris, Herford, Germany) primary antibodies.

Statistical Analysis

Data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, Ill.). Initial analyses of frequency and variation revealed that fibroblast and capillary counts and indices of scar elevation were distributed nonparametrically. The Kruskal-Wallis test (a nonparametric test) was chosen to ascertain whether there is a statistical difference between groups in fibroblast and capillary counts and scar elevation index. The Kruskal-Wallis test demonstrated statistically significant differences between groups. The Mann-Whitney *U* test was used to perform paired comparisons between variables. The Bonferroni correction was used, which required a value of $p < 0.005$ to reflect statistical significance.

RESULTS

Clinical Findings

All wounds in the early enalapril group were completely epithelialized by the third week. Scars were softer, paler, and less elevated than those of the control group (Fig. 4, *above*).

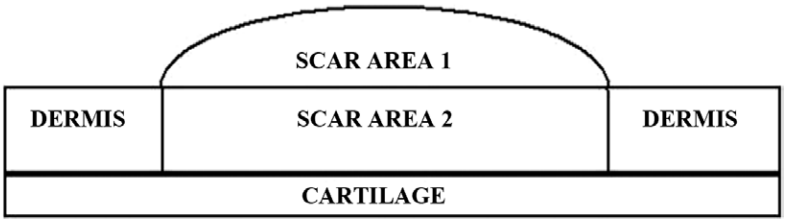
All wounds in the late enalapril group were completely epithelialized by day 14, and scar hypertrophy was obvious on day 28. Scars in this group were stiffer and more erythematous when compared with the early enalapril group, but softer than those in the control group (Fig. 4, *center*).

All wounds in the steroid group were completely epithelialized by day 14 and healed with hypertrophic scars by day 28. After steroid injections, scars lost their thickness and erythema by day 40. They were paler and softer than those in all other groups (Fig. 4, *below*).

All wounds in the control group were completely epithelialized by day 14 and healed with hypertrophic scar by day 28. Hypertrophic scars were erythematous, highly elevated from surrounding tissue, and stiff on palpation. They maintained these properties on day 40 (Fig. 5).

Histologic Observations

Scars in the early enalapril group had a thin epithelium, low fibroblast and capillary counts,



$$\text{SCAR ELEVATION INDEX} = \frac{\text{SCAR AREA 1} + \text{SCAR AREA 2}}{\text{SCAR AREA 2}}$$

Fig. 3. Estimation of scar elevation index.

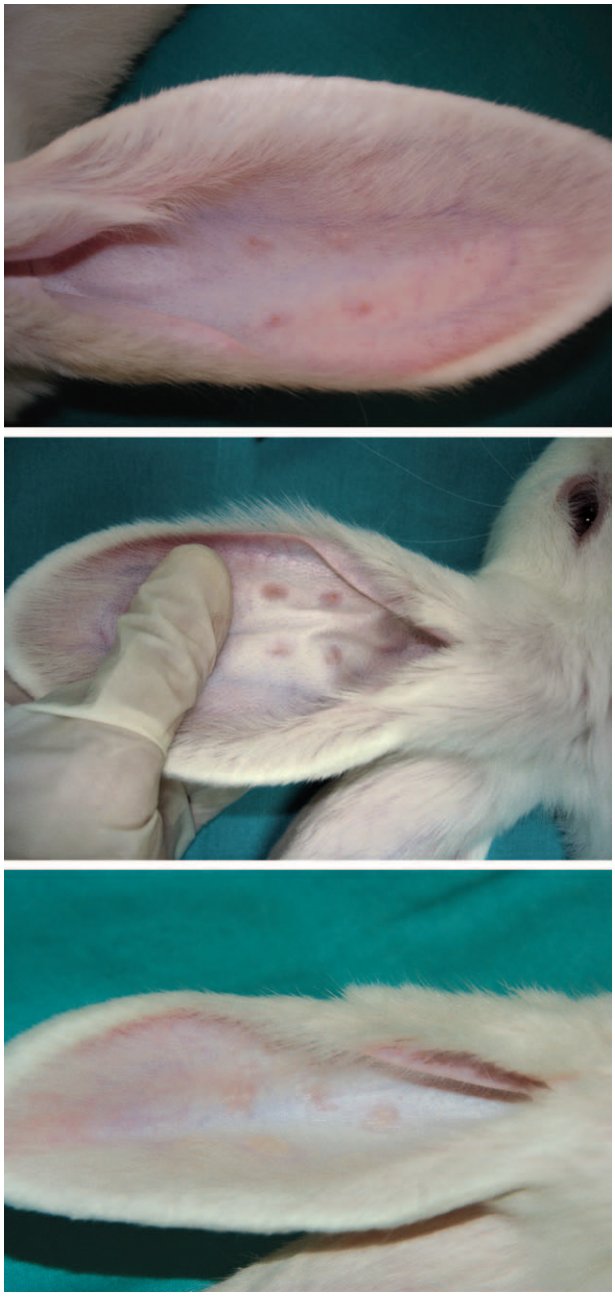


Fig. 4. (*Above*) Appearance of wounds in the early enalapril group at day 40. (*Center*) Appearance of wounds in the late enalapril group at day 40. (*Below*) Appearance of wounds in the steroid group at day 40.

and collagen-rich connective tissue (Fig. 6). Scars in the late enalapril group had a thin epithelium and collagen- and capillary-rich, dense, irregular connective tissue. Fibroblasts were diminished in number when compared with those in the control group (Figs. 7 and 8). Scars in the steroid group had loosely arranged, mature collagen bundles and low capillary and fibroblast counts (Fig. 9, *above*). Scars in the control group displayed classic

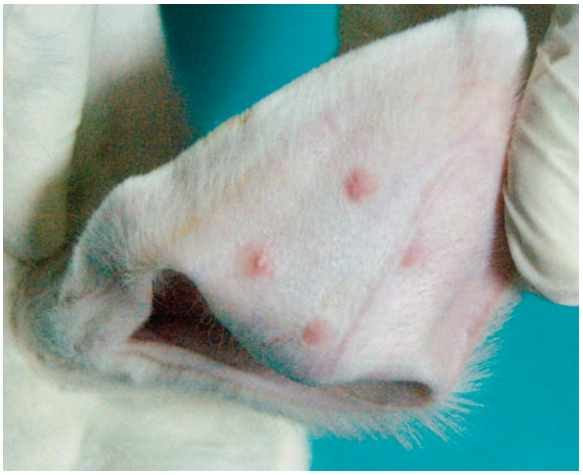


Fig. 5. Appearance of wounds in the control group at day 40.

characteristics of hypertrophy. The area between the thick stratified squamous epithelium and perichondrium was filled with dense, irregular connective tissue. This area of scar hypertrophy was rich in fibroblasts; capillaries; and thick, short collagen bundles (Fig. 9, *below*, and Table 1).

Fibroblast Counts

When compared with the control group, both early and late enalapril groups had lower fibroblast counts, and this difference was significant ($p < 0.005$). Although the median fibroblast count in the early enalapril group was lower than that of the late enalapril group (330 versus 405), the difference was insignificant ($p = 0.076$). The steroid group had the lowest median fibroblast count (269), and this was significant ($p < 0.005$) (Fig. 10 and Table 2).

Scar Elevation Indices

The early enalapril group displayed a significantly lower scar elevation index (median, 1.30) than the late enalapril (median, 1.56) and control groups (median, 1.09). Although the median scar elevation index of the late enalapril group (1.56) was lower than that of the control group (median, 1.98), the difference was not significant ($p = 0.014$).

The steroid group had the lowest median scar elevation index (1.25). Scar elevation index in the steroid group was significantly lower than those of the late enalapril and control groups ($p < 0.005$). There was no difference in scar elevation index between the steroid and early enalapril groups ($p = 0.546$) (Fig. 11 and Table 3).

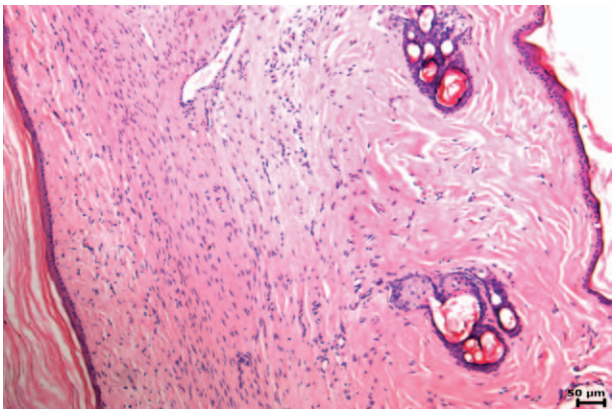


Fig. 6. The early enalapril group had loosely arranged collagen fibers, fewer capillaries, and thinner epithelium.

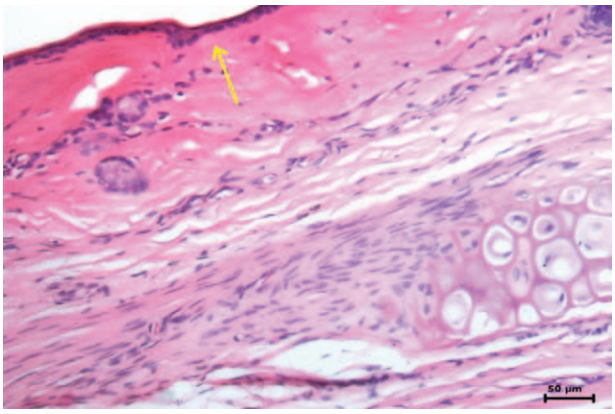


Fig. 8. The late enalapril group had thinner epithelium (*yellow arrow*) and fewer fibroblasts compared with the control group.

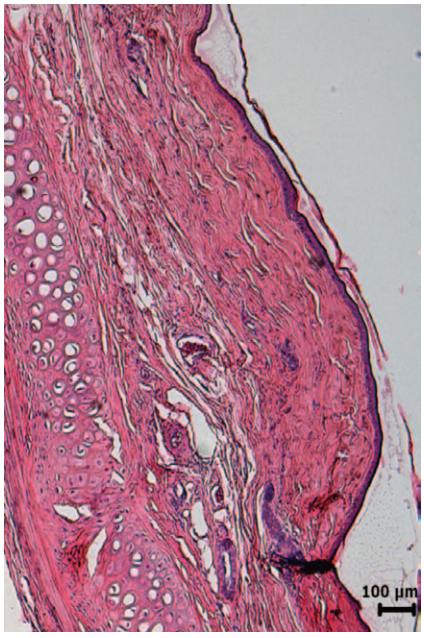
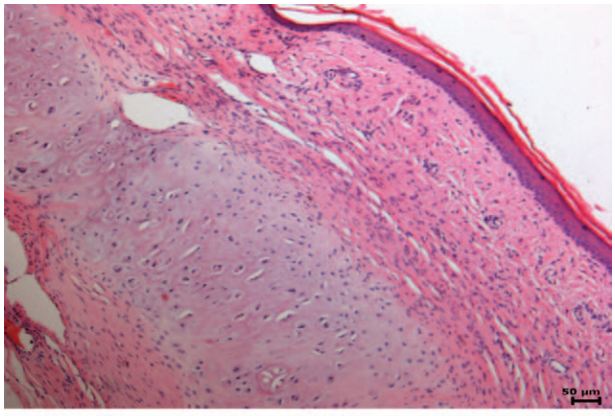


Fig. 7. The late enalapril group had collagen- and capillary-rich, dense, irregular connective tissue.



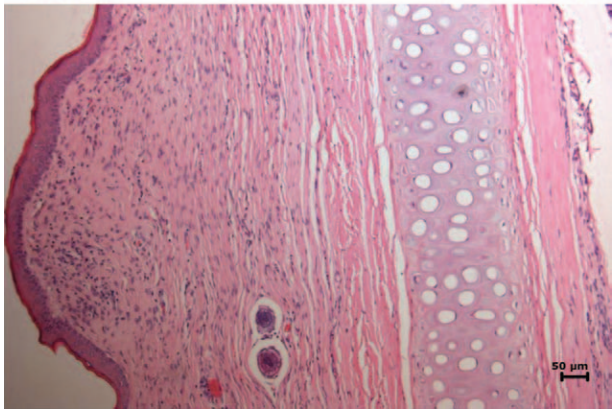


Fig. 9. (*Above*) The steroid group had loosely arranged mature collagen fibers. (*Below*) Scars in the control group had thick epithelium and the highest number of fibroblasts.

Capillary Counts

The early enalapril group had the lowest median capillary count (1.75). Capillary counts of the early enalapril group were significantly lower than those of the late enalapril and control groups ($p < 0.005$). Although the late enalapril group had a lower median capillary count than that of the control group (3.19 versus 3.9), the difference

Table 1. Histomorphometric Features

Groups	Fibroblast Count	Scar Elevation Index	Capillary Count	Collagen Structure	Epithelial Thickness
Early enalapril	Lower	Low	Lower	More organized	Lower
Late enalapril	Lower	High	High	Less organized	High
Steroid	Lowest	Lowest	Lower	Most organized	Lower
Control	Highest	Highest	Highest	Least organized	Highest

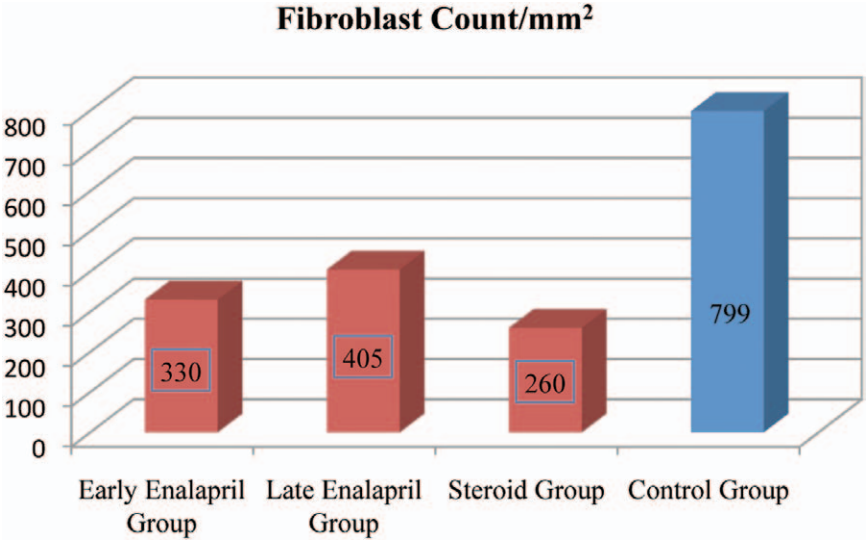


Fig. 10. Median fibroblast counts (Mann-Whitney *U* test, $p < 0.005$). Groups that are statistically different from the control group are represented in *red*.

was not significant ($p = 0.034$). Also, there were no significant differences between the early enalapril and steroid groups in terms of capillary count ($p = 0.49$) (Fig. 12 and Table 4).

Immunohistochemical Analysis

In the early enalapril group, collagen type I immunoreactivity was higher than that of the late enalapril and control groups. Type I immunoreactivity was not different between the early enalapril and steroid groups. Collagen type III fibers were less immunoreactive and shorter than those of the late enalapril, steroid, and control groups. Thus, early administration of enalapril decreased collagen type III content of scar tissue the most (Fig. 13, *above*).

Table 2. Fibroblast Counts*

Rabbit	Early Enalapril Group	Late Enalapril Group	Steroid Group	Control Group
1	432	442	384	757
2	330	389	389	752
3	442	346	277	906
4	453	309	304	794
5	474	506	234	688
6	320	330	282	917
7	330	448	213	656
8	304	421	176	693
9	266	314	234	709
10	250	288	192	826
11	320	458	213	805
12	330	464	266	789
13	341	496	272	853
14	373	464	288	896
15	282	357	256	805
16	400	336	309	810

*Values are given as cells per square millimeter.

In the late enalapril group, collagen type I immunoreactivity was more prominent than type III immunoreactivity. Type I fibers were still dense and started to form regular bundles. Type III fibers were arranged loosely (Fig. 13, *below*).

In the steroid group, collagen type I immunoreactivity was similar to that in the early enalapril group and higher than that in the late enalapril and control groups. However, bundles were more mature in appearance (Fig. 14, *above*). Collagen type III immunoreactivity was found to be moderate in the steroid group.

In the control group, collagen type III fibers were the dominant type because they were strikingly more immunoreactive than type I fibers. Type III fibers were also denser and thicker (Fig. 14, *below*). Although the steroid group displayed moderate immunoreactivity for collagen type III, the ratio of type III to type I immunoreactivity was highest in the control group. This ratio, in favor of collagen type III, is a typical characteristic of hypertrophic scars.

DISCUSSION

The transforming growth factor (TGF)-β family of cytokines plays a major role in wound healing and in the pathophysiology of hypertrophic scarring. Autocrine and paracrine stimulation of dermal fibroblasts by TGF-β increase the synthesis of collagen type I and other extracellular matrix proteins. Tang et al. showed that angiotensin II increases TGF-β expression, and that blockage of TGF-β inhibits angiotensin II–related collagen synthesis.[16] Angiotensin-converting enzyme

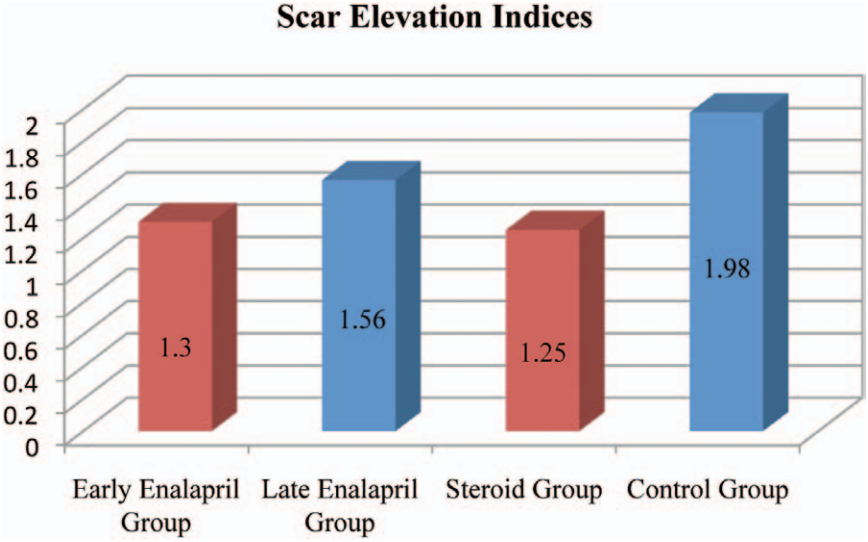


Fig. 11. Median scar elevation indices (Mann-Whitney *U* test, $p < 0.005$). Groups that are statistically different from the control group are represented in *red*.

inhibitors reduce the amount of angiotensin II and therefore decrease the expression of TGF-β.

Connective tissue growth factor increases synthesis of extracellular matrix in fibroblasts (particularly collagen type I, collagen type III, and fibronectin) by exerting a TGF-β–like profibrotic effect.[17,18] Zhang et al. showed that angiotensin II increases connective tissue growth factor synthesis in human dermal fibroblasts by binding to angiotensin type 1 receptors.[19] Also, human hypertrophic scar fibroblasts have increased expressions of Angiotensin-converting enzyme, angiotensin II and angiotensin type 1 receptors.[20]

The present study once again demonstrated that pharmacologic manipulation of this pathway, regardless of the steps involved, can alter internal scar dynamics. We were able to form hypertrophic scars using a rabbit ear model described previously[3,13,21] and showed the histologic and immunohistochemical properties of hypertrophic scars. When compared with the enalapril and steroid groups, the scars in the control group had the highest fibroblast density, vessel density, and scar elevation index, all of which are characteristic features of hypertrophic scars.

Enalapril was chosen because there are data from previous studies that used enalapril.[12] Its history in clinical use, documented safety profile, and off-the-shelf availability were also supportive.[22] In the present study, enalapril was administered orally. Oral drug therapy may potentially replace steroid injections in daily use if it can display equal or superior efficacy in the treatment of hypertrophic scars. This is why we preferred not to inject enalapril (although an injectable form is available).

Enalapril groups were designed as early and late groups to test whether enalapril has any regressive efficacy if applied after the development of a mature hypertrophic scar. Scar hypertrophy was evident in the fourth week in our experimental model, and that time was chosen as the initiation point of drug therapy for the late enalapril group. The steroid group was designed because any prospective treatment should outpace the current criterion standard: intralesional steroid injections. The present study showed that early oral administration of enalapril had significant effects on wound healing, which helped to improve the quality of scars clinically and also to reduce capillary counts, fibroblast counts, and scar elevation index.

Table 3. Scar Elevation Indices

Rabbit	Early Enalapril Group	Late Enalapril Group	Steroid Group	Control Group
1	1.22	1.48	1.39	2.61
2	1.43	1.70	1.36	1.62
3	1.40	1.55	1.00	1.99
4	1.15	1.37	1.61	2.33
5	1.14	1.95	1.43	2.74
6	1.25	2.39	1.41	1.31
7	1.21	1.55	1.18	2.17
8	1.31	1.94	1.22	1.82
9	1.55	1.48	1.32	1.46
10	1.29	1.71	1.27	1.50
11	1.30	1.38	1.11	2.29
12	1.34	1.53	1.23	1.99
13	1.45	1.56	1.32	1.87
14	1.26	1.65	1.22	1.97
15	1.55	1.66	1.18	1.76
16	1.19	1.34	1.20	2.01

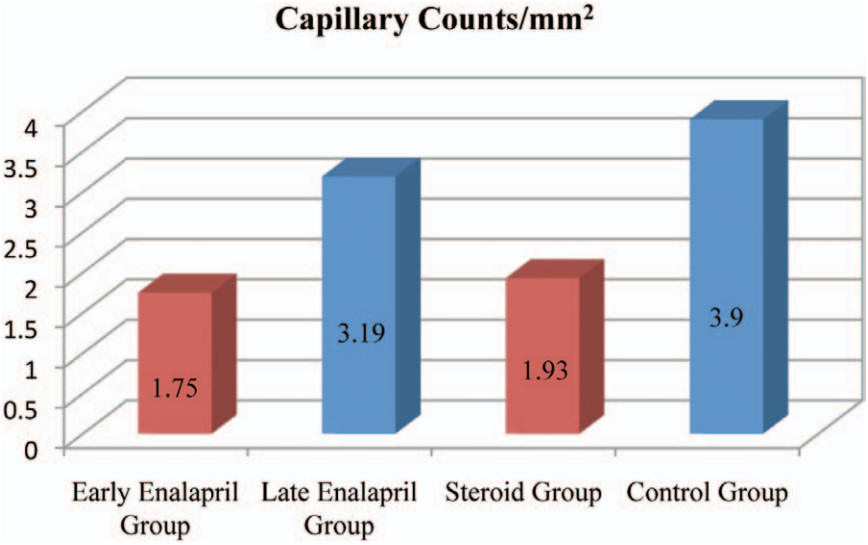


Fig. 12. Median capillary counts (Mann-Whitney *U* test, $p < 0.005$). Groups that are statistically different from the control group are represented in *red*.

The median scar elevation index in the early enalapril group was significantly lower than that of the late enalapril and control groups. Scar elevation index is a highly predictive measure with clinical correlative value. Our findings clearly demonstrated that angiotensin-converting enzyme inhibitor therapy significantly reduced the height and volume of the scar. Angiotensin-converting enzyme inhibitor therapy should be started as soon as possible to prevent hypertrophic scarring. Once the scars are fully hypertrophied, angiotensin-converting enzyme inhibitor administration does not significantly alter the course. Therefore, we conclude that angiotensin-converting enzyme inhibitor can be used as a preventive measure but not as a treatment option, which is supported by the immunohistochemical outcomes of our study.

Table 4. Capillary Counts*

Rabbit	Early Enalapril Group	Late Enalapril Group	Steroid Group	Control Group
1	1	4	2	3
2	2	3	2	5
3	2	1	1	4
4	1	2	2	3
5	2	3	2	5
6	2	5	2	4
7	1	4	3	3
8	2	5	2	5
9	2	3	1	4
10	2	3	1	4
11	1	5	2	3
12	1	4	3	4
13	2	1	1	5
14	2	3	2	3
15	2	2	2	4
16	3	3	3	4

*Values are given as capillaries per square millimeter.

The lower capillary count explains the paler appearance of scars in the early enalapril and steroid groups than in the control group. Scars in the late enalapril group were rich in capillaries, similar to the control group. Angiotensin II accelerates neovascularization, increases endothelial proliferation, and promotes the release of growth factors such as vascular endothelial growth factor and platelet-derived growth factor.[23,24] Although a paler appearance and a lower capillary count were expected in both enalapril groups, they was not observed in the late enalapril group. Neovascularization completes at 4 weeks, and this may be why late administration of enalapril was ineffective against capillary proliferation.[25]

Both enalapril groups had thinner epithelium and lower fibroblast density than the control group. In the early enalapril group, completion of epithelialization was delayed. These findings were thought to be related to the fact that angiotensin-converting enzyme inhibitors block the angiotensin II–mediated stimulation of keratinocyte and fibroblast migration and proliferation. Delayed epithelialization can be a problem in wounds with large surface areas, such as burns and venous ulcers. In surgical incisions, the delay in epithelialization is expected to be less of a problem because the gap to be reepithelialized is relatively small. In our opinion, in the management of large-surface-area wounds, a slight delay in wound healing would be reasonable if scar hypertrophy is reduced.

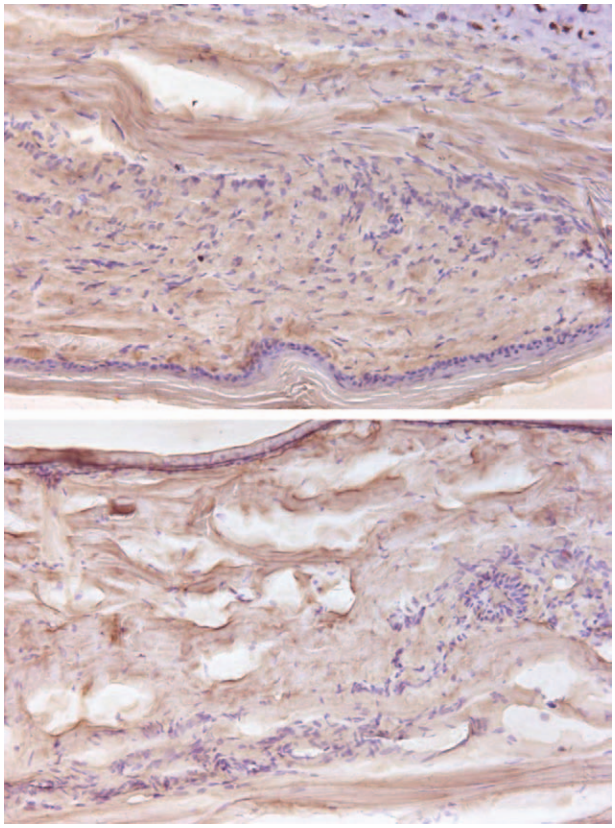


Fig. 13. (*Above*) Weak collagen type III immunoreactivity in the early enalapril group. (*Below*) Weak collagen type III immunoreactivity in the late enalapril group.

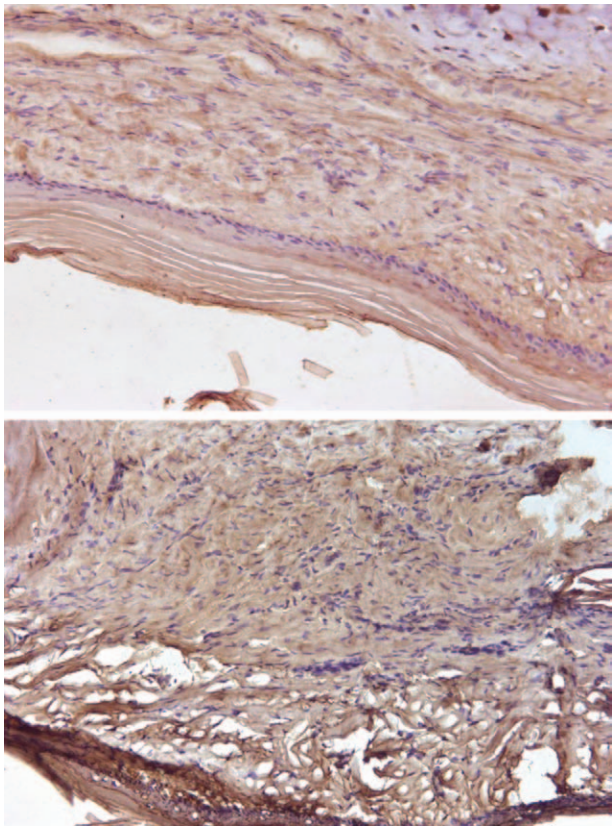


Fig. 14. (*Above*) Strong collagen type I immunoreactivity in the steroid group. (*Below*) Strong collagen type III immunoreactivity in the control group.

Enalapril administration significantly altered collagen composition in experimental scars. Inhibition of collagen type III synthesis appeared to be the most striking feature of enalapril action against hypertrophic scars. Decreased immunoreactivity of type III collagen in the early and late enalapril groups led us to conclude that enalapril down-regulates the synthesis of type III collagen.

During wound healing, levels of type I and type III collagen increase; during the remodeling phase, they decrease.[26] Hypertrophic scars have higher amounts of collagen type III, similar to fetal wounds.[27–29] Strong collagen type III immunoreactivity and weak type I immunoreactivity are typical features of hypertrophic scars as also reproduced in the control group of the present study. An increase in the collagen type I–to–type III ratio reflects the maturity of a scar; therefore, enalapril administration may result in enhanced scar maturity.

Earlier administration of enalapril was more effective in scar prevention. Angiotensin II acts primarily on the synthetic pathways involved in scar hypertrophy and fibrosis. Therefore, angiotensin-converting enzyme inhibitors are better suited for prevention rather than treatment.

Zimman et al. demonstrated a similar preventive efficacy of enalapril on implant capsules. They implanted smooth and textured miniature breast implants under rat skin.[30] They fed the control rats plain water and the test rats enalapril-added water. Regardless of the type of implant surface, groups that had been treated with enalapril displayed a significant reduction in the overall inflammatory reaction, thickness of the periprosthetic capsule, and amount of collagen type III within the capsule. Similarly, our results showed that early enalapril treatment decreased the thickness, fibroblast count, and vessel density of scars.

Recently, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) have been shown to reduce the formation of hypertrophic scars by means of connective tissue growth factor inhibition if administered at low doses.[31] Statins have well-known antifibrotic properties demonstrated in various fibrosis models.[32–35] This antifibrotic efficacy is displayed by means of modulation of TGF-β1 signaling.[36,37] This study deserves special

mention, because it has many similarities with the present study in terms of fundamental philosophy.

Avotermin (recombinant human TGF-β3) also deserves a special mention because prospective, randomized, double-blind, phase II clinical studies have demonstrated its antiscarring efficacy.[38,39] TGF-β3 is known to improve scar appearance in a range of mammalian species.[40] Intradermal prophylactic administration of recombinant TGF-β3 around newly formed wounds and hypertrophic scars has been shown to improve scar quality in human subjects.[38,41] Our approach of angiotensin-converting enzyme inhibition resulted in a decrease in angiotensin II and TGF-β levels. However, decreases in TGF-β levels were nonselective. A decrease in the syntheses of TGF-β1 and TGF-β2 is desirable in the prevention of hypertrophic scars, but a concomitant decrease in TGF-β3 synthesis is not. Therefore, addition of TGF-β3 to an angiotensin-converting enzyme–inhibited wound may be synergistic for scar improvement.

The regulatory effect of angiotensin II on connective tissue growth factor expression is dependent on its binding ability to angiotensin type 1 receptors. Theoretically, angiotensin type 1 receptor blockers can also interfere with the hypertrophic scarring pathway, similar to angiotensin-converting enzyme inhibitors, at the angiotensin II level.[42]

CONCLUSIONS

Early administration of enalapril after dermal injury reduced scar hypertrophy in the present experiment. Hypertrophic wound healing is a multifactorial, complex process. Numerous pathways have been described, but the exact mechanism and definitive cure are lacking. Similar complex multifactorial diseases such as asthma and cancer are treated with multidrug or multimodal strategies based on the pathways involved. Similarly, angiotensin-converting enzyme inhibitors may be used as noninvasive prophylactic modalities or as components of future combined regimens. Phase II studies are needed to test their efficacy in human subjects. Such a study is currently being designed in our institution.

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ACKNOWLEDGMENT

This research was supported by the Scientific Research Committee of Hacettepe University (No. 4133).

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