Effects of a Topical Angiotensin-Converting Enzyme Inhibitor and a Selective COX-2 Inhibitor on the Prevention of Hypertrophic Scarring in the Skin of a Rabbit Ear

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Abstract: Angiotensin-converting enzyme (ACE) inhibitors have been reported to inhibit fibrogenesis, and cyclooxygenase-2 (COX-2) inhibitors, to reduce scarring by reducing the initial inflammation. The authors reasoned that the topical application of these 2 agents may have a complementary effect on scar reduction. Methods. Captopril (ACE inhibitor), celecoxib (COX-2 inhibitor), or a combination of captopril and celecoxib were topically applied to a skin wound in a rabbit ear, and investigated for the effects on scar formation. Results. The level of scar elevation decreased in the captopril group and the level of infiltration of inflammatory cells decreased in the celecoxib group. In the group where a combination of the 2 drugs was used, the level of scar elevation decreased the most, and collagen deposition and organization returned to normal most rapidly. Celecoxib was found to inhibit the initial inflammation in the ear wound of the rabbit, and captopril inhibited scar elevation. Conclusion. Clinical application of these drugs will require further studies with regard to adverse events and their absorptivity as topical agents. However, these findings suggest that the combined topical administration of an ACE inhibitor and COX-2 inhibitor to a skin wound could be an effective treatment for the prevention of hypertrophic scarring.

Each year, more than 100 million people worldwide develop scars. More than $7 billion is spent annually in the United States alone for their treatment.¹,² Scar reduction is thus considered an important issue for the entire surgical field. Various methods have been attempted to inhibit excessive scarring. Corticosteroid, bleomycin, radiation, 5-fluorouracil, laser therapy, silicon sheets, pressure therapy, cryotherapy, and surgical treatment are currently used to inhibit abnormal scarring, despite reports of insufficient outcomes and limitations.³ Other scar-reducing therapies currently being investigated include treatment with transforming growth factor beta (TGF-β) superfamily, cyclooxygenase-2 inhibitors (COX-2 inhibitors), collagen synthesis inhibitors, angiotensin-converting enzyme (ACE) inhibitors, or minocycline, and gene therapy.⁴

Wound healing comprises 3 phases: inflammation, proliferation, and re-
modeling. Inflammation starts immediately after the appearance of the wound. The extent of inflammation in this first phase greatly influences the later formation of a scar. In addition, reactions that take place in the inflammation phase influence the migration and proliferation of fibroblasts in the proliferation phase. Scars may vary according to the amount of collagen produced by these fibroblasts and by the extent of degradation of collagen. Many treatments and prophylactic measures to prevent scars involve inhibition of initial inflammation, thus preventing the over activity of fibroblasts and controlling the degradation and reorientation of tissue.

ACE inhibitors are known to inhibit fibrogenesis by suppressing the production of angiotensin-II. Since the discovery of increased angiotensin-II in post-myocardial-infarction scar tissue, extensive studies have been performed on the link between angiotensin-II and fibrosis, revealing that angiotensin-II indeed contributes to tissue fibrogenesis. These findings prompted investigation of the use of ACE inhibitors to prevent hypertrophic scars. Despite a suggested role for angiotensin in the inflammatory reaction, there has been no evidence of reduction of the initial inflammatory reaction by ACE inhibitors. Topical application of a selected COX-2 inhibitor has been found to reduce wound inflammation, and at the same time, improve scarring. If an ACE inhibitor could be used to inhibit fibrogenesis and a selective COX-2 inhibitor to inhibit the initial inflammation, their combined use may be expected to more significantly prevent hypertrophic scarring. In addition, the combined use of these topical agents to reduce scarring and other adverse events is well worth studying as a cost-effective option for scar prevention. In this study, the authors compared the efficacy of celecoxib (a COX-2 inhibitor) or captopril (an ACE inhibitor) alone, and in combination, to reduce scarring.

**Keypoints**
- Each year, more than 100 million people worldwide develop scars. More than $7 billion is spent annually in the United States alone for their treatment.
- Many treatments and prophylactic measures to prevent scars involve inhibition of initial inflammation, thus preventing the over activity of fibroblasts and controlling the degradation and reorientation of tissue.
- In this study, the authors compared the efficacy of celecoxib (a COX-2 inhibitor) or captopril (an ACE inhibitor) alone, and in combination, to reduce scarring.

**Material and Methods**

Preparation of reagents. To allow topical application, the authors used polyethylene glycol (PEG) 400 (Sigma-Aldrich, St. Louis, MO), which has been certified as safe for skin application and for use as a solvent for injection by the United States Food and Drug Administration. The vehicle solution was prepared by mixing 30 ml of 100% ethanol (Merck Co, Whitehouse State, NJ) and 70 ml of PEG 400 (by shaking) to produce a 70% PEG 400 solution.

A Celebrex capsule (273 mg, consisting of 200 mg celecoxib, and 73 mg of excipients, consisting of lactose and a small amount of sodium lauryl sulfate, povidone iodine, and magnesium stearate [Pfizer, Collegeville, PA]) was added to 30 ml of the 70% PEG 400 solution and the mixture was shaken for 20 minutes. The volume was adjusted to 40 ml with 70% PEG 400 solution and shaken for another 10 minutes to yield a topical application that contained 1 mg of celecoxib per 200 μl. Captopril was prepared in a similar way, by the addition of 2 g to 30 ml 70% PEG 400 solution, vortexing, adjustment of the volume to 40 ml, and further vortexing for 10 minutes to yield a topical agent.

Experimental Animals and Methods. A total of 18 female New Zealand White Rabbits, each weighing about 3 kg, were used. The animal experiment was performed according to institutional guidelines after obtaining approval from the Ethics Committee on Animal Experiments of Kosin University (Busan, South Korea). Rabbits were randomly assigned to 3 groups, with 6 rabbits in each group. A COX-2 inhibitor was used in Group 1, an ACE inhibitor in Group 2, and both agents in Group 3. Anesthesia was administered through intramuscular injection of ketamine (50 mg/kg) and xylazine (7 mg/kg) into the femoral muscle. The ventral sides of both ears were then lit up with a pen light, after which the vertical thickest central vessel was located. Eight wounds were created using a 5 mm biopsy punch on the left and right points, both of which were 1 cm away from the thickest vessel. The wounds were made as deep as the cartilage. The full-thickness dermal tissue, including the perichondrium, was excised. After hemostasis, rabbits were randomized into the groups to receive topical application of the relevant test agent on one ear and control agent (vehicle only) on the other ear. All wounds after application of experimental agents or control agent were dressed using gauze and Tegaderm® (3M, St. Paul, MN). The topical application and dressing were performed daily for 2 weeks by the same investigators, who were blinded to the identity of the topical agents. Three rabbits from each group...
were sacrificed at 2 weeks and 4 weeks. The full-thickness of the skin, including the normal tissue around the scar, was collected.

**Histological Staining.** The collected tissue was fixed in a 10% formalin solution for 24 hours. The tissue was then embedded in paraffin, according to routine procedure, and cut into 4-μm-thick slices from the most protruded part of the scar (usually from the center of the scar). The slices were stained using hematoxylin and eosin (HE) and Masson’s trichrome.

**Scar elevation index.** The distance between the cartilage surface and epidermis of the normal tissue and the distance between the cartilage surface and the epidermis of the scar tissue was measured by Photoshop 8.0 (Adobe Systems, Inc. San Jose, CA) on digital photos taken under 40x magnified field microscope. After which the ratio of the former to the latter was calculated for use as the scar elevation index (SEI). An SEI of 1 indicated the wound was flat and as high as the surrounding normal tissues, while an index of 2 indicated the wound was twice as high as the surrounding normal tissues.

**Extent of Inflammatory Cell Infiltration.** Five random points on the HE-stained slide were observed at 400x magnification to investigate the level of infiltration of inflammatory cells, such as neutrophils and macrophages. The level of infiltration of inflammatory cells was graded as follows: < 10%, 1 point; 10% - 30%, 2 points; 30% - 70%, 3 points; and ≥ 70%, 4 points.

**Deposition and organization of collagen.** Collagens were stained blue by Masson’s trichrome and were observed at 40x and 400x magnification to examine the level of deposition and organization of collagen. A connective tissue assessment scale was formulated according to a modified version of the histological scar assessment scale 16 (Table 1). The orientation (wave, parallel, or non-orientation); density (gap between the collagen fibers); maturity of collagen (thickness of the collagen fibers); and the density of fibroblasts (fibroblasts counted per 400x field) were graded on a scale of 1 to 3 (close to normal, 1 point; significantly deviating from the normal, 3 points), and scores from each category were added together.

### Table 1. Histological connective tissue (collagen deposition and organization) assessment scale.

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<th>1</th>
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<tr>
<td>Collagen fiber bundle orientation</td>
<td>Normal basket-weave</td>
<td>Somewhat parallel</td>
<td>No orientation</td>
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<tr>
<td>Collagen fiber bundle density</td>
<td>Normal</td>
<td>Slightly abnormal</td>
<td>Very abnormal</td>
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<tr>
<td>Collagen fiber bundle maturity</td>
<td>Normal</td>
<td>Slightly abnormal</td>
<td>Very abnormal</td>
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<tr>
<td>Fibroblast density</td>
<td>Normal</td>
<td>Slightly more/less</td>
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**Statistical Analysis**

SPSS (version 12.0) for Windows was used to perform a Mann Whitney U test for the SEI, the level of infiltration of inflammatory cells, and the level of deposition and organization of collagen. The statistical significance level was set at \( P \leq 0.05 \).

**Results**

**Scar elevation index.** Two weeks after the creation of the wound in Group 1, the SEI did not differ significantly between the experimental ear (2.69 ± 0.41, representing a decrease to 88.2% compared with the control ear) where only celecoxib (COX-2 inhibitor: C2I) was applied, and the control ear (3.05 ± 0.37), \( P = 0.095 \). In Group 2, where captopril (ACE inhibitor: AI) was tested, the SEI was significantly different between the experimental ear (2.36 ± 0.12, representing a decrease to 71.5% compared with the control ears) and the control ear (3.30 ± 0.33), \( P = 0.008 \). In Group 3, where both agents (C2I + AI) were tested, the SEI was also significantly different between the experimental ear (1.90 ± 0.16, representing a decrease to 62.9%) and the control ear (3.02 ± 0.23), \( P = 0.008 \).

At 4 weeks in Group 1 (C2I), the SEI was still not significantly different between the experimental ear (2.45 ± 0.21) and control ears (2.54 ± 0.24); whereas in Group 2 (AI) a significant difference between the experimental ears (2.11 ± 0.15, decrease to 91.7% compared with the control ears) and control ears (2.30 ± 0.13), \( P = 0.008 \) was observed. In Group 3 (C2I + AI), the SEI was also significantly different between the experimental ears
(1.65 ± 0.31, a remarkable decrease to 73.3% compared with the control) and control ears (2.25 ± 0.13), \((P = 0.016)\) (Figures 1 and 2).

**Level of inflammation.** Two weeks after the creation of the wound, scar tissues that were distinct from the surrounding dermal tissues were observed at 40x magnification on slides stained with HE, and typical inflammatory reactions that involved the cartilage surface of the epidermis were observed in the wound. Closer to 4 weeks, the inflammatory cell counts remarkably decreased and became indistinct from those of the surrounding tissues.

In Group 1 (C2I), the level of infiltration of the inflammatory cells observed at 400x magnification after 2 weeks differed significantly between the experimental ears (2.2 ± 0.63) and control ears (3.00 ± 0.67), \((P = 0.029)\). In Group 2 (AI), the level of infiltration of the inflammatory cells did not significantly differ between the experimental and control ears (2.9 ± 0.76 and 3.10 ± 0.74, respectively). In Group 3 (C2I + AI), the level of infiltration of the inflammatory cells significantly differed between the experimental and control ears (2.1 ± 0.57 and 2.9 ± 0.74, respectively), \((P = 0.029)\). At 4 weeks, the level of inflammatory cell infiltration did not significantly differ between the experimental and control ears in Group 1 (1.8 ± 0.63 and 2.3 ± 0.67, respectively), Group 2 (2.1 ± 0.74 and 2.4 ± 0.67, respectively), or Group 3 (1.8 ± 0.42 and 2.3 ± 0.67, respectively) (Figures 3 and 4).

**Level of deposition and organization of collagen.** Two weeks after wound creation, the collagen was found to be thin and arranged irregularly in all the groups. In Group 1 (C2I), the level of deposition and organization of collagen did not differ sig-

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**Figure 1.** Gross findings 4 weeks after wound creation. Group 1 (topical application of celecoxib); Group 2 (topical application of captopril); Group 3 (topical application of celecoxib + captopril). In Groups 2 and 3, where captopril was topically applied, the scar elevation significantly decreased.

**Figure 2.** Quantitative analysis of scar elevation. In Groups 2 and 3, where captopril was topically applied, scar elevation significantly decreased at 2 and 4 weeks. In Group 3, where the combination of captopril and celecoxib was used, the decrease in the SEI was most remarkable: 62.9% and 73.3%, after 2 and 4 weeks, respectively, compared with the control ears. (E = experimental ear; C = control ear.) *Statistically significant
significantly between the experimental ears (10.1 ± 1.45) and control ears (11.1 ± 1.10), although the level of organization in the experimental ear was slightly closer to normal. In Group 2 (AI), the level of deposition and organization of collagen also did not differ significantly between the 2 ears (9.8 ± 1.56 and 10.7 ± 0.95, respectively), although it improved more significantly in the experimental ear. However, in Group 3 (C2I+AI) the level of deposition and organization of collagen was significantly lower in the experimental ears (9.5 ± 1.27) than in the control ears (10.7±1.06) (P = 0.035). At 4 weeks, the level of organization was closer to normal in the experimental ear than in the control ear in all groups (Group 1, 7.3 ± 1.16, and 8.5 ± 1.08, respectively; Group 2, 7.1 ± 0.88, and 8.4 ± 1.075, respectively; and Group 3, 6.9 ± 0.88, and 8.5 ± 0.97, respectively). This difference was highest in Group 3, and significant in all groups (Figure 5 and 6).

Discussion
Fibroblasts are activated by cytokines pro-
duced from various inflammatory cells or platelets, and form a scaffold called the extracellular matrix (ECM). Procollagen, elastin, proteoglycan, and hyaluronic acid are then deposited on the scaffold, promoting vascular ingrowth. When defective sites are filled with appropriate tissues and myofibroblasts promote contracture of the surrounding tissues, the wound becomes mature through appropriate rearrangement of collagen and degradation of excessive ECM.3

Histological overabundance of dermal collagen is observed in hypertrophic scars and keloids, and is associated with fibrogenesis, due to the excess of various proteins produced by fibroblasts and failure of the wound healing cells to appropriately down-regulate fibrogenesis.17 Many studies have shown that angiotensin-II is associated with fibrous disorder, and in particular with post-myocardial infarction and myocardial fibrosis. In addition, ACE inhibitors can reduce heart failure by inhibiting fibrogenesis during cardiac muscle remodeling after myocardial infarction.18,19 Experiments have clearly demonstrated that renal fibrosis is closely relat-
ed to angiotensin-II. In addition, the inhibition of ACE, which leads to the production of angiotensin-II, is helpful for the prevention of postoperative adhesion that occurs after intraperitoneal surgery. This fibrosis-preventing effect is also helpful for preventing encapsulating peritoneal sclerosis in patients undergoing peritoneal dialysis. Additional studies on ways to prevent fibrogenetic disorder by inhibiting angiotensin-converting enzyme are under way.

There have been numerous studies on angiotensin-II in relation to skin wounds. The rennin-angiotensin system (RAS) is known to play a local role in human skin wounds. In rats, the angiotensin-II level is high in wounded skin, and angiotensin-II was found to promote wound healing. In addition, cutaneous tissue ACE levels are increased in pathologic scars and in the cardiovascular system. Based on the idea that inhibition of angiotensin may reduce excessive skin scarring, a low-dose ACE inhibitor was orally administered to 2 patients with keloids, and their abnormal scars were cured. In addition, ACE inhibitors have been used as a topical agent in animal studies to reduce other side effects, and subsequently, also topically applied in humans. Theoretically, the inhibition of ACE leads to the inhibition of angiotensin-II production, which reduces the production of TGF-ß and interleukin 6, thereby decreasing fibroblast proliferation as well as the production of collagen and matrix metalloproteinase. However, the reduction of the initial inflammatory reaction by topical ACE inhibitors has not been shown to significantly influence the formation of scars in a skin wound until now. In this study the authors also showed that topical application of an ACE inhibitor (Groups 2 and 3) resulted in a more significant decrease in the scar elevation index, which represents the level of hypertrophy, in the experimental ears than in the control ears. In Group 2, where only the ACE inhibitor was used, the level of inflammatory cell infiltration did not significantly decrease.

In early wound healing, the activity of neutrophils, macrophages, and mast cells increases during the inflammatory reaction, which promotes the healing process. Neutrophils produce active oxygen, and synthesize and secrete various mediators that damage the surrounding tissues. Moreover, inflammatory mediators, active oxygen, and neutrophils are important sources of matrix-degrading enzymes such as matrix metalloproteinases. An excess of these inflammatory cells in tissue causes excessive tissue damage during inflammatory reactions, which results in more scars since a wider tissue area is replaced by scars during the healing process. In the past, the inflammatory reaction was considered a necessary process in wound healing; however, since wounds in rats that lacked neutrophils and macrophages healed faster, and the inhibition of inflammatory cells without the risk of infection was more effective for wound healing, some aspects of inflammation are considered unnecessary in the healing process. In addition, in a fetus, scarless wound healing occurs while inflammation and TGF-ß are suppressed. Based on such evidence, it can be expected that the inhibition of inflammation may reduce the formation of hypertrophic scars.

Selective COX-2 inhibitors prevent the deposition and function of inflammatory cells by inhibiting the expression of COX-2. COX-2 normally produces PEG2, which plays an important role in the initiation of inflammatory reactions in the early phase of a wound. Selective COX-2 inhibitors can thus reduce scarring by inhibiting the tissue damage that can occur as a result of excessive inflammation in the early phase of a wound. Polyethylene glycol 2 also increases fibroblast proliferation in the proliferation phase through a series of processes. Since angiotensin-II is also involved in the activity of inflammatory mediators such as prostaglandins, inhibition of both COX-2 and angiotensin-II is expected to result in a more effective reduction in scar formation. In Groups 1 and 3 of this study, where an inflammation-reaction-suppressing COX-2 inhibitor was topically applied to the wounds on rabbit ears, the inflammation reaction was inhibited in the experimental ears compared to the control ears after 2 weeks. The difference was particularly pronounced in Group 3, where both agents were
used. Inhibition of both the inflammatory reactions and fibrogenesis in Group 3 resulted in the most significant decrease in the SEI (to 73.3% of the control ear) in the experimental ears at 4 weeks. These data suggest that celecoxib inhibited early inflammation while captopril inhibited scar elevation in rabbit ear wounds.

Although the level of arrangement and maturity of the collagen fibers became closer to normal more quickly in the experimental ears compared to the control ears in all 3 groups, the effect was most pronounced in Group 3 where both agents were used. This finding indicates the experimental agents promoted maturation of the collagen fibers by reducing inflammatory reactions and promoting the healing process.

The results of this study show topical application of celecoxib and captopril to wounds in rabbit ears inhibited scar formation, and their combined application reduced scar formation more effectively than the single use of either agent. The authors therefore expect that topical application of celecoxib and captopril could be a cost-effective treatment to prevent scars. The clinical application of these findings would require further study on effects other than scar prevention, as well as the determination of optimum concentrations of the agents and absorptivity in the local tissue after topical application.

Conclusion

Various treatment methods have been investigated to reduce scars. This study reports that topical application of captopril reduced the level of inflammation, whereas celecoxib influenced the deposition and organization of collagen, and both agents reduced the level of scar elevation. In addition, the combined application of these 2 agents reduced the infiltration of inflammatory cells, promoted the deposition and organization of collagen, and reduced the level of scar elevation more effectively than either agent alone. Clinical application of these findings will require more studies on the mechanism of action of these agents, but these results clearly indicated that the topical application of a combination of a COX-2 inhibitor (celecoxib) and an ACE inhibitor (captopril) prevented excessive scarring in a wound scar model on rabbit ears, and that the effect of the 2 drugs were complementary.

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