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선택적 Cyclooxygenase-2 저해제 국소 도포가 토끼 귀의 창상반흔에 미치는 영향

김도엽[1] · 박진형[1] · 천봉권[2] · 한예식[1]

고신대학교 의과대학 성형외과학교실[1], 병리학교실[2]

The Effect of a Topical Selective Cyclooxygenase-2 Inhibitor on Skin-Wound Scarring of the Rabbit Ear

Do Yup Kim, M.D.[1], Jin Hyung Park, M.D.[1], Bong Kwon Chun, M.D.[2], Yea Sik Han, M.D., Ph.D.[1]

Departments of [1]Plastic and Reconstructive Surgery, [2]Pathology, Kosin University College of Medicine, Busan, Korea

Purpose: The inflammatory phase is considered an integral part of adult wound healing, but fetal wound healing studies have shown scarless healing results in the absence of the inflammation process. The COX-2 pathway is an essential component of inflammation. The purpose of this study is to identify the effect of a topical selective COX-2 inhibitor on inflammation in rabbit skin wound healing and scarring.

Methods: Full-thickness wounds were made on 6 New Zealand rabbits' ears. Topical 5% celecoxib + vehicle (experimental tissue) and vehicle only (controlled tissue) were applied daily for 14d on each side of the ears. Scar samples were harvested at 2 wks, 4 wks, and 8 wks after the wounding. Each sample was stained with hematoxylin and eosin and the Masson's trichrome stain to evaluate inflammation and scar formation.

Results: Histological analysis demonstrated a significant reduction of inflammation, neovascularization, and scar elevation in the experimental tissue as compared to the control. Additionally, experimental tissue exhibited faster improvement of collagen organization similar to that of normal tissue.

Conclusion: This study suggests that the topical application of a selective COX-2 inhibitor on a rabbit ear wound resulted in decreased inflammation and had a positive effect on the reduction of scar formation.

Key Words: Cyclooxygenase inhibitors, Scar

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Address Correspondence : Yea Sik Han, M.D., Ph.D., Department of Plastic Surgery, College of Medicine, Kosin University, 34 Amnam-dong, Seo-gu, 602-702 Busan, Korea. Tel: 051) 990-6131/Fax: 051) 990-3005/E-mail: hanplastic1@naver.com

I. INTRODUCTION

In the area of plastic surgery, skin scarring that occurs after wound healing is a very important issue. Various efforts are in progress to reduce scarring but any effective way to suppress scar formation does not yet exist. For this reason, the fetal wound healing process is drawing attention. Skin wounds that occur on the early fetus are regenerated to normal skin without scar formation as the healing process occurs without the deposition of excessive collagen in an environment where transforming growth factor (TGF-β) activity and inflammatory response are lowered.[1,2] The exact mechanism by which the inflammatory response promotes scarring is still unknown but there is no doubt that the inflammatory response makes some contribution to scar formation. While the inflammatory response is traditionally known to play an important function in the skin wound healing of adults, the fetal wound healing process suggests that excessive inflammation is involved in promoting scar formation.[3-6] Therefore, it is possible that controlling the inflammatory response phase may have a major impact on the formation of scars. Selective Cyclooxygenase-2 (COX-2) inhibitors can play an important role in controlling inflammation at the start of the inflammatory response because the COX-2 enzyme is activated by external inflammatory stimulation and leads to the production of inflammatory mediators such as prostaglandin E.

Thus, the purpose of this study is to investigate the effects on inflammation and scar formation by applying a selective COX-2 inhibitor (celecoxib) topically to the skin wounds of rabbits.

II. MATERIALS AND METHODS

A. Reagent production

To facilitate topical application of the medicine, a vehicle of polyethylene glycol (PEG) 400 (Sigma-Aldrich, St. Louis, U.S.A.) was prepared. The vehicle was a 70% PEG 400 solution consisting of 30 mL of 100% ethanol

(Hunterdon, Merck & Co., Inc., U.S.A.) and 70 mL of PEG 400 vortexed for 30 minutes. The drug was prepared by vortexing a capsule of celebrex® (Pfizer, Seoul, Republic of Korea) consisted with 200 mg of celecoxib and 73 mg of other additives in 30 mL of the 70% PEG 400 solution for 20 minutes. A total volume of 40 mL was obtained by adding 70% PEG 400 solution. The final concentration of Celebrex was 1 mg/200 μL of solution and was obtained after vortexing for 10 minutes more.

B. Experimental animals and drug testing method

Six New Zealand white female rabbits, each weighing about 3 kg, were anesthetized by injecting Ketamine (60 mg/kg) and Xylazine (5 mg/kg) into their gluteal muscles. Wounds were made on the ventral skin side 1 cm to the left or right of the thickest central blood vessel running vertically. The blood vessel was visualized using a penlight shone on the ventral surface of the rabbits' ears. Eight wounds were inflicted using a 5 mm skin biopsy punch, removing the full thickness of the skin, including the perichondrium. Bleeding was stopped by compression. The left or right ear was randomly assigned to the experimental group and the other ear received the control treatment. Those wounds receiving experimental treatment had 1 mg/200 μL of Celebrex applied topically. Control wounds received only the vehicle (70% PEG 400 solution). Both treatment and control wounds were dressed with gauze and TEGADERM®. The same person carried out medicinal application and wound dressing every day, and did not know what medicine was treatment or control. The rabbits were treated daily for up to 14 days with vehicle or drug. Two animals were sacrificed at each time-point 2 weeks, 4 weeks, and 8 weeks post-wounding and the full thickness of tissue, including the normal tissue surrounding the scar, was harvested.

C. Gross finding

The wounds were observed by the naked eye for redness and scar elevation at weeks 2, 4 and 8. The number of days until epithelialization fully proceeded was also recorded.

D. Tissue staining

The harvested tissues were fixed in 10% formalin solution for 24 hours. As per standard protocol, the fixed tissues were embedded in paraffin and cut to 4 μm thick. The sections were processed and subjected to hematoxylin-eosin staining and Masson's trichrome staining.

1) The degree of inflammation and number of vessels

Five random locations on each of the hematoxylin-eosin stained slides were observed at high magnification (400 ×) and the degree of inflammatory cell infiltration and the number of blood vessels was assessed. The degree of infiltration of inflammatory cells was given a score of 1~4 as follows: < 10%, 10~30%, 30~70%, or more than 70% of all cells observed were inflammatory cells. The number of vessels was compared between the experimental group and the control group by averaging the number of vessels observed in 5 sights.

2) Deposition and organization of collagen fiber

To identify the degree of organization and deposition of collagen, Masson's trichrome stained slides were observed at low (40 ×) and high (400 ×) magnifications. A connective tissue assessment scale was made by referring to "A modified version of the histological scar assessment scale"[7] for evaluation (Table I). The scale was divided into 3 scores by assessing the direction of the collagen fibers (evaluating the arrangement of non-orientation or parallel from waves), the density of collagen fibers (evaluating the spacing between collagen fibers), the fiber maturity (evaluating the thickness of collagen fibers), and the fibroblast density (evaluating the number of fibroblasts in a single field at 400 × magnification). A score of 1 was given if the parameters observed were closer to normal and a score of 3 was given if a big difference from normal was observed.

Table I. Hitstological Connective Tissue (Collagen deposition and organization) Assessment Scale by Scoring for Observed Items

	1	2	3
Collagen fiber bundle orientation	Normal basket-weave	Some parallel	No orientation
Collagen fiber bundle density	Normal	Slightly abnormal*	A lot abnormal†
Collagen fiber bundle maturity	Normal	Slightly abnormal*	A lot abnormal†
Fibroblast density	Normal	Slightly more/less*	A lot more/less†

*: < 50% abnormal finding, †: > 50% abnormal finding.

3) Scar elevation index

For comparing tissue elevation, the distance from the cartilage side to the epithelium of the normal tissue and the distance from the cartilage side to the epithelium of the scar tissue was measured. The ratio of these distances was the index. For example, if the scar elevation index is 1, a wound is flat and there is no difference between surrounding normal tissue and the degree of protrusion, while an index of 2 describes a protrusion 2 times the degree compared to normal tissue.

E. Statistical analysis

The Mann-Whitney U test was used for analysis of possible differences between the experimental group and control group in the degree of inflammation, the number of new vessels, the degree of deposition and organization of collagen, and the scar elevation index according to each period by using SPSS (ver.12.0) for Windows. A p value < 0.05 was considered statistically significant.

III. RESULTS

A. Gross finding

For the epithelialization of wounds to complete, it took on average 12.5 ± 0.98 days for the experimental group and 12.13 ± 1.36 days for control group ($p = 0.238$). Initially, the wounds of both groups exhibited redness that was distinct from the surrounding normal tissue and elevated scar tissue until the scar matured. At the second week, elevated scar tissue was easily observed in both the control and experimental groups. The degree of elevation was slightly more pronounced in the control group. At the fourth week, scar tissue was easily observed in both groups, but elevation and redness were reduced compared to findings from the second week. Additionally, the redness of the wounds in the experimental group was decreased as compared to the control group. At the eighth week, elevated scars were greatly decreased in both groups, redness was gone, but white, fibrous and

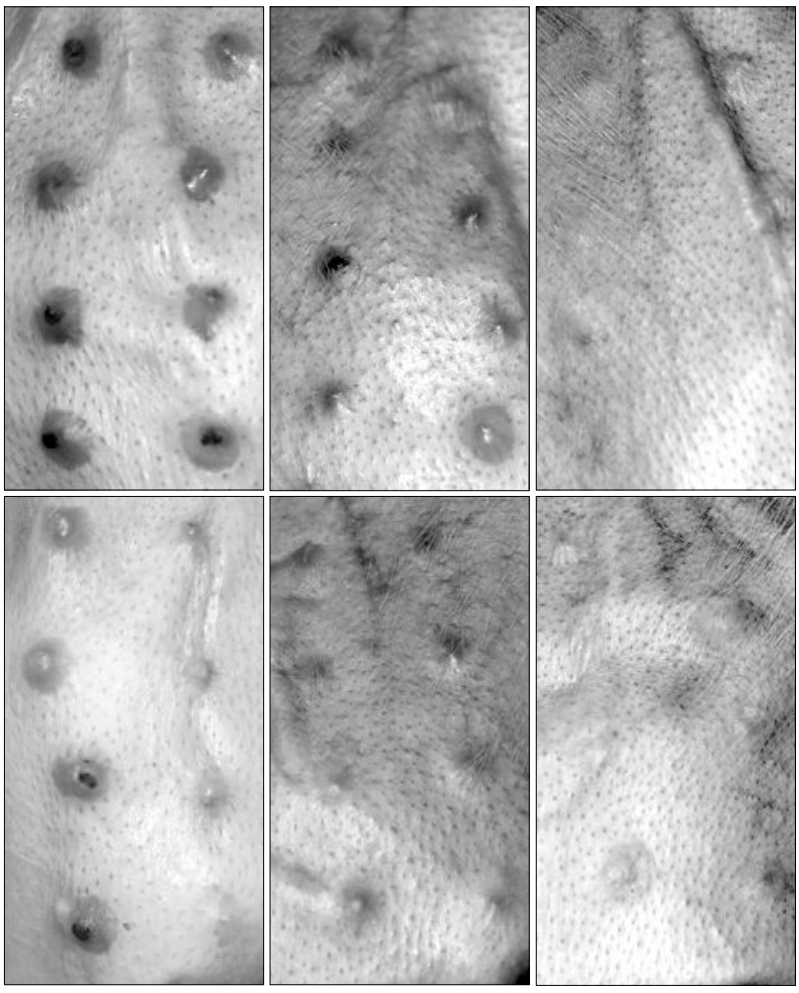

Fig. 1. Gross findings of the control group (Above, left) and experimental group (Below, left) in 2 weeks; control group (Above, center) and experimental group (Below, center) in 4 weeks; control group (Above, right) and experimental group (Below, right) in 8 weeks after wounding. Gross findings of experimental groups show significantly reduced redness and elevation as compared to control groups.

relatively stiff tissue was observed. There was no distinct difference between the groups as observed by the naked eye (Fig. 1).

B. Histological findings of scar tissue

1) Infiltration of Inflammatory cells

At two weeks, scar tissue that was clearly distinct from the surrounding tissue was observed at low magnification (40 ×) of hematoxylin-eosin stained slides in both the experimental and control groups (Fig. 2). A typical inflammatory response was observed extending from the cartilage side to the epidermal layer in both groups. At the fourth and eighth weeks, inflammatory cells decreased and it was hard to distinguish an inflammatory response from the surrounding tissue.

In the experimental group, the degree of deposition of inflammatory cells observed scored 2.2 ± 0.63 in the second week, while the control group exhibited a score of 3 ± 0.67, a difference that was statistically significant (p= 0.029). At 4 weeks, the degree of deposition of inflammatory cells observed in the experimental group was 1.8 ± 0.63 and 2.3 ± 0.67 in the control group. At 8 weeks, the experimental group scored 1.1 ± 0.32 and the control group scored 1.5 ± 0.53. While the degree of

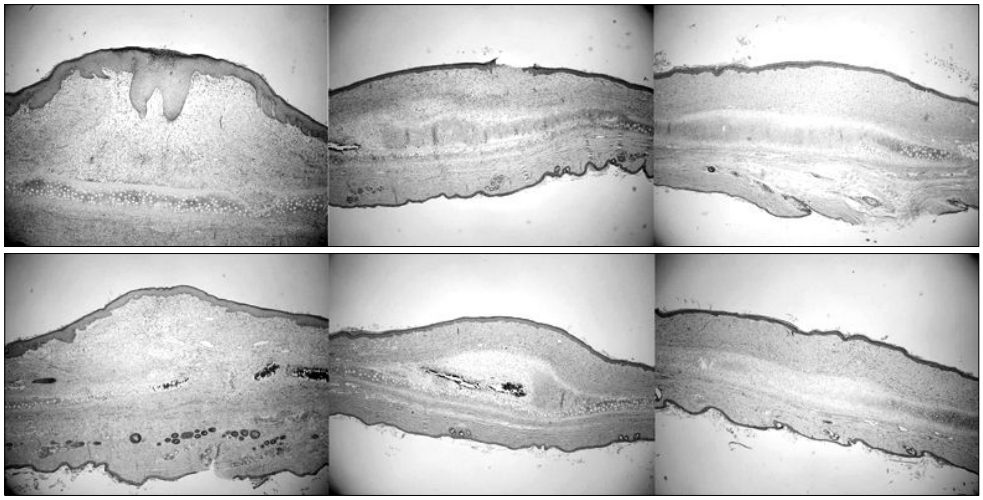

Fig. 2. Histological findings (Hematoxylin and eosin stain, × 40) of the control group (Above, left) and experimental group (Below, left) in 2 weeks; control group (Above, center) and experimental group (Below, center) in 4 weeks; control group (Above, right) and experimental group (Below, right) in 8 weeks after wounding. Experimental groups show significantly reduced scar elevation as compared to control groups.

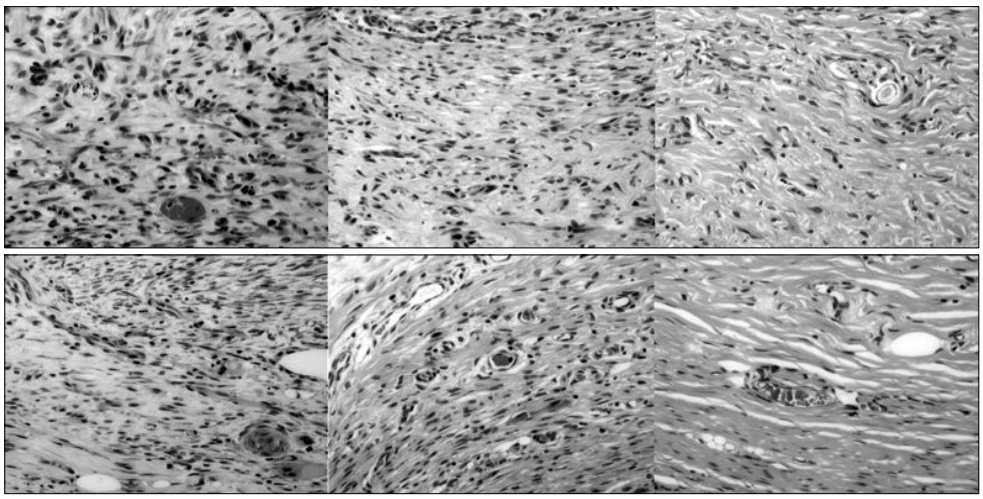

Fig. 3. Histological findings (Hematoxylin and eosin stain, × 400) of the control group (Above, left) and experimental group (Below, left) in 2 weeks; control group (Above, center) and experimental group (Below, center) in 4 weeks; control group (Above, right) and experimental group (Below, right) in 8 weeks after wounding. Experimental groups show significantly reduced inflammatory cell infiltration as compared to control groups.

Table II. Semi-quantitative Analysis of Inflammation*

	Control	Experimental	*p* value
2 weeks	3.0 ± 0.67	2.2 ± 0.63†	0.029
4 weeks	2.3 ± 0.67	1.8 ± 0.63	0.143
8 weeks	1.5 ± 0.53	1.1 ± 0.32	0.143

*: The degree of infiltration of inflammatory cells was given a score of 1~4 as follows: <10%, 10~30%, 30~70%, or more than 70% of all cells observed were inflammatory cells.
†: significant difference ($p < 0.05$).

Table III. Quantitative Analysis of Vascularity

	Control	Experimental	*p* value
2 weeks	18.5 ± 4.35	14.7 ± 3.86*	0.035
4 weeks	13.7 ± 3.27	11.6 ± 3.78	0.280
8 weeks	9.9 ± 3.96	6.0 ± 2.45*	0.015

*significant difference ($p<0.05$)

deposition of inflammatory cells in the experimental group was lower than the control at later time-points, there was no statistical significance (Fig. 3, Table II).

2) The number of vessels

At 2 weeks, the average number of blood vessels observed in the experimental group was 14.7 ± 3.86, while the control group had an average of 18.5 ± 4.35 vessels. The number of newly generated vessels in the experimental group was fewer and it was statistically different from the control group (p= 0.035). At 4 weeks, the experimental group had an average of 11.6 ± 3.78 vessels observed while the control group had 13.7 ± 3.27 vessels (there was no significant difference). The number of vessels was greatly reduced at the eighth week, with the experimental group showing an average of 6 ± 2.45 vessels and the control group showing 9.9 ± 3.96 vessels. By the eighth week, the number of vessels in the experimental group was fewer than the control and the difference between the groups was statistically significant (p= 0.015) (Table III).

3) The degree of deposition and organization of collagen

At 2 weeks, deposited collagen was thinly and irregularly arranged in both the experimental and control groups. The scores for the degree of deposition and organization of collagen were 11.1 ± 1.10 in the experimental group and 10.1 ± 1.45 in the control group (no significant difference). At 4 weeks, the amount of collagen stained blue clearly increased compared to the tissue observed at the second week. The pattern showed that compared to the control group, the collagen of the experimental group exhibited a partially constant direction and greater fiber thickness. The scores for the experimental and control groups were statistically different at 8.5 ± 1.08 and 7.3 ± 1.16, respectively (p= 0.043). At 8 weeks, the amount of collagen and its thickness increased in both groups. Additionally, the arrangement of fibers in a constant direction or in a pattern similar to the normal wave pattern was frequently observed in both groups. However, the normal wavy patterns were more definite in the experimental group: the score for that group was 3.2

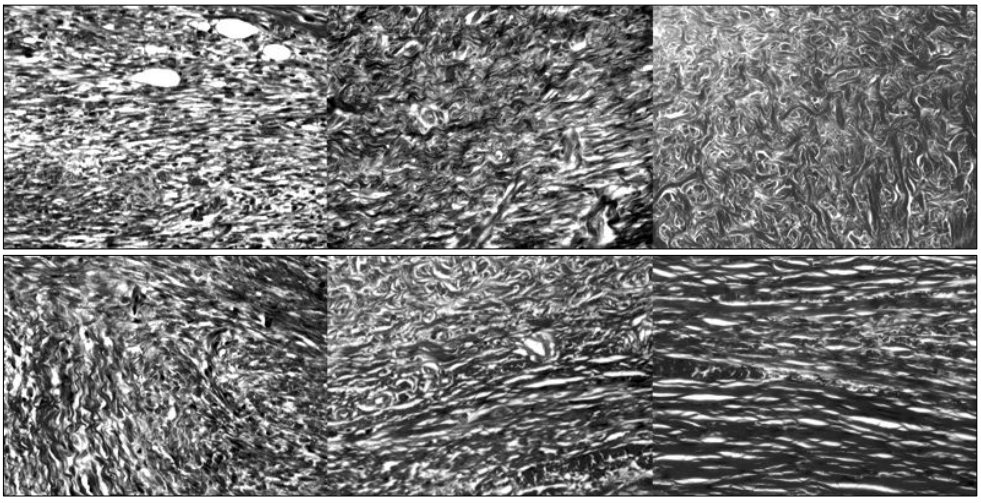

Fig. 4. Histological findings (Masson's trichrome stain, × 400) of the control group (Above, left) and experimental group (Below, left) in 2 weeks; control group (Above, center) and experimental group (Below, center) in 4 weeks; control group (Above, right) and experimental group (Below, right) in 8 weeks after wounding. Histological analysis demonstrated greater improvement of dermal collagen organization in experimental groups than in control groups.

± 1.14 while the control group scored 5.3 ± 0.82 showing a significant difference (p= 0.000) (Fig. 4, Table IV).

Table IV. Semi-quantitative Analysis of Collagen Deposition and Organization

	Control	Experimental	p value
2 weeks	11.1 ± 1.10	10.1 ± 1.45	0.123
4 weeks	8.5 ± 1.08	7.3 ± 1.16*	0.043
8 weeks	5.3 ± 0.82	3.2 ± 1.14*	0.000

*: significant difference (p < 0.05)

4) Scar elevation index

At 2 weeks, the scar elevation index for the experimental group was 2.69 ± 0.41, while the index for the control group was 3.05 ± 0.37. The degree of elevation of the experimental group was not sufficient but there was no statistical significance between the two groups (p= 0.095). At 4 weeks, the experimental group's index was 2.45 ± 0.21 and the control group's index was 2.55 ± 0.24. Again, the degree of scar elevation of the experimental group was not sufficient but the difference was not significant. At 8 weeks, the scar elevation index for the experimental group was 1.86 ± 0.16 and 2.19 ± 0.18 in the control group. As before, we could see that the degree of scar elevation in the experimental group was decreased compared to the control group and the difference was statistically significant (p= 0.032) (Table V).

Table V. Quantitative Analysis of Scar Elevation

	Control	Experimental	p value
2 weeks	3.05 ± 0.37	2.69 ± 0.41	0.095
4 weeks	2.55 ± 0.24	2.45 ± 0.21	0.310
8 weeks	2.19 ± 0.18	1.86 ± 0.16*	0.032

*: significant difference (p < 0.05)

IV. DISCUSSION

The healing process of adult wounds includes the process of scar formation that occurs through a complicated series of steps involving various mediators and cells. It goes through the inflammatory phase, proliferative phase, and remodeling phase. Cyclooxygenase-2 is produced in response to external inflammatory stimulation, such as occurs after wounding, and it becomes involved in the beginning phase of the inflammatory response by promoting generation of prostaglandin. Prostaglandin, in turn, controls many inflammatory cells, in addition to vascular permeability, especially promoting immediate generation of prostaglandin E. After this, the immunological defense system is engaged as blood vessels are expanded by the action of growth factors and various cytokines, the synthesis and secretion of which are promoted by prostaglandin E. These cytokines, along with plasma and white blood cells, gather in wound tissue due to the increased blood vessel permeability, and function to remove damaged or contaminated tissue. Like prostaglandin, transforming growth factor (TGF)-β, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), among others, are secreted by mediators and many inflammatory cells, including fibroblasts and epidermal cells. During the proliferative phase, re-epithelialization, matrix cell proliferation, extracellular matrix synthesis of collagen, wound contraction and new vascularization occur. The deposition of irregular and excessive collagen that occurs at this time is the main cause of scarring. During several months after this, synthesis and degradation of synthesized collagen becomes balanced and the scar enters a remodeling phase resulting in a matured scar. Unlike in adults, skin wounds that occur in early embryos are almost completely healed without forming a scar. In the fetal scar tissue restoration process, the inflammatory response is hardly noticeable and the activity of TGF-β is reduced.[1,2]

The early inflammatory response in the wounds of adults and the subsequent healing process is facilitated by inflammatory cells such as mast cells, macrophages, and neutrophils which play an important role in the inflammatory response.[3] Neutrophils make oxygen-derived free radicals and synthesize and secrete many other mediators which play a role in damaging surrounding tissue.[4] In addition to producing inflammatory mediators and oxygen-derived free radicals, neutrophils are an important source of matrix degrading enzymes such as matrix metalloproteinase-8. If these inflammatory cells exist excessively in tissue, disproportionate tissue loss during the inflammatory response phase results, leading to a larger portion of the tissue being replaced with scar, and thus, a larger scar. The inflammatory response has been thought necessary in wound healing, but results of skin wound healing in mice whose neutrophil and macrophages are insufficient show that they healed more quickly than did normal mice.[5] This shows that the suppression of inflammatory cells in a state in which there is no danger of infection was more effective for wound healing and that all parts of the inflammatory response are not necessary for the healing process.[6]

Keloid tissues in human represent a remarkable overexpression of COX-2 in a dermis scar lesion and in the overlying epidermis.[14] It suggests that COX-2 inhibition may affect to abnormal wound healing. Cyclooxygenase (COX)-2 is the first enzyme in a cascade of inflammation which are involved in inflammation, cancer, pro-angiogenic property and embryonic development. COX-2 is associated with sustained inflammatory condition unlike COX-1 plays many physiological roles, such as maintaining the integrity of the stomach lining as well as regulating renal function and platelet aggregation. The non-selective non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen do not inhibit only the inflammatory COX-2 but also the homeostatic COX-1. Because of the side effects associated with the nonselective NSAIDs like gastric ulcer and inhibition of platelet aggregation, specific COX-2 inhibitors were developed. Previous work conducted by Traci A. Wilgus et al. has shown that topical treatment with celecoxib following UVB irradiation which is a kind of inflammatory stimuli inhibits several parameters of acute inflammation including vascular permeability, the infiltration and activation of inflammatory cells, and the production of prostaglandin E2. And long term studies illustrated the effectiveness of topical COX-2 inhibitor in reducing chronic inflammation.[15]

Selective COX-2 inhibitors suppress the deposition and function of inflammatory cells by inhibiting the COX-2 mediated PGE2 cascade that plays an important role in the beginning of the inflammatory response in early wound healing. Since a selective COX-2 inhibitor can suppress tissue damage caused by excessive inflammation in early wound healing, use of such a drug may also result in scar reduction. Additionally, PGE2 affects the early inflammatory response as well as the following proliferative phase through a series of processes.[3] If exogenous PGE2 is provided to fetal skin tissue, fibroblasts proliferate.[8] If PGE2 expression induced by COX-2 in wound tissue increases, then the deposition and proliferation of collagen by fibroblasts increases, leading to excessive scar tissue. Since expression of PGE2 is controlled by COX-2, inhibition of COX-2 blocks PGE2 release; we therefore hypothesized that COX-2 inhibition would prevent excessive proliferation of fibroblasts and abnormal scar formation.

In wound healing, many cytokines and growth factors are involved, but TGF-β is involved in the entire process of the inflammatory response and matrix deposition. TGF-β has a main influence on scar formation by stimulating the generation of collagen fibers by fibroblasts. Since TGF-β is hardly expressed in embryos, the characteristic scar-free embryonic wound healing is facilitated by its absence. It is thought that fibrosis can be controlled by inhibiting TGF-β based on several experimental results.[2] This growth factor is secreted by the surrounding platelets, skin cells and various inflammatory cells, and if deposition of inflammatory cells is inhibited, generation of TGF-β is lowered. Thus, a selective COX-2 inhibitor would decrease the activity of TGF-β through inhibiting production of PGE2 and thereby reducing the number of inflammatory cells in the region.[9] TGF-β also functions to increase expression of its own mRNA through an autocrine loop, so COX-2 inhibitors not only suppress the deposition of inflammatory cells, but decrease the available TGF-β, further hindering its production.[10] Therefore, COX-2 inhibitors suppress scar formation by hindering generation of TGF-β and by inhibiting deposition of inflammatory cells in wounds.

By the second week of our study, the degree of deposition of inflammatory cells was significantly decreased in the experimental group relative to the control group. This shows that the topical application of a COX-2 inhibitor suppresses the inflammatory response by inhibiting the activity of COX-2. At both the fourth and eighth weeks, the inflammatory response was decreased in the experimental group relative to the control, but there was no statistical significance. The period encompassing the fourth through eighth weeks is one in which the healing process progresses into the proliferative and remodeling phases so it is thought that the deposition of inflammatory cells may be generally reduced compared to the second week. Since COX-2 inhibitors act on the early inflammatory response to affect synthesis and deposition of collagen downstream, it follows that the subsequent series of processes leading to the elevation degree of the scar would be significantly reduced. This was observed at the eighth week, as the experimental group had a significantly decreased elevation of scar and maturity and structuring of collagen was advanced relative to the control group.

The density of new vessels is also associated with scar formation. Previous studies show that the number of vessels is increased significantly at excessively formed scar sites or keloids as compared to normal tissue.[11] On the other hand, in fetal wound repair the number of vessels is decreased as compared to adults. Notably, wounds in mucosal tissues leave less of a scar than in other tissues, and the number of new vessels is decreased, with wound repair rapidly normalizing as compared to skin wounds.[12] The exact mechanism by which excessive

generation of blood vessels affects severe scar formation is unknown. However, we hypothesize that excessive angiogenesis is a product of the early inflammatory response aimed at providing nutrients and inflammatory cells to the site, gathering in excess around the forming scar so that deposition of the extracellular matrix is more activated. Through all time-points, the number of vessels in our experimental group was significantly reduced compared to the control group. We hypothesize that since inhibition of the inflammatory response decreased the expression of several cytokines and growth factors, angiogenesis was reduced.

A previous study showed that if COX-2 is inhibited, generation of the gastric mucous membrane and bones is hindered, but according to recent studies, selective COX-2 inhibitors do not delay skin scar healing and do not affect re-epithelialization or recovery of tissue tension.[13] However, since a selective COX-2 inhibitor did not show an inhibition effect on platelet cohesion as was the case in the use of a non-selective COX-2 inhibitor, it may have a side-effect of cardiovascular thrombosis depending on the amount given. Hence, efforts to resolve the cardiovascular side-effects of selective COX-2 inhibitors are in progress but clinically meaningful outcomes have not yet been reported. It is thought that if selective COX-2 inhibitors are used topically on skin wounds, they may reduce the inflammatory response and inhibit scarring, while minimizing the impact on cardiovascular side-effects.

V. CONCLUSION

Through topical application of a selective COX-2 inhibitor (Celecoxib) after inducing wounds in the ears of rabbits, the subsequent generation of new vessels and the inflammatory response typically associated with adult wound healing were inhibited, and the degree of deposition and organization of collagen matured more quickly as compared to the control group. Additionally, the elevation degree of the resultant scars was reduced by treatment. These results suggest that topical application of a selective COX-2 inhibitor to skin wounds can affect to reducing the scar formation.

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