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Inhibition of Wound TGF Beta-1 by Celecoxib: A Possible Therapeutic Route for Scar Free Wound

Seham Abd El-Raouf Abd El-Aleem*[1] and Edward Jude[2]

[1]Department of Histology, Minia Faculty of Medicine, UK
[2]Tameside Hospital NHS Foundation Trust and the University of Manchester, UK

Abstract

Background: Wound healing is a highly ordered dynamic process associated with inflammation at early stages and with permanent scarring at late stages. Scars could be disfiguring and could advance to be hypertrophic or keloid scars, this would have a strong physical and psychological impact on the patients afterward. The role of inflammatory mediators which could be pro- or anti-inflammatory, pro-or anti-fibrotic was the focus of wound healing research for decades and the balance between them is the key factor determining the outcome of healing.

Aims: In this study**,** we investigate the correlation and the inter-relation between the pro-inflammatory cyclooxygenase-2 (COX-2) and the pro fibrotic (TGF-Beta-1) in an in vivo model of incisional dermal wound healing and the effect of selective COX-2 inhibition on the progression of wound healing and scar formation.

Materials and methods: Adult male Sprague-Dawley rats received four full thickness dermal wounds. a selective COX-2 inhibitor (Celecoxib) was applied to the wounds immediately postwounding twice daily for two days. Wounds and scars were then harvested at different time points and processed for COX-2 and TGF Beta-1 immunostaining and for collagen staining. Immunoreactivity was semi quantifed using Image J software.

Results: We have shown upregulation of COX-2, co-upregulation and co-localization of TGF-Beta-1 and COX-2 two days postwounding during the inflammatory phase. Celecoxib application significantly inhibited wound COX-2 ($P<0.01$) and TGF Beta-1 ($P<0.001$). It improved wound healing microscopically and macroscopically, through reducing inflammatory cell infiltrate, granulation tissues formation and early closure of the incision. Additionally, there was marked improvement in the postwounding scarring. There was a significant ($P<0.01$) correlation between COX-2 and TGF Beta-1 (Pearson Correlation=0.94).

Conclusion: The overall effect of COX-2 inhibition was shortening of the inflammatory phase of wound healing with subsequent minimization of the associated tissue destruction and consequently improvement of the scar quality. COX-2 inhibitors regulate inflammatory phase of the wound. They could regulate collagen deposition by regulating the production of the pro fibrotic TGF Beta-1, through autocrine/paracrine effect. Therefore, early application of COX-2 inhibitors to wounds immediately after injury/surgery could enhance the repair and more importantly,improve the quality of the postwounding scar.

Introduction

Wound repair and inflammatory mediators

Wound repair is a complex multi-steps process, consists of overlapping cellular and biochemical events. These events include inflammation, re-epithelialisation and matrix deposition [1]. Inflammation is an important event in determining the outcome of the healing since persistent inflammation can lead to a chronic wound [2]. The outcome of healing is determined by the balance between inflammatory mediators which have different physiological roles, pro-inflammatory or anti-inflammatory pro-fibrotic or anti-fibrotic [3- 5]. Prostaglandins play a key role in inflammation and they could be pro-inflammatory or anti-inflammatory [5,6].

COX in inflammation

COX is the rate-limiting enzyme in Prostaglandins synthesis [6]. They are the classic targets for therapeutic intervention [7]. Inhibition of COX is the mode of action of a wide range of non-steroidal anti-inflammatory drugs (NSAIDs) [3,8]. COX is present in two isoforms; COX-1 normally expressed in the body [9,10]. It is important for many physiological functions [11,12]. The second isoform is COX-2, is not normally present in most cells [13,14] but is rapidly induced during inflammation [15,16]. COX-2 products particularly prostaglandin E2 (PGE_2) are responsible for many of the cytotoxic effects of inflammation [17].

TGF Beta-1 and postwounding scarring

While wounds in adult heal by scar formation, fetal wounds heal without scars [18,19]. This was attributed to lack of inflammatory response [20,21] and low levels of TGF Beta-1 in fetal wounds [22,23]. TGF Beta-1 is secreted by dermal cells and by inflammatory cells infiltrating the wound; it promotes collagen deposition and remodelling [24-26]. Interestingly, exogenous application of TGF Beta-1 or PGE2 COX product to the fetal wound resulted in induction of

*Corresponding author: Abd El-Aleem SA, Department of Histology, Minia Faculty of Medicine, Tameside Hospital NHS Foundation Trust University and University of Manchester, UK, Tel: 0020102997270; E-mail: sehamns@yahoo.com

inflammation and scar formation [27-32]. Therefore, the inflammatory mediators including COX product and TGF Beta-1 are determent factors affecting the postwounding scarring. We have shown that while, TGF Beta-1 downregulation in wound was associated with persistence inflammation, lack of scarring and impaired healing [33] its upregulation was associated with excessive scarring [34].

Interrelation between inflammatory mediators in wound healing and scarring

Inflammatory mediators are produced and operate in a network with different roles pro-inflammatory and anti-inflammatory, pro-fibrotic and anti-fibrotic, thus there could be inter-regulation [35-41]. Our ongoing work investigate the regulation of wound TGF Beta-1 by other inflammatory mediators mainly COX and iNOS. Recently, Roman-Souz et al. [42] have shown inter-regulation between COX-2 and iNOS in a model of wound healing and we have shown inter-regulation of TGF Beta-1 and NOS in keloids [34] and in acute wounds (manuscript in preparation).

Aims and Hypothesis

The current study was designed to examine the colocalization and correlation between the pro-inflammatory COX-2 and the profibrotic TGF Beta-1 in wound tissues in a rat model of incisional dermal wound. This would provide a precise elucidation of the role of these factors in wound healing. To further elucidate the possible interrelation, we examined the effect of a selective COX-2 inhibitor on TGF Beta-1 production in wound and the effect on the repair progression and scar quality. The main concern following injury was enhancing healing by using anti-inflammatory and antiseptics to avoid chronicity of wound. However, in the last few decades attention was paid not only to get healing but to improve the quality of postwounding scarring. Therefore, our main target is to find the best choice of the available anti-inflammatory that not only relieve postwounding inflammation but reduce the pro-fibrotic TGF Beta-1 and subsequently minimise post traumatic scarring.

Materials and Methods

Specimens

Adult male Sprague Dawley rats, 225 to 250 g, age and weight matched, (4-6 per group) were housed singly for a week prior to the experiment. Standard rat diet and water were allowed. In the back of each animal, four full thickness incisions 1 cm each were made under halothane anesthesia. Two groups of rats (Sham groups) were wounded and left untreated to study COX-2 expression during the inflammatory phase one and two days postwounding. Six groups of rats were wounded and treated immediately either with 200 microliter of K-Y Jelly (Biovea) the vehicle control (3 vehicle groups; two days, 4 weeks and 8 weeks) or with Celecoxib (Pfizer) dissolved in the vehicle so that 200 microliters contained 1 mg of active drug (3 groups; two days, 4 weeks and 8 weeks). The treatment was applied topically to the wounds twice daily for two days. The wounds were unsutured and allowed to heal by secondary intention. The animals were housed in individual cages and allowed to recover. Animals were kept under the same conditions until the end of the experiment. Control untreated animals were killed one and two days postwounding. The vehicle and Celecoxib treated groups were killed two days, four weeks and 8 weeks post-wounding. Animals were killed by chloroform overdose followed by cervical dislocation. The dorsal skin was then removed from the animals by using a sterile surgical blade down to and including the panniculus carnosus muscle; wounds were dissected, fixed in 10% formalin for 48 hours and processed for paraffin embedding. 5-micron sections were used for running the staining. Sampling of the wounds was standardized by selecting at least 3 sections from identical sites within the wounds for each staining.

Immunoperoxidase staining

Sections were deparaffinized, hydrated then washed in 0.1 M phosphate buffer saline (PBS). Sections were then treated with trypsin 0.01% for 10 minutes at 37°C then washed with PBS for 5 minutes. Endogenous peroxidases were quenched by treatment with 0.5% H_2O_2 in methanol and non-specific binding was blocked in normal goat serum diluted 1:50 in 0.1 M PBS. Sections were incubated in the diluted primary antibody of interest overnight at 4°C. Sections were washed and incubated in biotinylated goat anti-rabbit secondary antibody (Vector laboratory 1:2000) for 30 minutes. The substrate, diaminobenzidine tetrahydrochloride in distilled water (Sigma) was added for the appropriate period (5-10 min). Positive cells were labelled brown. For the negative control, primary antiserum was replaced with normal serum of the host species of the secondary antibody. Sections were dehydrated and mounted in mounting media. Specimens were viewed using a Leica DRRB microscope and images were captured using a Spot RT Slider digital camera (Image Solutions) using Spot RT software run on a PC. The antibodies used were: polyclonal rabbit anti-COX-2 (1:200 Cayman, aa 570-598), Polyclonal rabbit anti-TGF Beta-1 (1:500 ABCAM, ab92486).

Double immunofluorescence

Sections were prepared and incubated with antibody to COX-2 (1:200) for 1 hour at room temperature. Then, they were washed and incubated for a further 30 minutes with a FITC conjugated goat anti-rabbit secondary antibody diluted 1:200 in TBS. Sections were incubated with an antibody to TGF-Beta-1 for 1 hour at room temperature. Then, they were washed and incubated with TRITC conjugated goat anti-mouse secondary antibody 1:100 in TBS for 30 minutes at room temperature. Hoechst No. 33258 (bis-benzimide fluorescent DNA stain that intercalates in A-T regions of DNA) was used as a nuclear counterstain. Sections were incubated in Hoechst No. 33258 1: 1000 for 15 min at room temperature to counterstain the nuclei. Sections were then mounted in polyvinyl alcohol. Viewed using the Leica DMRB microscope operating in fluorescence mode with appropriate filter sets and images were captured as above. The antibodies used were: Polyclonal rabbit anti-COX-2 (1:200 Cayman, aa 570-598), Monoclonal mouse anti-TGF Beta-1 (1:100 ABCAM, ab64715).

Masson's trichrome stain

Sections were stained with Masson's trichrome to examine the dermal connective tissue. Deparaffinized, rehydrated sections were processed for the staining and distilled water was used through all the procedure for washing. Sections were stained in Harris' Haematoxylin for 3 minutes and left in running water for 2 minutes. Sections were then stained in picric acid for 1 minute followed by washing for 1 minute. Sections were then stained with 25% Biebrisch scarlet for 1 minute followed by washing for 1 minute. Sections were stained with phosphotungstic/ phosphomolybdic acid solution for 3 minutes, then stained in fast green for 10 minutes. Slides were dehydrated and mounted in Pertex mounting media and images were captured as above.

COX-2 and TGF Beta-1 assessment in wound

Image J software (developed at US National Institutes of Health

and available on the Internet at http://rsb.info.nih.gov/nih-image/) was used to assess COX-2 and TGF Beta-1 immunoreactivities on immunofluorescence stained slides in the field of a 20x objective. The immunoreactivity was assessed by measuring the surface area covered by the positive staining. Assessment was done at the wound site in 6 adjacent areas from each section, 3 areas on each side of the midline of the wound. In each case 4 sections were scored and the distance between sections were 50 mm. Before starting the analysis, the setting was adjusted on a test slide and the same setting parameters were used throughout the whole experiment on all slides as we previously described [34]. Below figure demonstrates the method of assessing and scoring the staining.

Statistics

Statistical analyses were performed using IBM SPSS 19 statistical package. Results were expressed as the mean+SEM. One-way AVNOVA was used, with $P < 0.05$ being considered as statistically significant.

Results

Expression of COX-2 in the wounds during the inflammatory phase

COX-2 was expressed in the wound site during the inflammatory phase one and two days postwounding (Figure 1). One-day wound showed inflammatory cell infiltrate and new vascularization in the wound site particularly around the impeded hair follicles. COX-2 immunoreactivity was seen mainly in the infiltrating inflammatory cells and vascular endothelial lining in the wound site (Figure 1A). Condensation of neutrophils within the granulation tissues was seen on the surface of the wound, neutrophils were identified by their characteristic nuclear morphology and they did not show immunoreactivity (Figure 1A). Two days wound showed wide wound gap and massive inflammatory infiltrate (Figure 1B). COX-2 immunoreactivity markedly increased almost in all the infiltrating inflammatory cells and in the newly formed blood vessels (Figure 1B). Two days wound showed strong COX-2 immunoreactivity in the deep wound tissues in the infiltering inflammatory cells and vascular endothelial lining (Figure 1C and 1D). Thus, two days wound was a good representation of the inflammatory phase of wound so was chosen for studing the effect of COX-2 inhibiton.

Colocalization of COX-2 and TGF Beta-1 during the inflammatory phase

Double immunofluorescent showed that COX-2 and TGF Beta-1 were co-expressed in the wound both in the vehicle control wounds (Figure 2A-2D) and Celecoxib treated wounds (Figure 3A-3D). COX-2 was expressed in the infiltrating inflammatory cells at the wound areas (Figure 2A and 3A). TGF Beta-1 was expressed in most of cells expressing COX-2 (Figures 2B and 3B). Merging COX-2 and TGF Beta-1 images showed that both mediators are colocalized in the same cells in the wound area (Figures 2C, 2D and 3C).

Effect of Celecoxib on wound TGF Beta-1 production during the inflammatory phase

The vehicle control wounds showed formation of granulation tissues with massive inflammatory cell infiltrate two days postwounding (Figure 2). COX-2 and TGF Beta-1 immunoreactivity was seen in the infiltrating inflammatory cell (Figure 2A and 2B). They were expressed in same cells and this was confirmed in the merged the images (Figure 2C and 2D). Topical application of Celecoxib,
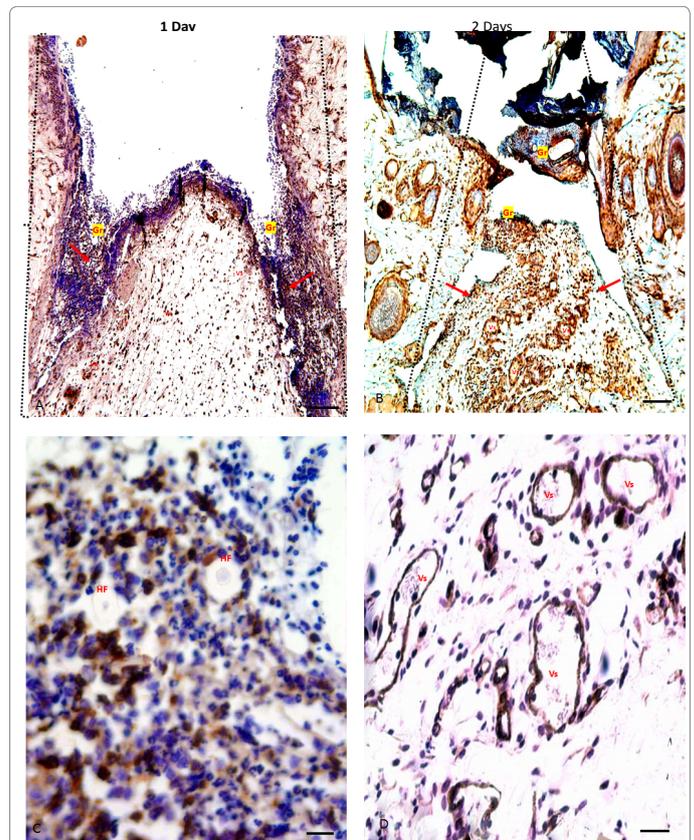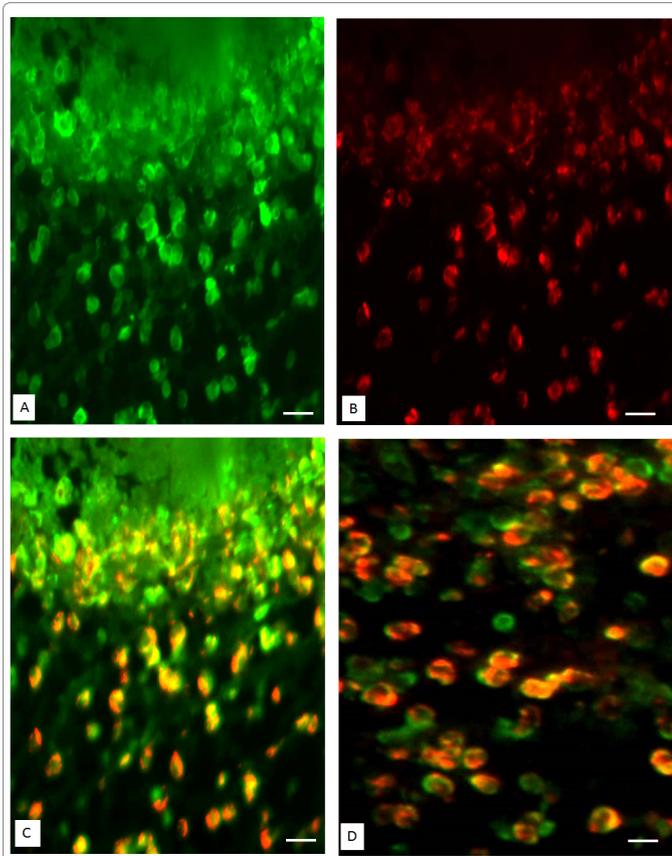

Figure 1: Representative images of immunoperoxidase staining showing expression of COX-2 in acute incisional rat dermal wounds during the inflammatory phase of healing: A) One day wound showing inflammatory cell infiltration (arrows) and newly formed blood vessels (Vs) at the wound site (Between the brackets). COX-2 immunoreactivity is seen in the infiltrating inflammatory cells (arrows) and few blood vessels (Vs). There is large number of neutrophils (identified by their characteristic segmented nuclei that deeply stained with haematoxylin counter stain) in the inflammatory infiltrate. COX-2 immunoreactivity is seen in many of the infiltrating cells but not in the neutrophils. B) Two days wound showing massive infiltration with inflammatory cells and many newly formed blood vessels at the wound site. COX-2 immunoreactivity is seen at the wound site (Between the brackets). in the granulation tissues (Gr) filling up the wound gap, in the inflammatory cell infiltrate and in most of the newly formed blood vessels (Vs). C) Higher magnification of the centre of a two-day wound showing massive inflammatory cell infiltration around the impeded hair follicle deep in the wound (HF). There is large number of neutrophils (identified by their characteristic segmented nuclei deeply stained with haematoxylin counter stain) in the inflammatory infiltrate. COX-2 immunoreactivity is seen in many of the infiltrating cells but not in the neutrophils. D) Higher magnification of a two days wound showing many blood vessels (Vs) of different sizes, deep in the wound with COX-2 immunoreactivity seen in the endothelial lining and in dermal inflammatory cells. Scale Bars; A, B = 400µm C, D =100µm.

immediately postwounding twice daily resulted in marked reduction in the inflammatory infiltrate at the wound site (Figure 3). There was marked decrease in the COX-2 and TGF Beta-1 immunoreactivity at the wound site (Figure 3A and 3B) and they were mostly colocalized in the infiltrating inflammatory cells (Figure 3C).

Effect of Celecoxib on wound COX-2 and TGF Beta-1 levels during the inflammatory phase

Scoring the immunoreactivity in two days wounds showed that Celecoxib application to the wound significantly reduced COX-2 ($P < 0.01$) and TGF Beta-1 ($P < 0.001$) levels (Figure 4B) by comparison to the sham control and vehicle control groups. There were no

Figure 2: Representative images of double immunofluorescence staining showing colocalization of COX-2 and TGF Beta-1 in the vehicle control wounds. Wound gap is filled up by granulation tissues formed of massive inflammatory cell infiltrate showing A) COX-2 immunoreactivity (Green). B) TGF Beta-1 immunoreactivity (Red). C) Merging A and B shows that COX-2 (Green) is widely distributed in the wound and TGF Beta-1 is seen in some but not all COX-2 positive cells. Many of cells expressing COX-2 are also expressing TGF-Beta-1. Thus, COX-2 and TGF Beta-1 are colocalised in some cells. D) Higher magnification of a merged images showing that some of the COX-2 positive cells express TGF Beta-1 (Yellow/Orange or partially green and partially red). Scale Bars; A-C = 40µm, D = 25.
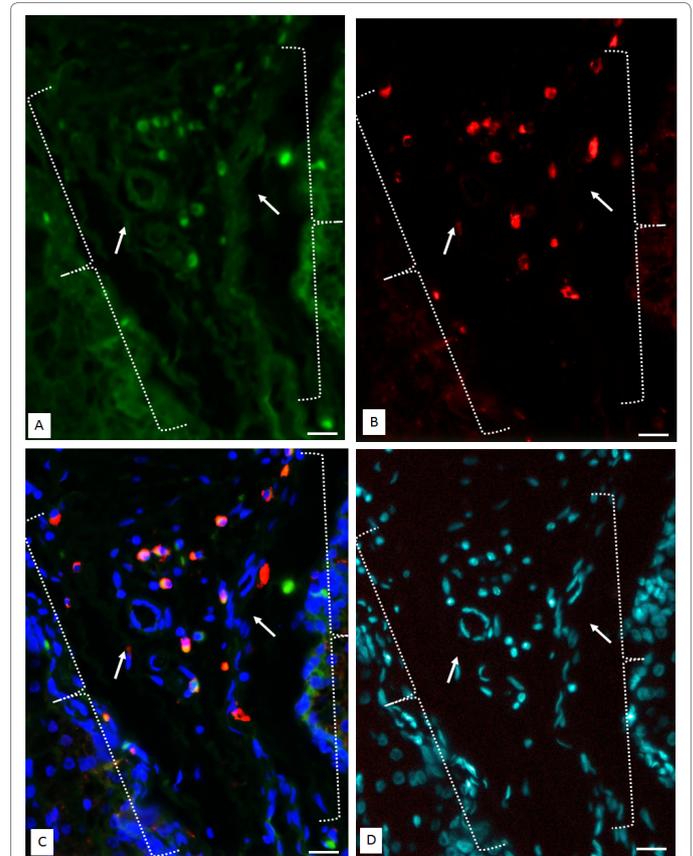

Figure 3: Representative images of double immunofluorescence staining showing colocalization of COX-2 and TGF Beta-1 in Celecoxib treated wounds. A) Wound (Between the brackets), showing few inflammatory cells infiltrate at the centre of the wound gap. COX-2 immunoreactivity (Green) is seen in the infiltrating inflammatory cells. B) TGF Beta-1 immunoreactivity (Red). C) Merging A and B shows that COX-2 and TGF Beta-1 are colocalised in few inflammatory cells within the granulation tissues in the wound gap. Nuclei (Blue) are counterstained. D) Fluorescent DNA stain (a nuclear counterstain) showing marked reduction in the wound gap (Between the brackets) with marked decrease in the granulation tissues mass in the wound gap. Scale Bars; A-D = 100µm.

significant changes between the sham control and the vehicle control wounds (Figure 4B).

Correlation between wound COX-2 and TGF Beta-1 during the inflammatory phase

From the colocalization study of COX-2 and TGF Beta-1 seen in Figures 2 and 3 we have demonstrated that they showed a similar pattern both in the vehicle control and Celecoxib treated wounds. However, to elucidate this pattern of relation between COX-2 and TF Beta-1, we ran a correlation analysis including the three groups; sham control, vehicle control and treated wounds. Interestingly COX-2 and TGF Beta-1 showed a similar pattern of expression. They get coupregulated in sham and vehicle groups and codownregulated in the treated group in a similar pattern (Figure 4C). Statistically, there was a significant ($P<0.01$) strong correlation (Pearson Correlation=0.94) (Figure 4C and 4D). between COX-2 and TGF Beta-1.

Effect of Celecoxib on collagen deposition and the quality of postwounding scar

Masson's trichrome was used to assess the dermal architecture four and eight weeks postwounding to evaluate the effect of COX-2 inhibition

on scar quality. The main criteria considered for assessing scars were; differences in the spacing between collagen bundle orientation and organisation of collagen fibres. Four weeks postwounding the vehicle control showed a well-defined distinct mass of collagen filling up the wound gap. The scar showed obliteration of spaces between collagen bundles and between collagen fibres, and a distinct difference in the dermal architecture in the scar tissues at the wound site by comparison to the adjacent unwounded dermis (Figure 5A). On the other hand, Celecoxib treated wounds showed spacing between collagen bundles and between collagen fibres (Figure 5B). Eight weeks postwounding, the vehicle control showed dense collagen that was deposited compactly in an abnormal pattern with lack of spacing between fibres (Figure 6A and 6C). By contrast, in Celecoxib treated wounds the scar width was considerably reduced, and collagen fibres were deposited in a reticular pattern with spacing in between resembling the dermal architecture of the adjacent unwounded dermis (Figure 6B and 6D).

Effect of COX-2 on the macroscopic appearance of the wounds and scars

Two days postwounding the vehicle control showed widely open wounds, with inflammation and excessive granulation tissues
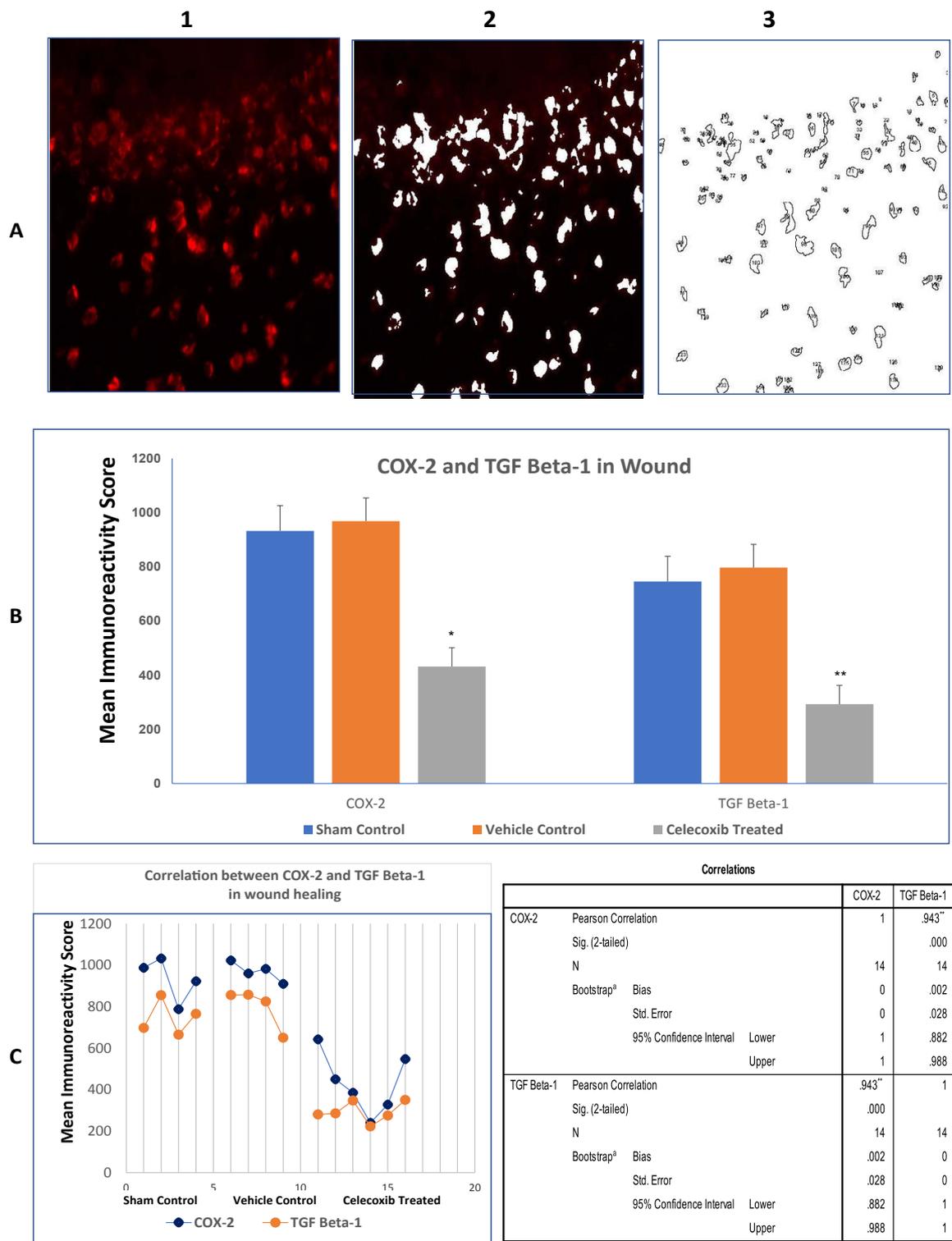
Figure 4: A) Photomicrographs demonstrating image J analysis of immunofluorescence staining: 1) Original image before processing. 2) Converted image immunoreactive structures were converted to a distinct colour (White) that the software can score with exclusion of background staining. 3) Outlined image showing that the software scored the specific staining (Immunoreactivity=Outlined structures) only with exclusion of any background staining. B) A graph showing COX-2 and TGF Beta-1 levels in two days wounds in sham control, vehicle control and Celecoxib treated wounds. Celecoxib treated wound shows a significant decrease in COX-2 (P<0.01) and TGF Beta-1 levels (P<0.001) by comparison to the sham control and vehicle control. C) A graph showing a significant (P<0.01) and strong correlation between COX-2 and TGF Beta-1 in wounds in the sham control, vehicle control and Celecoxib treated (Pearson Correlation=0.94). This indicate that the changes in COX-2 was associated with similar changes in TGF Beta-1. Thus, COX-2 and TGF Beta-1 mediators get up-regulated or down-regulated simultaneously.
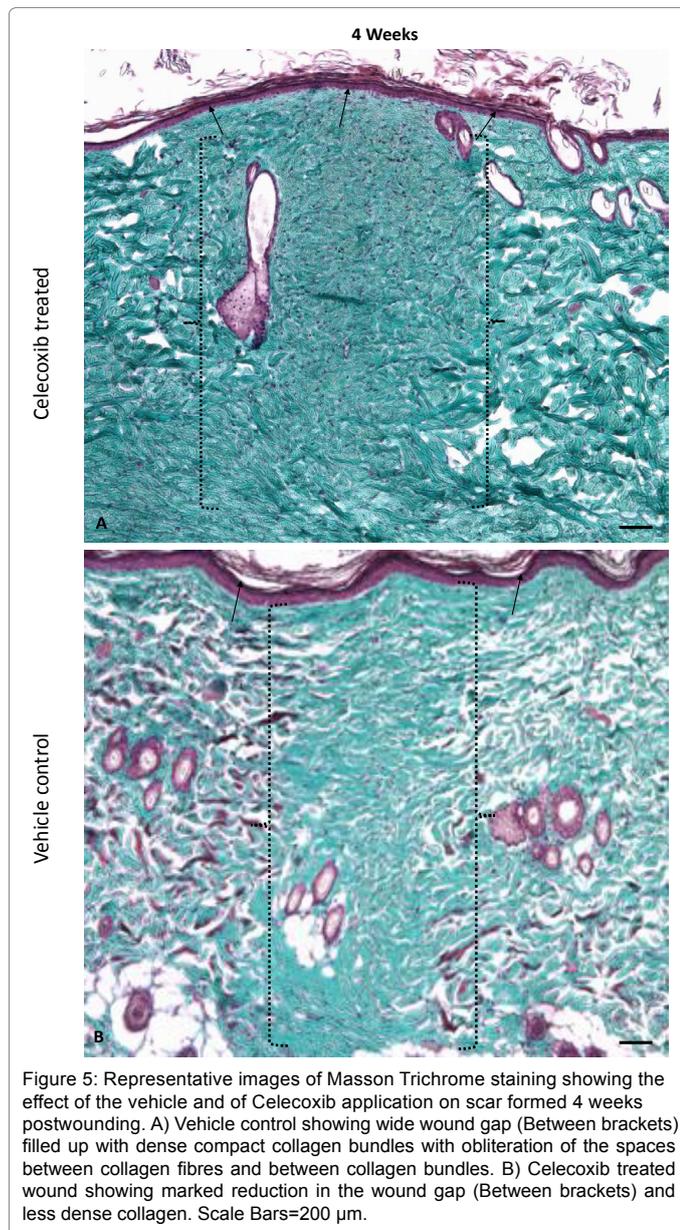
Figure 5: Representative images of Masson Trichrome staining showing the effect of the vehicle and of Celecoxib application on scar formed 4 weeks postwounding. A) Vehicle control showing wide wound gap (Between brackets) filled up with dense compact collagen bundles with obliteration of the spaces between collagen fibres and between collagen bundles. B) Celecoxib treated wound showing marked reduction in the wound gap (Between brackets) and less dense collagen. Scale Bars=200 μm.
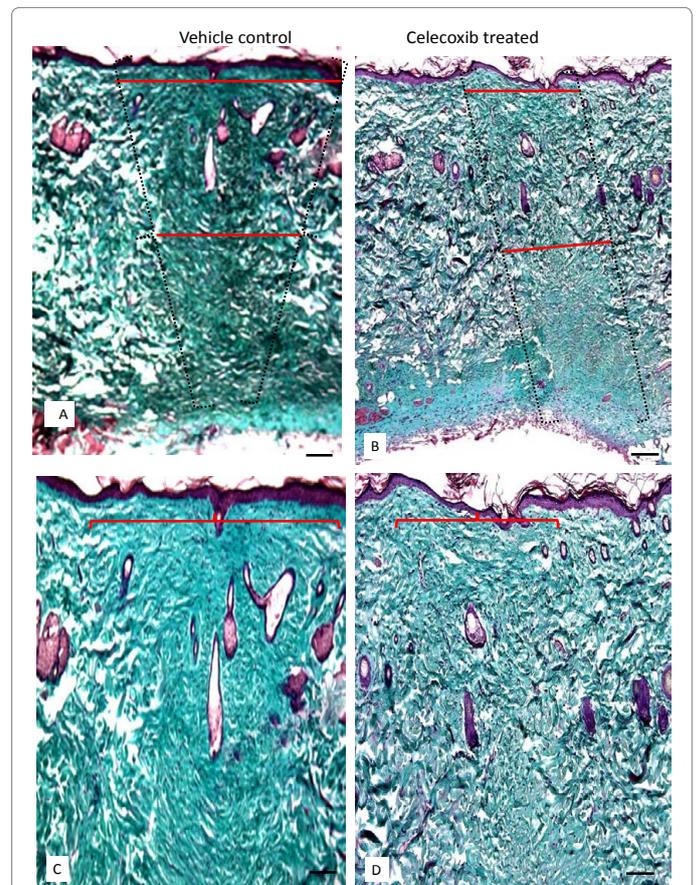

Figure 6: Representative images of Masson Trichrome staining showing the effect of the vehicle and of Celecoxib application on the scar formed 8 weeks postwounding. A) Vehicle control showing a wide wound gap (Between brackets) filled up with a mass of disorganized collagen. collagen bundles arranged compactly in an abnormal pattern with lack of spacing in between B) Celecoxib treated wound showing marked reduction in the wound gap (Between brackets). Collagen bundles arranged in a reticular pattern with spacing in between resembling that of the normal dermis. C) Higher magnification of A showing a wound gap filled with dense collagen with collagen fibres compactly arranged in an abnormal pattern resulting in a distinct scarring D) Higher magnification of B showing marked reduction in scarring. Collagen bundles arranged in a reticular pattern closely resembling that of the normal dermis resulting in dermis indistinguishable from that of the surrounding unwounded skin. Scale Bars; A, B=400 μm, C, D=200 μm.

formation (Figure 7A). While, the Celecoxib treated group showed narrow wounds with less inflammation and less granulation tissues (Figure 7B). The macroscopic appearance of the postwounding formed scars was directly related to the microscopic differences in the dermal architecture between the vehicle control and treated wounds. The Celecoxib treated group showed no obvious scarring and the wound sites were indistinguishable from the surrounding unwounded skin (Figure 7D) by comparison to the vehicle control wound (Figure 7C).

Discussion

There are several biological mediators produced at the wound site that control the healing progression. They have either agonising or antagonising effects on phases of healing, they can be pro-inflammatory or anti-inflammatory, profibrotic or antifibrotic, pro-proliferative or anti-proliferative…etc [41-48]. The Wound healing requires a balance between these endogenously produced mediators [43-48] to progress in a coordinated manner without complications [2,33,34]. The basic

idea of using exogenous medication is to restore the balance between the endogenous mediators to obtain optimal condition for healing. Previously we have studied several human disorders associated with abnormal wound healing such as chronic venous ulcers [49-51] diabetic ulcers [2,33] and keloids [34] and we have reported that the abnormality in healing was attributed primarily to the imbalance between endogenous mediators in the wound environment. Here we extended our study to an animal model of wound healing with interest in investigation of the postwounding scars and the role of COX-2 and TGF Beta-1, two mediators believed to have a crucial role in scar formation [24-32]. At time of injury or surgery, the main concern of both the patient and the surgeon is relieving inflammation and achieving wound closure, thus, NSAIDs are routinely used postoperative to achieve this short-term target [52-55]. However, wound closure is usually associated with scar formation [56] and this itself is disturbing and could be disfiguring or develop to hypertrophic scars [57] or keloid [34]. Unfortunately, postwounding scars do not receive much concern as they develop late
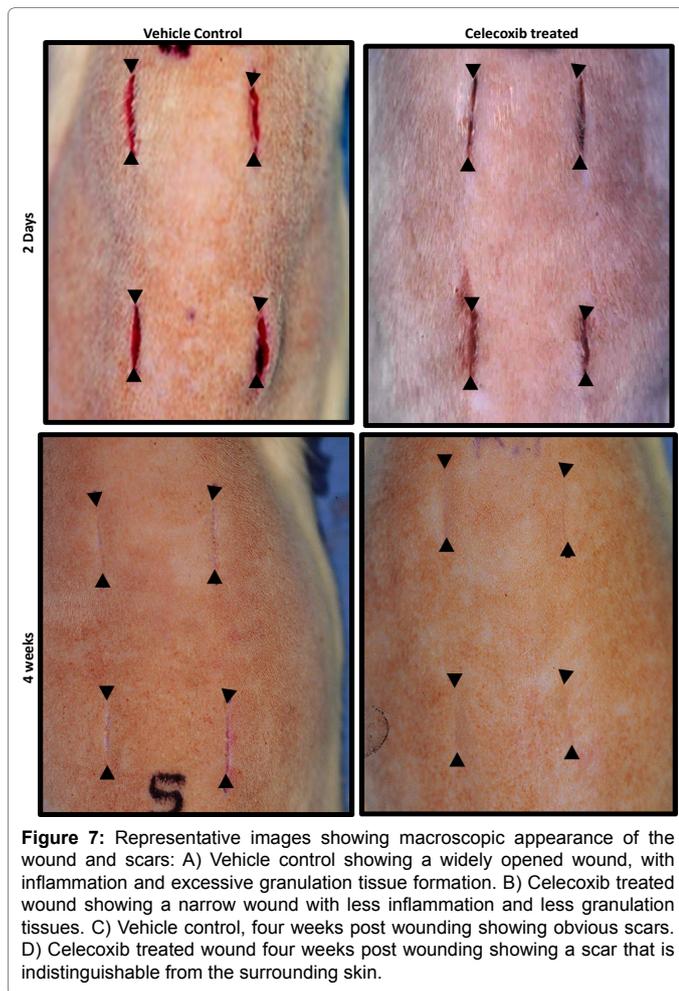
Figure 7: Representative images showing macroscopic appearance of the wound and scars: A) Vehicle control showing a widely opened wound, with inflammation and excessive granulation tissue formation. B) Celecoxib treated wound showing a narrow wound with less inflammation and less granulation tissues. C) Vehicle control, four weeks post wounding showing obvious scars. D) Celecoxib treated wound four weeks post wounding showing a scar that is indistinguishable from the surrounding skin.

after wound closure but on the long term this could have psychological impact. Therefore, our long-term target in this study is achieving a scar free wound. Here, we have shown upregulation of wound COX-2 and TGF Beta-1 during the inflammatory phase of wound. Indeed, high levels of COX-2 results in increase wound PGE2 levels which account for the inflammatory changes associated with wound [58-60], COX-2 levels were restored by the usage of anti-inflammatories to relieve post-traumatic inflammation [52-55]. Investigating the interrelation between the proinflammatory COX-2 and the profibrotic TGF Beta-1 in wound might offer an opportunity to find one of the available anti-inflammatory drugs that can also be antiscarring. This would achieve the short-term target, wound closure and our long-term target, scar free wound. From this study, we present evidences that there is an inter-relation and inter-regulation between the pro-inflammatory COX-2 and the profibrotic TGF Beta-1 in wound tissues. *Firstly*, we demonstrated that COX-2 and TGF Beta-1 showed similar cellular profiles as both were expressed mainly by the infiltrating inflammatory cells in the wound area, this was in line with other studies [52-55], however, we endorsed our finding by showing for the first time actual colocalization of Cox-2 and TGF Beta-1 on the cellular level in the wound area. *Secondarily*, demonstrating that both mediators showed a similar pattern of expression in the wound, where COX-2 upregulation and downregulation was associated with a concomitant similar change in TGF Beta-1, this was in line with other studies in different animal models of inflammation [61-70]. *Thirdly*, we endorsed this COX-2 and

TGF Beta-1 coordinated relation results further by a correlation studies that showed for the first time a strong correlation between COX-2 and TGF Beta-1 in dermal wounds. In summary, these mounting evidences; COX-2 and TGF Beta-1 coexpression, endorsed by their colocalization and their coordinated relation, endorsed by their correlation would indicate a strong interrelation and interregulation between them in wound. The colocalization finding indicates that COX-2 produced by the infiltrating inflammatory cells at the wound site could induce TGF Beta-1 synthesis and production by the same cells through autocrine mechanism or the nearby cells through a paracrine mechanism or both. Paracrine/autocrine signalling between COX-2 and TGF Beta-has been extensively studied in several *in vitro* models [71] and this COX-2 and TGF Beta-1 interregulation was shown in fibroblast [72], in macrophages and intestinal epithelial lining [73,74], in bronchial epithelium [75] in lung fibroblasts [76] and in keratinocytes [77]. Paracrine/autocrine regulation between COX-2 and TGF Beta and other growth factor has been a hot area of research in oncology as this inter-regulation was proposed to play a critical role in cancer invasion and metastasis [74-78].

As our long-term target is achieving scar free wound by finding an antiinflammatory drug that can be antiscarring; we investigated the effect of Celecoxib on wound TGF Beta-1 and on wound progression and more importantly on scar quality. Celecoxib is a NSAID that selectively inhibits COX-2 with anti-inflammatory effect but lesser toxicity than other NSAIDs [66]. We have shown that inhibition of wound COX-2 resulted in inhibition of wound TGF Beta-1 and improvement in wound repair progression as demonstrated by reducing inflammation and early wound closure. Interestingly, Celecoxib application improved the postwounding scarring and we almost achieved our target in getting scar free wound. The effect of COX-2 inhibition on wound healing is controversial. In line with our finding COX-2 inhibition was reported to relieve inflammatory response in sponge implants model of wound healing and promotes the closure of excisional lesions [42,79]. However, Other studies, showed that COX inhibition does not affect wound healing [80,81] and other studies showed that COX-2 inhibition reduces the wound closure delay the healing [82,83]. This discrepancy in the results could be due to the differences in models, in species and in the route of administration; Salcido et al. and Dong et al. [81,82] have used a pressure ulcer or a burn model of wound healing, the lack of effect of COX inhibition on inflammation in these models could be attributed to the excessive damage and epidermal loss associated with these models [84,85], unlike our incisional wound model. Additionally, the response to COX inhibition varied in different species, while COX inhibitors did not affect wound healing in hairless SKH-1 mice, they delayed healing in C57BL/6J mouse [83] and this could be explained by differences in genetic background [86] between species and even intra-species. Furthermore, the route of administration of the drugs would another factor explaining the discrepancy in the results; oral administration of COX inhibitor were found not to improve healing [82,83], indeed topical application would be more effective in wound healing than systemic [87,88].

Indeed, inflammatory response is necessary for proper wound healing, but our finding suggests that high levels of inflammation may not be an essential requirement for healing, and may promote tissue destruction and scar formation. This idea is in line with the fetal scarless healing characterised by a lack of inflammatory response and a lack of scar formation [19-31]. Interestingly, in this study we have shown macroscopic and microscopic reduction of postwounding inflammatory response and postwounding scarring simulating fetal wound without completely interfering with the inflammation.

Our findings suggest that COX-2 is critical determinants of the post wounding scar and that this might be through promotion of the excessive production of the profibrotic TGF Beta-1 in wounds. Inflammatory phase of dermal wound determines the quality of the post wounding scars. While the reduction of inflammatory response with early wound closure achieved by Celecoxib application at early time point could be attributed to its direct anti-inflammatory effect [62-64]. The improved scar quality could be attributed to an indirect antifibrotic effect of Celecoxib through reducing wound TGF Beta-1 [61,62] with subsequent reduction in collagen deposition and scar formation [89].

Conclusion

In summary, controlling inflammation in the wound by Celecoxib could decrease the postwounding scarring without jeopardizing the healing process itself. Therefore, proper wound management by using treatment that control inflammation and pain at early stages and minimize the postwounding scar would be of great impact economically and psychologically. Celecoxib, could be one of the already available anti-inflammatory that can also be antiscarring. It would be one of the good choices to achieve our target early wound closure with scar free wound. Interestingly, our results from the animal model of wound healing is supported by a recently released publication showing that simultaneous silencing of TGF-β1 and COX-2 reduces human skin hypertrophic scar. Probably, this was the mechanism by which Celecoxib improved wound healing progression and scar quality in our current study.

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