

ORIGINAL RESEARCH

Thermal Burn Wounds Produce Greater Scars Compared to Similarly Sized Excisional Wounds and Topical Amiloride Applied to Burn-Induced Scars Shows Scar Reduction

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ABSTRACT

Background: Victims of severe traumatic injuries such as large surface area lacerations and thermal burns require substantial medical care that primarily promotes healing. And although this care is essential, there is a lack of pharmacological treatments that reduce the resulting scars, consequently leaving many traumatic victims with profusely disfigured skin.

Methods: A rabbit-ear injury model was used to compare scar progression in adjacently paired contact thermal burns (n=24) and excisional wounds (n=16). Once that model revealed significant differences in scar hypertrophy between these two types of injuries, a succeeding study involved solely inducing burns, with the resulting wounds undergoing scar elevation index (SEI) and gene expression analysis after unilateral topical treatment with either amiloride (n=12), celecoxib (n=11) or contralateral vehicle control (n=10 for each of the two control groups).

Results: In the initial burn and excisional wound comparison study, thermal burns showed significantly larger scars, both in scar height measured at four timepoints ($P < 0.0001$, < 0.01 , < 0.05 , and < 0.05) and histologically by analyzing the SEI ($P < 0.05$). In the succeeding project, burn-induced scars treated with amiloride also demonstrated a significantly reduced histological SEI ($P < 0.05$) compared to scars receiving vehicle control. However, relative *PTGS2*, *ACTA2* and *COL1A1* expression was not significantly different in scar tissues treated with amiloride compared to those receiving vehicle control. Also, no significant differences in SEI were determined in scars treated with celecoxib compared to vehicle control.

Conclusions: Contact thermal burn injuries create profusive hypertrophic scars compared to similarly sized excisional wounds. Topical application of amiloride to burn-induced scars reduce scar formation, yet this finding necessitates further studies to comprehend the mechanism behind its scar-reducing effect.

INTRODUCTION

Traumatic skin injuries such as large lacerations and severe thermal burns

commonly conclude with pathological scars.^{1,2} Skin sequela such as hypertrophic scars, keloids and contractures frequently form after skin re-epithelialization, with all three manifestations harboring abundant

fibrotic extracellular matrix deposition,^{3,4} and often times accompanied with pain, pruritus, paresthesia, limited mobility and/or psychosocial distress.⁵⁻⁷ Although various skin injuries — chemical, electrical, scald, abrasions and lacerations — have the potential to heal with pathological scars, some research has found that contact thermal burn injuries heal with more substantial scars when compared to similarly sized excisional wounds.⁸⁻¹⁰

With such findings, and having a scar reduction study to pursue, we opted to first test whether considerable differences in scar development materialize between two similarly sized adjacent injuries: contact thermal burns and excisional wounds. The objective being that data from this study would help us select a significant scar-producing injury, and that such selected injury would be well-matched for a secondary project aimed at testing the scar-reducing potential of amiloride and celecoxib. This was the basis of these two experiments.

However, the principle behind the potential for these drugs to minimize scar development arose from previously published *in vitro* work involving amiloride and fibroblasts. Amiloride, a widely used drug to treat hypertension, heart failure and, in some instances, treat or prevent hypokalemia, functions through selective inhibition of epithelial sodium channels (ENaC), Na⁺/H⁺ exchangers and Na⁺/Ca²⁺ exchangers in kidney tubules, effectively reducing intracellular sodium and enhancing the tubule diuretic effect.¹¹ Although mainly recognized as a nephron-targeting drug, amiloride has also shown activity outside of the kidney. In a former study, *in vitro* induction of ENaC-mediated sodium flux by reduced hydration activated fibroblasts through the cyclooxygenase-2/prostaglandin

E2 (COX-2/PGE₂) pathway, successively promoting collagen deposition from human dermal fibroblasts.¹² However, amiloride-induced inhibition of ENaC in keratinocytes prevented fibroblasts from generating collagen deposition, thereby demonstrating that ENaC promotes fibroblast activation via ENaC-dependent paracrine signaling in response to reduced hydration. Given that skin injuries disrupt the integumentary barrier and consequently produce local dehydration by increasing transepidermal water loss (TEWL),¹³ and since burn scars also exhibit increased TEWL compared to normal skin,¹⁴ we hypothesized that amiloride (and celecoxib due to its COX-2 antagonistic effect) could reduce scar formation in an *in vivo* study by hampering collagen production in a rabbit-ear injury model.

METHODS

This study complied with National Research Council's Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee. The project utilized New Zealand White female rabbits (Envigo, Indianapolis, IN) aged 17-22 weeks and weighing 2.7-3.2 kilograms at the start of the study. During discomforting procedures, animals were placed under an anesthetic plane (ketamine 40 mg/kg, xylazine 5 mg/kg) and received systemic (buprenorphine SR 0.2 mg/kg) and local (lidocaine 4 mg/kg, except during burn induction) pain control along with core body temperature assistance. The use of E-collars was also used to safeguard wounds.

Wounding Process: Excisional Injuries

Surgical excisional wounds were performed on the ventral surface of ears using a 7 mm-

diameter biopsy punch, whereby a circular skin incision was made until the depth was tangential to the underlying perichondrium. Once a circular incised border was visible, forceps were used to grasp the incised tissue and traction was applied to bluntly free the skin and complete the excision. Tegaderm film dressing (3M, Maplewood, MN) was immediately applied over the excised wounds and remained until complete re-epithelialization, with scar-tissue harvesting taking place upon conclusion of the study.

Wounding Process: Contact Thermal Burns

Burns were performed with a 1-cm diameter cylindrical 90-gram brass rod, heated within an Isotemp™ dry block and heater (Fisher Scientific, Pittsburg, PA). Once the temperature on the heater stabilized, the rod was removed from the dry block and placed on an ambient temperature K-Type TL0225 thermocouple (Perfect Prime, Far Rockaway, NY) so that real-time temperature readings could be displayed on a Digi-Sense 20250-08 thermometer (Cole-Parmer, Vernon Hills, IL). Once the temperature declined to 94° C, the rod was immediately transferred to the ventral ear for an 8 second freestanding burn induction. Five days later, debridement of burn-induced eschar buildup was performed tangential to the underlying perichondrium with a 7 mm-diameter biopsy punch and forceps. Tegaderm film dressing was then applied over the debrided wounds and remained until complete re-epithelialization, with scar-tissue harvesting taking place upon conclusion of the study.

Scar Comparison of Burn and Excisional Wounds

Eight rabbits (one ear from each animal) were used to compare scar formation from paired burn and excisional injuries that progressed free from pharmacological intervention. On post-operation day (POD) 0, burns (n=24) were introduced with eschar debridement occurring on POD 5. On this same day, soon after debridement, excisional wounds (n=16) were made adjacent to burn-induced wounds. As healing progressed, scar height was measured in both types of injuries on POD 12, 21, 26 and 28 with calipers by directly contacting the scar (ventral surface) and the normal skin directly underneath the scar (dorsal surface). On POD 29 the scar tissues were harvested for scar elevation index (SEI) analysis.

Pharmacological Application to Burn-Induced Scars

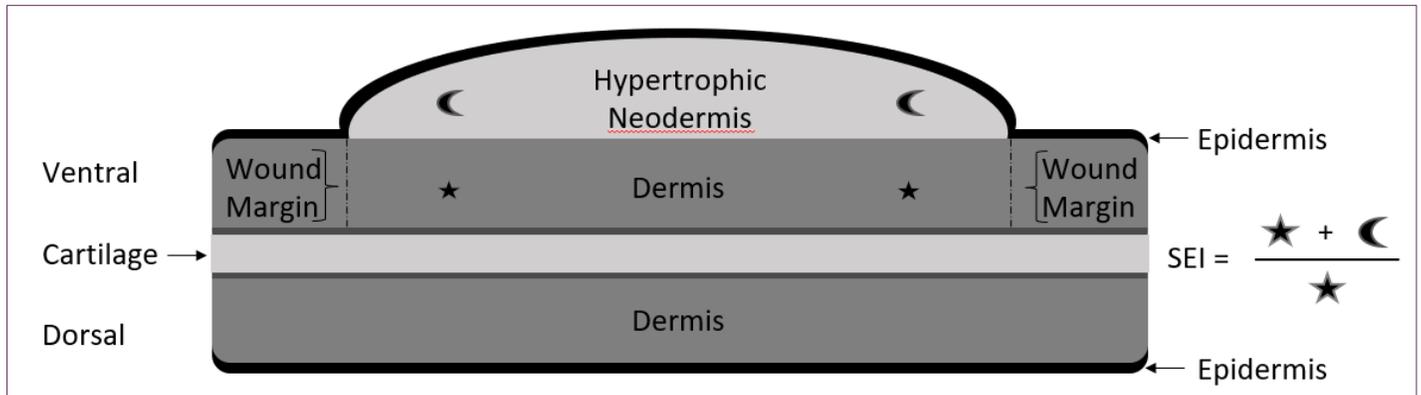


Figure 1. Method used for calculating the scar elevation index (SEI). Sketch of the dorsal and ventral skin layers found in the rabbit ear, portrayed with a developing hypertrophic scar (hypertrophic neodermis). The SEI is calculated with histological images, whereby the area of the ventral dermis (stars) within the wound margins is summed with the area of the hypertrophic neodermis (moons), and subsequently divided by the area of ventral dermis (stars).

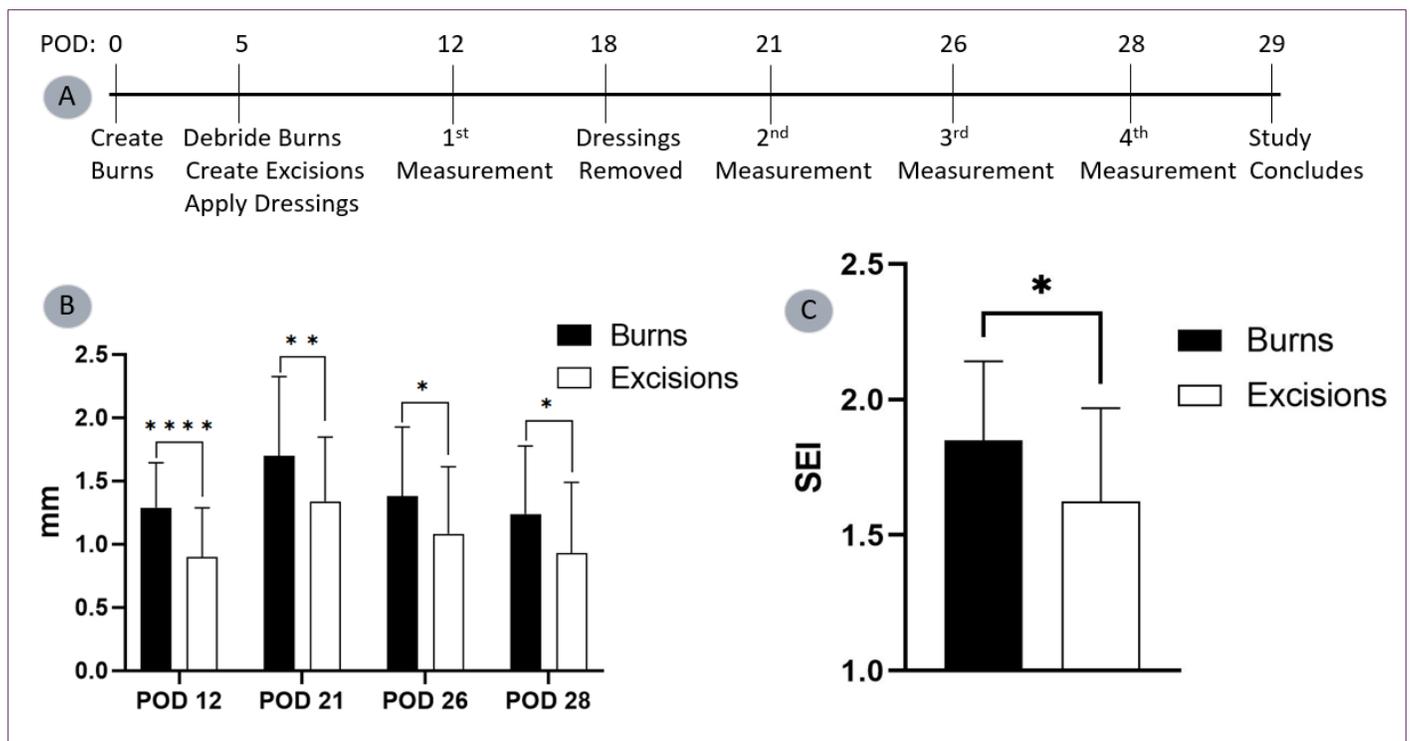


Figure 2. Timeline and data from study comparing scars from paired burn and excisional wounds. **A)** Timeline of study undergoing both burns and excisional wounds, with top values indicating post-operation day (POD) and bottom content relating to events. **B)** Bar graphs illustrating data from the four days caliper scar measurements were obtained. **C)** Bar graph comparing the histological scar elevation index (SEI) between burns and excisions at POD 29. Bar graphs represent mean ± standard deviation.

Eight rabbits divided into two groups (4 allocated to receive amiloride to one ear and vehicle control to the contralateral ear, and 4 allocated receive celecoxib to one ear and vehicle control to the contralateral ear) were used to test the scar-reducing capabilities of selected drugs and vehicle controls.

Animals underwent burns to both ears with subsequent eschar debridement. Under each group tested, scars from a unilateral ear underwent topical treatment (amiloride n=12, or celecoxib n=11) while scars on the contralateral ear underwent vehicle control application (n=10 for each of the two control groups). Treatments and vehicle controls were applied five times over the course of ten days (POD 18, 20, 22, 25, 27) with scar tissue harvested on POD 29.

Drug Preparation, Histology, and qPCR Gene Expression

Amiloride (Sigma-Aldrich, St. Louis, MO) and celecoxib (Sigma-Aldrich) were used to treat the developing scars. To increase penetration of these drugs through skin, each was dissolved in a microemulsion vehicle consisting of transcitol (as a surfactant) and capmul MCM (as an emulsifier) at a ratio of 1:1 (v:v).^{15,16} Dissolved drugs, 2% amiloride and 4% celecoxib, were then mixed with Vaseline lotion (Unilever, Trumbull, CT) in a ratio of 1:2 (v:v), and placed inside syringes for subsequent application of roughly 20 μ L onto each scar of either drug or vehicle control. On day 29, scar tissues from burns and excisions were harvested, fixed in 10% neutral-buffered formalin, and subsequently dehydrated in serial ethanol and embedded in paraffin for microtome sectioning. Sectioned samples (5 μ m-thick) were stained with hematoxylin and eosin (H&E) according to standard protocols, and histological images and measurements were captured using a Nikon Eclipse 50i (Nikon

Instruments, Melville, NY). Using these image captures, the scar elevation index (SEI) was determined by adding the area within the ventral dermis wound margins and hypertrophic neodermis and dividing by the area of the ventral dermis (Figure 1). RNA isolation was conducted from whole tissue scars for real-time PCR analyses with primers specific to rabbit type I collagen (*COL1A1*), alpha-smooth muscle actin (*ACTA2*), cyclooxygenase-2 (*PTGS2*) and *GAPDH* as a reference control. To test for significant differences between groups, mean values were compared with two-tailed unpaired student's t-tests on data from wound height caliper measurements, histological SEI values and qPCR expression markers. Graphical data are expressed as the mean \pm standard deviation, n = number of wounds. Differences were considered significant when *P* values were < 0.05 (*), <0.01 (**), <0.001 (***), <0.0001(****) and not significant (ns) when *P* values were > 0.05. Statistical analysis was performed with GraphPad Prism 9 (Graphpad, San Diego, CA).

RESULTS

Scar Comparison of Burn and Excisional Wounds

Scar comparison among paired burn and excisional-induced injuries revealed that burn wounds produce scars with greater height when compared to equally sized excision-induced scars measured at the same time point. Among the four timepoints measured with calipers, POD 12 showed the greatest difference (*P* <0.0001), with the final two days, POD 26 and 28, showing consecutively thicker scars (*P* <0.05 for both days) among burn-induced wounds (Figure 2, A and B). Moreover, the SEI calculated

from these scar tissues also complemented the caliper measurements, revealing that burn-induced scars are significantly more elevated ($P < 0.05$) than excisional-induced scars (Figure 2C). These quantitative findings were also consistent with gross observations, whereby the appearance of burn-induced scars was visibly more exacerbated than excisional-induced scars (Figure 3).

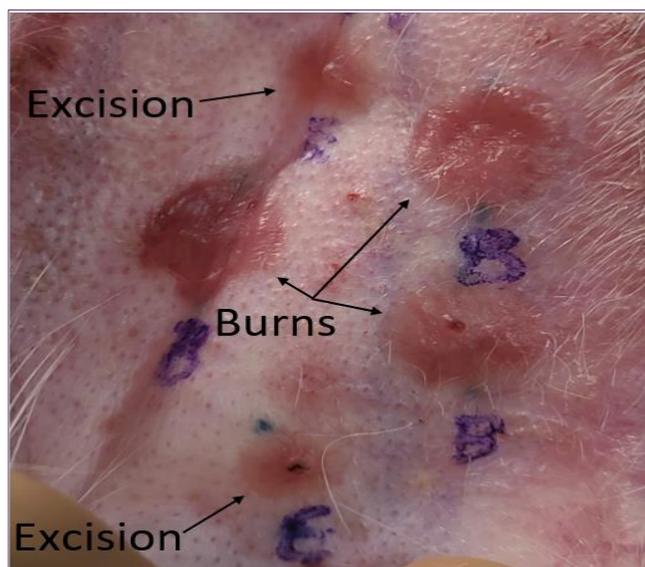


Figure 3. Gross image of a rabbit ear with two excisional-induced scars (contain the letter “E”) and three hypertrophic burn-induced scars (contain the letter “B”) on POD 29.

Pharmacological Application to Burn-Induced Scars

Burn-induced wounds under the amiloride group of animals showed that scars treated with topical amiloride were characterized by a significantly reduced SEI ($P < 0.05$) compared to the contralateral scars allocated to vehicle control (Figure 4, A and B); with representative histological images of both treatment and vehicle control supportive of this finding (Figure 5, A and B). Gene expression analysis for *PTGS2*, *ACTA2* and *COL1A1* was performed by

qRT-PCR on whole-scar tissues that underwent amiloride and vehicle application, but significant gene expression differences could not be determined in this group (Figure 6, A-C).

Under the group of animals allocated to test celecoxib and vehicle control, only a small decline in the SEI emerged under scars treated with celecoxib, with the fall being statistically insignificant (Figure 4C). The histological images also complemented the SEI results, showing only a minor visibly evident reduction. (Figure 5, C and D). Given the negative results with celecoxib, tissue gene expression analysis was not performed in this group.

DISCUSSION

After pairing both burn and excisional wounds onto individual ears, we found our data supportive of previously reported results. Encouraging as this was, even more worthwhile was that there was a selectable superior injury that reliably materialized reproducible scars: one with worsened, more exacerbated scars, therefore making it remarkably suited for a scar reduction study.

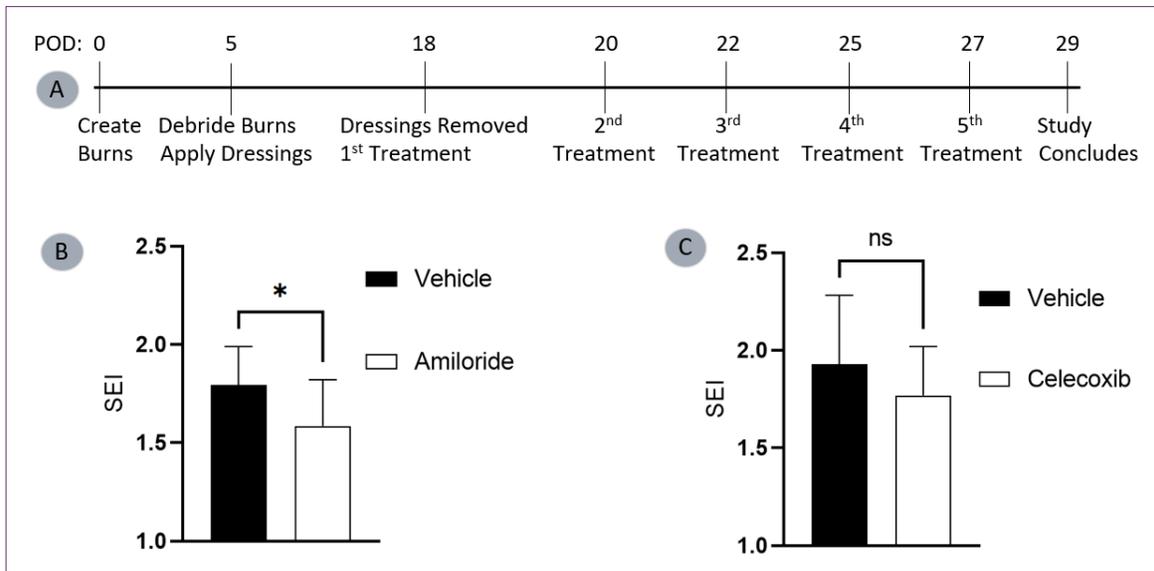


Figure 4. Timeline and data from study pharmacologically treating burn-induced scars. **A)** Timeline of study undergoing pharmacological treatment of burn-induced scars, with top numerical values indicating post-operation day (POD) and bottom content relating to events and treatments. **B)** Bar graph illustrating a significantly reduced SEI in burn scars treated with amiloride compared to vehicle control, and **C)** no significant difference in SEI in scars treated with celecoxib when compared to vehicle control scars. Bar graphs represent mean \pm standard deviation.

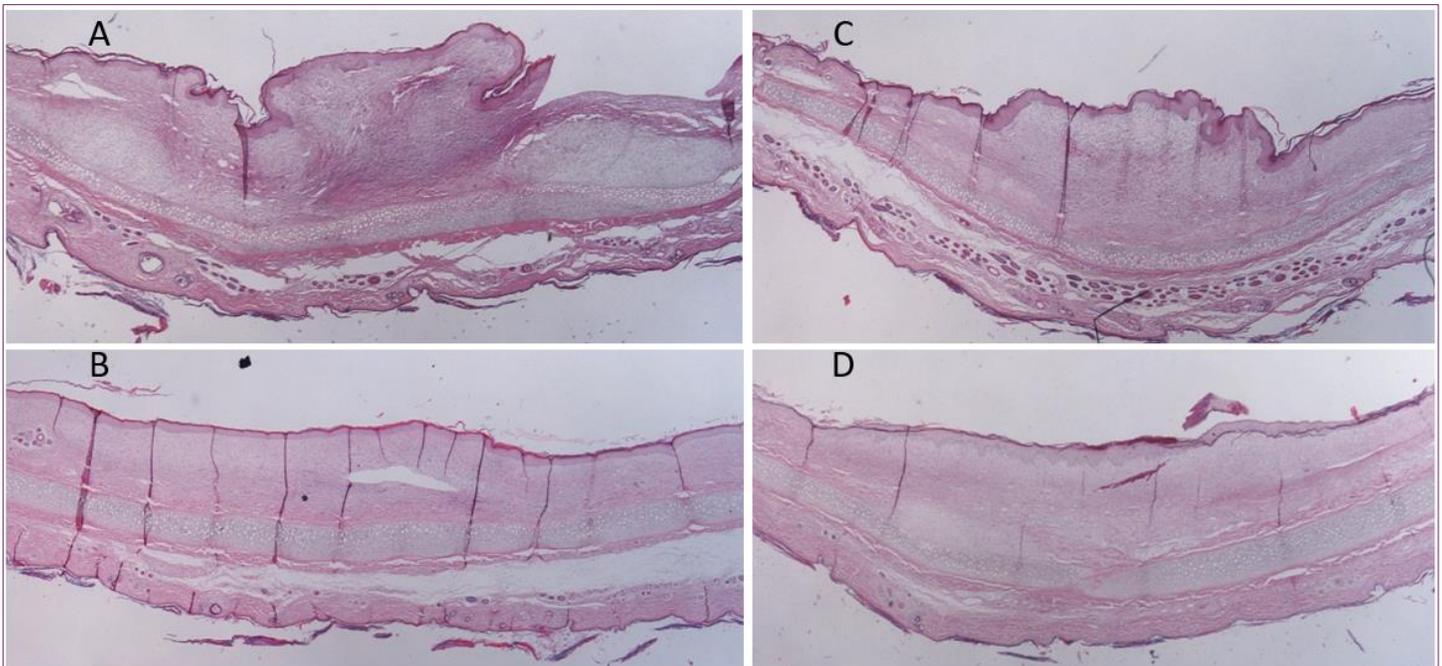


Figure 5. Histological images of scars harvested on post-operation day 29. **A)** Unilateral scar that underwent vehicle control application, and **B)** its corresponding contralateral amiloride treated scar, showing an enlarged epidermis yet overall reduced scar. **C)** Unilateral scar that underwent vehicle control application, and **D)** its corresponding contralateral celecoxib treated scar, also showing an enlarged epidermis with an overall minor scar reduction.

Since we now had a consistent model that demonstrated hypertrophic scars with burns, we opted to test whether such scars could be reduced with amiloride or celecoxib. Upon conclusion, celecoxib-treated scars were insignificantly reduced. However, the data did reveal a significant SEI reduction with amiloride, yet complementing this finding with a downregulated expression of COX-2 (*PTGS2*) — a finding that holds importance in previous wound studies including those analyzing scars^{12,17,18}, and a mechanistic step by which in vitro work revealed collagen hinderance¹²— was not possible in this study. Similarly, myofibroblast cells — which correlate with alpha smooth muscle actin (*ACTA2*) expression and are well-known contributors of collagen deposition — were also insignificantly present in these tissues. And lastly, type I collagen (*COL1A1*), a protein found in abundance within mature scars, was also not differentially expressed between amiloride-treated and vehicle control scars.

Given the unsupportive gene expression data, we cannot comment with any certainty on amiloride's mechanistic scar reduction pathway. We can only speculate that antagonizing ENaC likely cascades a unique scar reduction mechanism, and that such mechanism may be at least partially independent of COX-2. Clearly, in vivo burn-scar production, and in our case reduction, is complex. So much so, that other researchers who have analyzed bulk gene transcription in hypertrophic burn scars have reported a large number of genes that are either down or up regulated,¹⁹ while other studies have reported distinctive, yet complex, inflammatory responses in scar tissues.^{8,10,20}

Still, regardless of their complexities, such injuries warrant further studies. Although the

wound healing phases along with key cellular and protein signaling are well-understood in some injury modalities,²¹ the full mechanisms by which pathological scars arise is relatively obscure. While tension and infection are well-known hypertrophic scar contributors, keloids and contractures remain the most elusive because they are relatively human traits (particularly keloids).²² For now, the best treatments for some type of scars involves an invasive approach, such as cryosurgery or carbon dioxide laser ablation.^{23,24} Future studies however, should aim to couple both invasive and pharmacological approaches to assess the best outcome, and to better understand subsets of scar-forming wounds that preferentially respond to various treatment modalities, including biomarkers that may be used to distinguish tested subsets.

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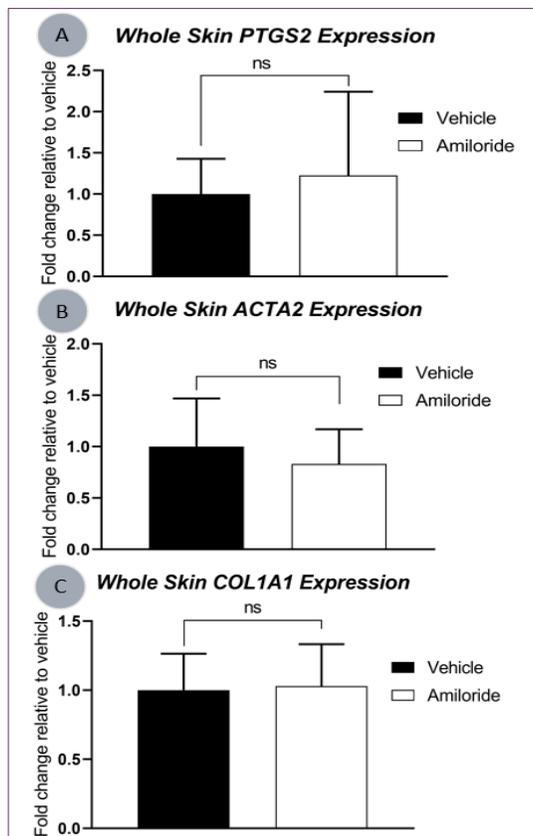


Figure 6. qRT-PCR gene expression analysis comparing scars that underwent amiloride and vehicle control application. **A)** No significance in PTGS2 (cyclooxygenase-2) quantification relative to GAPDH in whole skin scar samples between vehicle and amiloride-treated wounds. **B)** No significance in ACTA2 (α -smooth muscle actin) quantification in whole skin scar samples between vehicle and amiloride-treated wounds. **C)** No significance in COL1A1 (type I collagen) quantification in whole skin scar samples between vehicle and amiloride-treated wounds. Bar graphs represent mean \pm standard deviation.

Conflict of Interest Disclosures: None

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