Article:
Tiganescu, A orcid.org/0000-0003-3688-2204, Hupe, M, Uchida, Y et al. (3 more authors) (2018) Topical 11β-Hydroxysteroid Dehydrogenase Type 1 Inhibition Corrects Cutaneous Features of Systemic Glucocorticoid Excess in Female Mice. Endocrinology, 159 (1). pp. 547-556. ISSN 0013-7227

https://doi.org/10.1210/en.2017-00607

Topical 11β-hydroxysteroid dehydrogenase type 1 inhibition corrects cutaneous features of systemic glucocorticoid excess in female mice

Ana Tiganescu[1,2], Melanie Hupe[2], Yoshikazu Uchida[2], Theadora Mauro[2], Peter M Elias[2], Walter M Holleran[2]

[1]University of Leeds, Leeds Institute of Rheumatic and Musculoskeletal Medicine, Leeds, UK

[2]University of California San Francisco, Department of Dermatology, San Francisco, CA, USA (institute where the work was conducted)

Short title: Skin 11β-HSD1 blockade corrects steroid effects

Keywords: Glucocorticoid
Skin
Wound healing
11 beta hydroxysteroid dehydrogenase type 1
Epidermal barrier
Trans-epidermal water loss

Corresponding author: Ana Tiganescu
Leeds Institute of Rheumatic and Musculoskeletal Medicine
Level 8, Wellcome Trust Brenner Building
St James's University Hospital, Leeds, LS9 7TF
0113 34 38336
a.tiganescu@leeds.ac.uk

Abstract

Glucocorticoid (GC) excess drives multiple cutaneous adverse effects including skin thinning and poor wound healing. The ubiquitously-expressed enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) activates mouse corticosterone from 11-dehydrocorticosterone (and human cortisol from cortisone). We previously demonstrated elevated 11β-HSD1 activity during mouse wound healing but the interplay between cutaneous 11β-HSD1 and systemic GC excess is unexplored. Here, we examined effects of 11β-HSD1 inhibition by carbenoxolone (CBX) in mice treated with corticosterone (CORT) or vehicle for 6 weeks. Mice were treated bi-daily with topical CBX or vehicle 7 days before wounding and during wound healing. CORT mice displayed skin thinning and impaired wound healing but also increased epidermal integrity. 11β-HSD1 activity was elevated in unwounded CORT skin and was inhibited by CBX. CORT mice treated with CBX displayed 51%, 59% and 100% normalization of wound healing, epidermal thickness and epidermal integrity, respectively. Gene expression studies revealed normalization of interleukin-6, keratinocyte growth factor, collagen-1, collagen-3, matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-4 by CBX during wound healing. Importantly, pro-inflammatory cytokine expression and resolution of inflammation was unaffected by 11β-HSD1 inhibition. CBX did not regulate skin function or wound healing in the absence of CORT. Our findings demonstrate that 11β-HSD1 inhibition can limit the cutaneous effects of GC excess which may improve the safety profile of systemic steroids and the prognosis of chronic wounds.

Précis

Topical 11β-hydroxysteroid dehydrogenase type 1 inhibition normalizes steroid-induced skin thinning, epidermal integrity, poor healing and gene dysregulation despite ongoing systemic steroid exposure.

Introduction

Systemic glucocorticoid (GC) therapy remains mainstream treatment for many inflammatory diseases e.g. lupus, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, multiple sclerosis, polymyalgia rheumatica and giant cell arteritis. Recent estimates indicate that 1.2% of the US population (>2.5 million people) were prescribed oral GC between 1999 and 2008, 28% of whom reported use of >5 years (1). Despite anti-inflammatory benefits, chronic GC excess drives adverse side-effects including weight gain, hypertension, hyperglycemia, osteoporosis, muscle weakness, glaucoma and depression (2). GC therapy also substantially increases healthcare costs. In patients with systemic lupus erythematosus, GC use was associated with a three-fold increase in annual expenditure (3). In a study of patients with severe asthma, high dose GC use was associated with annual increments of $5479 relative to low GC exposure (4). Moreover, the incidence and impact of GC-related side-effects is under-reported (5) and further compounded by GC over-prescription (6).

In skin, GC excess causes acne, thinning, dryness, atrophic striae, telangiectasia, bruising, impaired wound healing (WH) and increased infection risk (2,7). Skin bruising and thinning have also been reported with low-dose (<7.5 mg/day) GC therapy (8). In a survey of asthma physicians, cutaneous manifestations were the second most frequently reported complication of inhaled GC (9). Despite recent advances in GC mimetics and GC-independent immune suppressants, the “holy grail” of dissociating GC anti-inflammatory benefits from side-effects remains elusive (10).

It is now established that human skin has corticosteroidogenic capability that is regulated in a complex way (11,12), although this function is lacking in mouse skin (13). Peripheral GC availability is also regulated by 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which converts biologically inert cortisone and 11-dehydrocorticosterone to cortisol and corticosterone in humans and mice, respectively. In intact tissues and cells, 11β-HSD1 functions predominantly as a reductase in an NADPH-dependent manner (14). We previously reported increased 11β-HSD1 activity during

mouse skin WH (13), in aged mouse and human skin (15,16) and accelerated WH in aged 11β-HSD1-null mice (16). However, the role of 11β-HSD1 in impaired WH is unknown.

We propose that 11β-HSD1 is a causative agent of cutaneous side-effects during systemic GC excess such as skin atrophy and impaired WH. Since human 11β-HSD1 inhibitors are readily available, such a demonstration could lead to rapid progress in ameliorating this major clinical problem. Here, we provide the first empirical evaluation of this causal link. We investigate effects of topical 11β-HSD1 inhibition by carbenoxolone (CBX) on skin function in mice treated with oral corticosterone and examine inflammatory, growth factor and extracellular matrix gene expression responses during WH. We demonstrate that 11β-HSD1 inhibition accelerates WH and reduces skin damage in steroid-treated mice without compromising the inflammatory response.

Materials and Methods

Declarations

Studies presented in this paper were approved by the Institutional Animal Care and Use Committee and San Francisco Veterans Affairs Medical Center Veterinary Medical Unit. Materials were obtained from Sigma-Aldrich unless otherwise stated.

Animal Studies

Female SKH1 hairless mice (8–10 weeks old) were obtained from Charles River Labs (Wilmington, MA, USA) and acclimatized for 2 weeks. Mice were group housed, supplied with a basal chow diet *ad libitum* and exposed to a standard 12h light/dark cycle. Drinking water was supplemented with corticosterone (100µg/ml) or vehicle (0.66% ethanol) for 6 weeks (replaced twice weekly). 7 days prior to wounding, 100µg/cm^2 topical CBX (20µl) or vehicle (70% propylene glycol, 30% ethanol) was massaged into 2cm^2 skin until fully absorbed. Treatments were repeated bi-daily for the remainder of the experiment and replaced with 10µl per wound post-wounding.

24h prior to wounding, epidermal barrier function was measured using a Tewameter 300 (Courage + Khazaka, Cologne, Germany) to evaluate trans-epidermal water loss (TEWL). Epidermal barrier recovery was determined at 2 and 4h following disruption by sequential D-Squame tape stripping to a TEWL level of >30g/h/m^2. Epidermal integrity was defined as the number of tape strips required to achieve barrier disruption.

WH was conducted as previously described (13). Briefly, mice anesthetized under 2% isoflurane were wounded on both dorsal flanks by 5mm punch biopsy (Acuderm, Fort Lauderdale, FL, USA). Wounds received 20µl bupivacaine 0.25% (analgesic) and monitored daily.

At 2, 4 and 9 days post-wounding, mice were culled by cervical dislocation, wounds were digitally imaged and excised for 11β-HSD1 activity assay, RNA extraction or histology. Wound areas were determined by ImageJ (NIH, Bethesda, MD, USA).

Experiments were also replicated in control mice (untreated drinking water) treated with vehicle or CBX.

11β-HSD1 activity assay

Radioactive conversion of tritiated 11β-HSD1 substrate is the gold standard measure of 11β-HSD1 activity as tissue steroid extraction efficiencies are variable and less sensitive.

Freshly isolated skin (20-40mg) was incubated immediately in 1ml high glucose and pyruvate DMEM with 100nM 11-dehydrocorticosterone (Steraloids, Newport, RI, USA) and ~1500cpm [3H] 11-dehydrocorticosterone, generated in-house as previously described (13). Samples were incubated at 37°C for 13h. Subsequently, tissues were weighed and steroids extracted and separated by TLC in 186ml chloroform and 14ml ethanol for 90min (co-migrated with 10mM 11-dehydrocorticosterone / corticosterone standards). Plate regions identified under UV were excised and percentage conversion of 11-dehydrocorticosterone to corticosterone was determined following liquid scintillation.

Quantitative PCR

Fresh skin tissue (20-40mg) was snap-frozen and stored at -80°C. Samples were homogenized in 1ml Trizol and RNA extracted using a PureLink RNA Mini Kit (Life Technologies, Grand Island, NY, USA). cDNA was generated from 1.2µg RNA using a Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA). qPCR was conducted in 10µl reactions using a SensiFAST Probe Kit (Bioline) with 900nM TaqMan primers (with 250nM FAM probe) or 50nM 18S rRNA primers (with 200nM VIC probe) mix (Life Technologies) and 10ng cDNA. Duplicate PCRs were performed and analyzed, as previously described (13).

Serum corticosterone

For serum corticosterone, 400µl of blood was obtained by terminal cardiac puncture at 11 AM and incubated for 1h at 5°C before centrifuging at 1000 x RCF for 5min. Costicosterone levels were determined using a corticosterone EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).

Histology

Freshly isolated samples were stored in Formalde-Fresh (Fisher Scientific, Pittsburgh, PA, USA) and processed into paraffin blocks. 5µm sections were de-waxed, rehydrated, and stained with hematoxylin and eosin (Leica, Buffalo Grove, IL, USA and Thermo Scientific, Kalamazoo, MI, USA). Sections were examined with a Zeiss microscope (Jena, Germany) and digital images captured with AxioVision software (Carl Zeiss Vision, Munich, Germany).

For epidermal thickness, the average of 4 measurements was taken per image and dermal area was quantified using ImageJ. For quantification of epidermal cellularity (>90% of which is composed of keratinocytes), the total number of epidermal nuclei were counted per field of view and normalized to epidermal length (to account for rete ridge undulation).

Proliferation was evaluated by incubating rehydrated sections with biotinylated primary antibody against Proliferating cell nuclear antigen (PCNA), CalTag Laboratories, Burlingame, CA, USA) overnight at 4°C. After 3 x TBS washes, staining was detected with ABC-peroxidase Kit (Vector Lab, Burlingame, CA, USA) and quantified using ImageJ.

Statistical Analysis

Following confirmation of data displaying a normal distribution, significance levels were determined by Student's t-test, one-way or two-way ANOVA using GraphPad Prism (La Jolla, CA, USA) on untransformed data. For two-way ANOVA, post hoc testing included analysis of differences between time-points in each treatment group and differences between treatment groups at each time-point with p-values adjusted for multiple testing as detailed in Figure legends. Variation is displayed as standard error based on at least three biological replicates.

Results

Cutaneous 11β-HSD1 expression and activity increase during wound healing and systemic glucocorticoid excess

In agreement with known suppressive effects of GC treatment on hypothalamic-pituitary-adrenal axis signaling, systemic GC treatment (CORT) reduced circulating corticosterone levels by 70% compared to vehicle mice (Fig. 1A) with body weight unaffected between groups (data not shown).

The effect of GC excess on 11β-HSD1 expression and activity during mouse skin WH were previously unexplored. At baseline, 11β-HSD1 mRNA was 10-fold higher in CORT skin compared to vehicle (Fig. 1B). Hexose-6-phosphate dehydrogenase (H6PDH), which supplies 11β-HSD1 co-factor, showed a similar increase in expression at baseline (Fig. 1C). Correspondingly, 11β-HSD1 activity was also 42% greater in unwounded CORT skin compared to vehicle (Fig. 1D).

In vehicle-treated mice, 11β-HSD1 mRNA and activity increased by 43-fold and 2.6-fold at day 2 and by 17-fold and 1.7-fold at day 4 post-wounding, respectively (Fig. 1B, 1D). H6PDH mRNA was also increased by 7-fold at days 2 and 4 post-wounding (Fig. 1C). CORT further increased 11β-HSD1 mRNA by 11-fold and 20-fold (independently of CBX) at days 4 and 9 post-wounding, respectively (Fig. 1B). H6PDH mRNA was also further upregulated by 5-fold at day 4 and 9-fold (independently of CBX) at day 9 post-wounding in CORT mice (Fig. 1C). However, 11β-HSD1 activity was not affected by CORT treatment during WH (Fig. 1D).

In CORT mice, CBX treatment inhibited 11β-HSD1 activity by 61% in unwounded skin and by 91% and 55% at days 2 and 4 post-wounding, respectively (Fig. 1D). In control mice (untreated drinking water), CBX inhibited 11β-HSD1 activity by 30% in unwounded skin and by 93%, 94% and 73% at days 2, 4 and 9 post-wounding (Supplemental Fig. 1).

These findings indicate that GC excess induces cutaneous 11β-HSD1 expression and activity in unwounded skin which is inhibited effectively by CBX treatment.

11β-HSD1 inhibition limits glucocorticoid-mediated skin thinning

GC cause skin thinning and dermal atrophy. However, the effect of 11β-HSD1 inhibition on epidermal thinning and proliferation following GC exposure was previously unreported. Epidermal thickness was reduced by 36% in CORT mice compared to vehicle and was restored by 59% with CBX (Fig. 2A, 2B). A 27% CORT-mediated reduction in epidermal cellularity was also prevented by CBX (Fig. 2A, 2C) with a similar trend in PCNA staining (Supplemental Fig. 2). Dermal area decreased by 21% with CORT treatment compared to vehicle and this was also prevented by CBX (Fig. 2D). No difference in epidermal thickness or epidermal cellularity was observed in control mice treated with CBX (Supplemental Fig. 3).

These results suggest that 11β-HSD1 inhibition can limit GC-mediated skin thinning despite sustained exposure to systemic GC excess.

Impact of glucocorticoid excess and 11β-HSD1 inhibition on epidermal barrier function

A functional epidermal barrier is indispensable for protection against dehydration, exposure to irritants and invasion by microorganisms. The regulation of epidermal barrier function, integrity and recovery by corticosterone excess or 11β-HSD1 inhibition has not been investigated.

Trans-epidermal water loss (TEWL) is the gold standard measure of epidermal barrier function. Baseline TEWL was largely unaffected by CORT or CBX (Fig. 3A). Interestingly, the number of tape strips required to disrupt the barrier (indicative of barrier integrity) was 50% greater in CORT mice and this was prevented by CBX treatment (Fig. 3B). We observed a modest increase in barrier recovery in the CORT/CBX group 2 hours after barrier disruption but no difference was observed between CORT and CORT/CBX or between any of the groups 4 hours after disruption (Fig. 3C). No statistically significant effect of CBX on barrier function (baseline TEWL), barrier integrity or barrier recovery was observed in control mice (Supplemental Fig. 4).

These findings suggest that 11β-HSD1 mediates increased epidermal integrity during systemic GC excess.

11β-HSD1 inhibition improves skin wound healing during glucocorticoid excess

GC excess drives impaired WH and chronic wound management remains an unmet clinical need in diabetic and elderly patients. However, the regulation of impaired WH during GC excess by 11β-HSD1 is unknown. Systemic GC excess induced a striking WH delay which was improved by CBX treatment (Fig. 4A).

Initially, WH progressed at similar rates between all groups with a 36%, 39% and 33% reduction in wound area at day 4 compared to day 2 in vehicle, CORT and CORT/CBX mice, respectively (Fig. 4B). No change in wound area was observed between days 4 and 6 for either group. Although healing continued from day 6 for vehicle and CORT/CBX mice, no further healing was observed for CORT/VEH mice. By day 9, wound areas were 94% and 48% healed compared to day 4 in vehicle and CORT/CBX mice, respectively (Fig. 4B).

H+E staining of day 9 wounds revealed more advanced granulation tissue formation, improved structural organization and extracellular matrix deposition in CORT/CBX compared to CORT mice (Fig. 4C). CBX treatment did not affect WH in control mice (Supplemental Fig. 5).

In summary, 11β-HSD1 inhibition improved mouse skin WH despite sustained systemic GC exposure.

Effect of systemic glucocorticoid excess and 11β-HSD1 inhibition on cytokine, extracellular matrix and growth factor gene expression during wound healing

To further investigate the mechanisms underlying improved WH with 11β-HSD1 inhibition, we analyzed changes in cytokine, extracellular matrix and growth factor gene expression.

Pro-inflammatory cytokine interleukin (IL)-1β mRNA increased at days 2, 4 and 9 post-wounding by 750-fold, 587-fold and 90-fold respectively, independently of CORT or CBX treatment (Fig. 5A). A similar expression profile was seen for tumor necrosis factor alpha (TNFα) mRNA (Fig. 5B).

The immunomodulatory cytokine IL-6 regulates a broad range of biological functions including antibody-secreting B-cell differentiation and generation of Th17 cells. IL-6 mRNA expression was undetectable in the majority of unwounded skin samples but increased at day 2 and day 4 before declining at day 9 post-wounding in vehicle mice (Fig. 5C). IL-6 displayed a trend towards higher expression at day 2 post-wounding in CORT compared to vehicle mice which was prevented by CBX (Fig. 5C). IL-6 mRNA was also 14-fold greater in CORT mice at day 9 post-wounding compared to vehicle mice (Fig. 5C).

Keratinocyte growth factor (KGF) is produced during WH and stimulates wound re-epithelialization. KGF mRNA expression peaked at day 4 with a 9.9-fold and a 3.5-fold induction in vehicle and CORT/CBX mice compared to unwounded skin, respectively (Fig. 5D). No induction in KGF was seen at day 4 in CORT mice. At day 9 post-wounding, KGF was 17-fold higher in CORT compared to vehicle mice and this was reversed by CBX (Fig. 5D).

Type I and III collagen constitute >70% of the dermis by dry weight. Collagen type I alpha 1 (COL-1α1) and collagen type III alpha 1 (COL-3α1) mRNA expression increased 15-fold at day 4 post-wounding in vehicle mice compared to unwounded skin (Fig. 5E, 5F). This was delayed in CORT mice, which displayed a 3.5-fold and 14-fold induction of COL-1α1 and COL-3α1 at day 9 post-wounding compared to vehicle controls both reversed by CBX (Fig. 5E, 5F).

Extracellular matrix turnover is regulated by collagen-degrading matrix metalloproteinases (MMP) and their inhibitors, tissue inhibitor of matrix metalloproteinases (TIMP). MMP-9 mRNA in vehicle mice increased 7.7-fold and 9.5-fold at day 2 and day 4 post-wounding, respectively, before returning to baseline levels by day 9 (Fig. 5G). However, in CORT (but not CORT/CBX) mice, MMP-9

expression at day 9 remained elevated at 7.2-fold higher than vehicle mice (Fig. 5G). TIMP-1 displayed a similar mRNA profile, with 20-fold and 33-fold higher expression at day 2 and day 4 post-wounding respectively, in vehicle mice compared to unwounded skin (Fig. 5H). At day 9 post-wounding, MMP-9 mRNA was also 10-fold greater in CORT (but not CORT/CBX) compared to vehicle mice (Fig. 5H).

These findings oppose reports of increased inflammatory cytokine expression following 11β-HSD1 blockade in other models. Improved WH appears to be mediated, in part, by restoration of KGF signaling and appropriate collagen, MMP and TIMP expression.

Discussion

Although GC have been used for over 65 years for their anti-inflammatory properties (17), adverse side-effects limit their use to low-dose, short-term intervention (2). GC-mediated skin atrophy, bruising, impaired WH and increased infection risk are of concern to both patients and physicians alike (18). In a survey of patients taking GC for >60 days, skin bruising or thinning was the second most prevalent self-reported side-effect (8). We have demonstrated for the first time that topical 11β-HSD1 inhibition can limit these cutaneous side-effects. This key insight opens a wide range of avenues for 11β-HSD1 inhibitors as adjunct therapies to safely increase steroid doses or treatment duration.

Morgan *et al*. recently demonstrated that global 11β-HSD1-null mice were protected from the systemic side-effects of oral corticosterone (19). However, this study did not evaluate inflammation, which others report to be exacerbated by 11β-HSD1 inhibition (20). Here, we evaluated the ability of 11β-HSD1 inhibition to *reverse* cutaneous effects of GC excess.

Reduced morning serum corticosterone levels in CORT mice are consistent with the reported adrenal suppression at this sampling time (21) and total body weight was unaffected by GC treatment as previously described (19). The forward-feedback induction of 11β-HSD1 mRNA and activity by GC in

unwounded skin is also in agreement with our previous observations in human skin (15) and may exacerbate the 11β-HSD1-mediated effects observed in unwounded CORT mouse skin.

Our results demonstrate that 11β-HSD1 inhibition prevents GC-mediated reductions in epidermal cellularity and dermal area, despite sustained exposure to systemic GC excess. Interestingly, we observed that CBX also reversed a GC-mediated induction in epidermal integrity. This is supported by studies demonstrating that exogenous GC treatment promote epidermal keratinocyte differentiation and increase corneocyte numbers (22), reflecting a greater degree of disruption required for epidermal barrier perturbation. Conversely, endogenous GC excess or topical GC treatment cause GC-mediated decreases in epidermal integrity indicating that model-specific differences should also be taken into consideration (23-26).

Baseline TEWL (epidermal barrier function) was unaffected by CORT treatment in agreement with previous reports (23-26). In contrast to our findings, other studies reported impaired barrier recovery following endogenous GC excess and GC treatment, although this was associated with impaired barrier integrity (23,25,26). In our model, integrity and barrier recovery were both stimulated by systemic GC excess. This may be due to differences in GC potency, formulation, treatment duration, administration, strain, gender, age, diet or environment. Whilst integrity appeared to be 11β-HSD1-mediated, the modest induction in barrier recovery was not reversed by CBX. This may be due to regulation by other hypothalamic-pituitary-adrenal axis pathways such as β2-adrenergic receptor signaling (27).

Between days 0 to 4, wound closure was similar between all groups, suggesting that the initial contractile phase of WH (28) was unaffected. We observed termination of WH kinetics from day 4 post-wounding in CORT mice, supported by previous reports of GC-mediated impairment of keratinocyte proliferation, migration and increased epidermal differentiation (29,30). Strikingly, our results demonstrate that CBX treatment improved WH in CORT mice. The lack of complete WH rescue by CBX may be due to exposure to serum corticosterone before cellular

compartmentalization is fully restored. Others have also demonstrated benefits of 11β-HSD1 blockade on cutaneous WH in animal models of metabolic disease and endogenous GC excess (31,32), however, 11β-HSD1 inhibition, inflammatory effects and mechanistic insights were lacking in these studies.

Our findings indicate that the cutaneous side-effects of GC excess are mediated through increased 11β-HSD1 substrate availability as 11β-HSD1 activity was not further increased by CORT treatment and CBX treatment did not affect skin thickness, epidermal integrity or WH in control mice not exposed to systemic GC excess. This suggests that 11-dehydrocorticosterone availability is insufficient in young, healthy mice to regulate skin function.

Recent reports have raised concerns over increased inflammation as consequence of 11β-HSD1 blockade (33-36). We found no evidence of this our model; there was no exacerbation of IL-1β, TNF-α or IL-6 mRNA expression during early WH and no delay in resolution of these pro-inflammatory cytokines in CBX mice. On the contrary, IL-6 expression increased during CORT mouse WH. This is supported by reports of pro-inflammatory GC actions (37,38), indicating greater regulatory complexity than previously thought (39-41).

KGF is a key factor that promotes wound re-epithelialization and is indispensable to the WH process (30,42,43). We found a lack of induction of KGF at day 4 post-wounding in CORT mice (coinciding with the cessation of wound closure) which was restored by CBX. The induction of collagen type I and III mRNA during the granulation phase of WH were attenuated in CORT mice, consistent with previous reports (30,44) and were also normalized by CBX, further supported by our histological findings.

MMP-9 and TIMP-1 mRNA expression were elevated at day 9 post-wounding in CORT mice and were normalized by CBX treatment. Although the underlying mechanisms remain poorly understood,

elevated MMP-9 and TIMP-1 mRNA are associated with slower healing in mouse wounds (45) and MMP-9 is elevated in diabetic foot ulcers (46).

GC are able to activate the mineralocorticoid receptor (MR) with strong affinity and recent work by Boix *et al.* elegantly demonstrated that MR epidermal knockout mice were resistant to GC-induced epidermal thinning (47). Differentiating between glucocorticoid receptor and MR-mediated effects was beyond the scope of the current study, but is an area of ongoing interest.

CBX has been used extensively in human and mammalian models. Administered systemically, it also inhibits 11β-HSD2 (which conducts the opposing reaction to 11β-HSD1) resulting in renal mineralocorticoid excess due to GC-mediated MR activation (48). However, 11β-HSD2 is not expressed in unwounded mouse skin or during WH (13) and topical administration limits the risk of systemic exposure. CBX is also able to block gap junctions but at several orders of magnitude less potently than 11β-HSD1 (48). Further, we observed no difference between CBX and vehicle mice not exposed to CORT, suggesting our findings are most likely due to cutaneous 11β-HSD1 inhibition.

In summary, we report the ability of topical 11β-HSD1 inhibition to limit cutaneous side-effects of systemic GC excess including skin thinning and delayed WH. Our findings may improve the safety profile of systemic steroids and the prognosis of chronic wounds, particularly those associated with elevated circulating GC levels (49).

Acknowledgements

Author contributions are as follows; AT, YU and WMH designed the research study. AT performed the experiments, analyzed the data and prepared the manuscript. MH prepared tissue sections for histology. TM and PME contributed essential equipment. All authors evaluated and approved the final manuscript.

References

1. Overman RA, Yeh JY, Deal CL. Prevalence of oral glucocorticoid usage in the United States: a general population perspective. *Arthritis care & research.* 2013;65(2):294-298.
2. Harris E, Tiganescu A, Tubeuf S, Mackie SL. The prediction and monitoring of toxicity associated with long-term systemic glucocorticoid therapy. *Current rheumatology reports.* 2015;17(6):513.
3. Chen SY, Choi CB, Li Q, Yeh WS, Lee YC, Kao AH, Liang MH. Glucocorticoid Use in Patients With Systemic Lupus Erythematosus: Association Between Dose and Health Care Utilization and Costs. *Arthritis care & research.* 2015;67(8):1086-1094.
4. Lefebvre P, Duh MS, Lafeuille MH, Gozalo L, Desai U, Robitaille MN, Albers F, Yancey S, Ortega H, Forshag M, Lin X, Dalal AA. Acute and chronic systemic corticosteroid-related complications in patients with severe asthma. *The Journal of allergy and clinical immunology.* 2015;136(6):1488-1495.
5. Hyland ME, Whalley B, Jones RC, Masoli M. A qualitative study of the impact of severe asthma and its treatment showing that treatment burden is neglected in existing asthma assessment scales. *Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation.* 2015;24(3):631-639.
6. White P, Thornton H, Pinnock H, Georgopoulou S, Booth HP. Overtreatment of COPD with inhaled corticosteroids--implications for safety and costs: cross-sectional observational study. *PloS one.* 2013;8(10):e75221.
7. Schoepe S, Schacke H, May E, Asadullah K. Glucocorticoid therapy-induced skin atrophy. *Experimental dermatology.* 2006;15(6):406-420.
8. Curtis JR, Westfall AO, Allison J, Bijlsma JW, Freeman A, George V, Kovac SH, Spettell CM, Saag KG. Population-based assessment of adverse events associated with long-term glucocorticoid use. *Arthritis and rheumatism.* 2006;55(3):420-426.
9. Storms WW, Theen C. Clinical adverse effects of inhaled corticosteroids: results of a questionnaire survey of asthma specialists. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 1998;80(5):391-394.
10. Jones SA, Morand EF. Glucocorticoids in 2015: New answers to old problems. *Nature reviews Rheumatology.* 2016;12(2):73-74.
11. Slominski A, Zbytek B, Nikolakis G, Manna PR, Skobowiat C, Zmijewski M, Li W, Janjetovic Z, Postlethwaite A, Zouboulis CC, Tuckey RC. Steroidogenesis in the skin: implications for local immune functions. *The Journal of steroid biochemistry and molecular biology.* 2013;137:107-123.
12. Slominski AT, Manna PR, Tuckey RC. On the role of skin in the regulation of local and systemic steroidogenic activities. *Steroids.* 2015;103:72-88.
13. Tiganescu A, Hupe M, Uchida Y, Mauro T, Elias PM, Holleran WM. Increased glucocorticoid activation during mouse skin wound healing. *The Journal of endocrinology.* 2014;221(1):51-61.
14. Bujalska IJ, Draper N, Michailidou Z, Tomlinson JW, White PC, Chapman KE, Walker EA, Stewart PM. Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 beta-hydroxysteroid dehydrogenase type 1. *Journal of molecular endocrinology.* 2005;34(3):675-684.
15. Tiganescu A, Walker EA, Hardy RS, Mayes AE, Stewart PM. Localization, age- and site-dependent expression, and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in skin. *The Journal of investigative dermatology.* 2011;131(1):30-36.
16. Tiganescu A, Tahrani AA, Morgan SA, Otranto M, Desmouliere A, Abrahams L, Hassan-Smith Z, Walker EA, Rabbitt EH, Cooper MS, Amrein K, Lavery GG, Stewart PM. 11beta-

Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects. *The Journal of clinical investigation.* 2013;123(7):3051-3060.

17. Benedek TG. History of the development of corticosteroid therapy. *Clinical and experimental rheumatology.* 2011;29(5 Suppl 68):S-5-12.

18. van der Goes MC, Jacobs JW, Boers M, Andrews T, Blom-Bakkers MA, Buttgereit F, Caeyers N, Choy EH, Cutolo M, Da Silva JA, Guillevin L, Holland M, Kirwan JR, Rovensky J, Saag KG, Severijns G, Webber S, Westhovens R, Bijlsma JW. Patient and rheumatologist perspectives on glucocorticoids: an exercise to improve the implementation of the European League Against Rheumatism (EULAR) recommendations on the management of systemic glucocorticoid therapy in rheumatic diseases. *Annals of the rheumatic diseases.* 2010;69(6):1015-1021.

19. Morgan SA, McCabe EL, Gathercole LL, Hassan-Smith ZK, Larner DP, Bujalska IJ, Stewart PM, Tomlinson JW, Lavery GG. 11beta-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(24):E2482-2491.

20. Chapman KE, Coutinho AE, Zhang Z, Kipari T, Savill JS, Seckl JR. Changing glucocorticoid action: 11beta-hydroxysteroid dehydrogenase type 1 in acute and chronic inflammation. *The Journal of steroid biochemistry and molecular biology.* 2013;137:82-92.

21. Karatsoreos IN, Bhagat SM, Bowles NP, Weil ZM, Pfaff DW, McEwen BS. Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse. *Endocrinology.* 2010;151(5):2117-2127.

22. Laurence EB, Christophers E. Selective action of hydrocortisone on postmitotic epidermal cells in vivo. *The Journal of investigative dermatology.* 1976;66(4):222-229.

23. Choi EH, Brown BE, Crumrine D, Chang S, Man MQ, Elias PM, Feingold KR. Mechanisms by which psychologic stress alters cutaneous permeability barrier homeostasis and stratum corneum integrity. *The Journal of investigative dermatology.* 2005;124(3):587-595.

24. Kim HJ, Park HJ, Yun JN, Jeong SK, Ahn SK, Lee SH. Pseudoceramide-containing physiological lipid mixture reduces adverse effects of topical steroids. *Allergy, asthma & immunology research.* 2011;3(2):96-102.

25. Choi EH, Demerjian M, Crumrine D, Brown BE, Mauro T, Elias PM, Feingold KR. Glucocorticoid blockade reverses psychological stress-induced abnormalities in epidermal structure and function. *American journal of physiology Regulatory, integrative and comparative physiology.* 2006;291(6):R1657-1662.

26. Kao JS, Fluhr JW, Man MQ, Fowler AJ, Hachem JP, Crumrine D, Ahn SK, Brown BE, Elias PM, Feingold KR. Short-term glucocorticoid treatment compromises both permeability barrier homeostasis and stratum corneum integrity: inhibition of epidermal lipid synthesis accounts for functional abnormalities. *The Journal of investigative dermatology.* 2003;120(3):456-464.

27. Denda M, Fuziwara S, Inoue K. Beta2-adrenergic receptor antagonist accelerates skin barrier recovery and reduces epidermal hyperplasia induced by barrier disruption. *The Journal of investigative dermatology.* 2003;121(1):142-148.

28. Davidson JM. Animal models for wound repair. *Archives of dermatological research.* 1998;290 Suppl:S1-11.

29. Lee B, Vouthounis C, Stojadinovic O, Brem H, Im M, Tomic-Canic M. From an enhanceosome to a repressosome: molecular antagonism between glucocorticoids and EGF leads to inhibition of wound healing. *Journal of molecular biology.* 2005;345(5):1083-1097.

30. Sanchis A, Alba L, Latorre V, Sevilla LM, Perez P. Keratinocyte-targeted overexpression of the glucocorticoid receptor delays cutaneous wound healing. *PloS one.* 2012;7(1):e29701.

31. Terao M, Murota H, Kimura A, Kato A, Ishikawa A, Igawa K, Miyoshi E, Katayama I. 11beta-Hydroxysteroid dehydrogenase-1 is a novel regulator of skin homeostasis and a candidate target for promoting tissue repair. *PloS one.* 2011;6(9):e25039.

32. Youm JK, Park K, Uchida Y, Chan A, Mauro TM, Holleran WM, Elias PM. Local blockade of glucocorticoid activation reverses stress- and glucocorticoid-induced delays in cutaneous wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society.* 2013;21(5):715-722.

33. Coutinho AE, Kipari TM, Zhang Z, Esteves CL, Lucas CD, Gilmour JS, Webster SP, Walker BR, Hughes J, Savill JS, Seckl JR, Rossi AG, Chapman KE. 11beta-Hydroxysteroid Dehydrogenase Type 1 Is Expressed in Neutrophils and Restrains an Inflammatory Response in Male Mice. *Endocrinology.* 2016;157(7):2928-2936.

34. Coutinho AE, Gray M, Brownstein DG, Salter DM, Sawatzky DA, Clay S, Gilmour JS, Seckl JR, Savill JS, Chapman KE. 11beta-Hydroxysteroid dehydrogenase type 1, but not type 2, deficiency worsens acute inflammation and experimental arthritis in mice. *Endocrinology.* 2012;153(1):234-240.

35. McSweeney SJ, Hadoke PW, Kozak AM, Small GR, Khaled H, Walker BR, Gray GA. Improved heart function follows enhanced inflammatory cell recruitment and angiogenesis in 11betaHSD1-deficient mice post-MI. *Cardiovascular research.* 2010;88(1):159-167.

36. Chapman K, Holmes M, Seckl J. 11beta-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. *Physiological reviews.* 2013;93(3):1139-1206.

37. Cruz-Topete D, Cidlowski JA. One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids. *Neuroimmunomodulation.* 2015;22(1-2):20-32.

38. Busillo JM, Cidlowski JA. The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends in endocrinology and metabolism: TEM.* 2013;24(3):109-119.

39. Homma T, Kato A, Hashimoto N, Batchelor J, Yoshikawa M, Imai S, Wakiguchi H, Saito H, Matsumoto K. Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *American journal of respiratory cell and molecular biology.* 2004;31(4):463-469.

40. Busillo JM, Azzam KM, Cidlowski JA. Glucocorticoids sensitize the innate immune system through regulation of the NLRP3 inflammasome. *The Journal of biological chemistry.* 2011;286(44):38703-38713.

41. Ding Y, Gao ZG, Jacobson KA, Suffredini AF. Dexamethasone enhances ATP-induced inflammatory responses in endothelial cells. *J Pharmacol Exp Ther.* 2010;335(3):693-702.

42. Pyter LM, Yang L, da Rocha JM, Engeland CG. The effects of social isolation on wound healing mechanisms in female mice. *Physiology & behavior.* 2014;127:64-70.

43. Brauchle M, Fassler R, Werner S. Suppression of keratinocyte growth factor expression by glucocorticoids in vitro and during wound healing. *The Journal of investigative dermatology.* 1995;105(4):579-584.

44. Gallucci RM, Sugawara T, Yucesoy B, Berryann K, Simeonova PP, Matheson JM, Luster MI. Interleukin-6 treatment augments cutaneous wound healing in immunosuppressed mice. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research.* 2001;21(8):603-609.

45. El-Hamoly T, Hegedus C, Lakatos P, Kovacs K, Bai P, El-Ghazaly MA, El-Denshary ES, Szabo E, Virag L. Activation of poly(ADP-ribose) polymerase-1 delays wound healing by regulating keratinocyte migration and production of inflammatory mediators. *Molecular medicine.* 2014;20:363-371.

46. Liu Y, Min D, Bolton T, Nube V, Twigg SM, Yue DK, McLennan SV. Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. *Diabetes care.* 2009;32(1):117-119.

47. Boix J, Sevilla LM, Saez Z, Carceller E, Perez P. Epidermal Mineralocorticoid Receptor Plays Beneficial and Adverse Effects in Skin and Mediates Glucocorticoid Responses. *The Journal of investigative dermatology.* 2016;136(12):2417-2426.

48. Sandeep TC, Yau JL, MacLullich AM, Noble J, Deary IJ, Walker BR, Seckl JR. 11Beta-hydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(17):6734-6739.

49. Bitar MS. Glucocorticoid dynamics and impaired wound healing in diabetes mellitus. *The American journal of pathology.* 1998;152(2):547-554.

Figure Legends

Figure 1 Cutaneous 11β-HSD1 expression and activity increase during wound healing and systemic glucocorticoid excess

A) Serum corticosterone levels were downregulated in mice treated with oral corticosterone (CORT) compared to vehicle (VEH, n=3-6, Student's t-test), B) 11β-HSD1 and C) H6PD mRNA expression were upregulated by CORT in unwounded skin compared to vehicle mice (VEH). Increased expression post-wounding was exacerbated by CORT independently of 11β-HSD1 inhibitor (CBX) treatment (n=3-6, two-way ANOVA), D) 11β-HSD1 enzyme activity was elevated by CORT in unwounded skin and increased during healing independently of CORT. Activity was inhibited by CBX in unwounded skin and during healing (n=3-5, two-way ANOVA). Multiple comparisons for 1B-1D: * = vs. baseline (day 0) in each treatment group (Dunnet's test), # = vs. VEH/VEH at each time-point and † = vs. VEH/CORT at each time-point (Tukey's test). Significance: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 2 11β-HSD1 inhibition limits glucocorticoid-mediated skin thinning

A) Representative H+E staining showing decreased epidermal thickness and epidermal cellularity in CORT mice and improvement following 7 days CBX treatment (n=4, one-way ANOVA), B) Quantification of epidermal thickness (n=4), C) Quantification of epidermal cellularity (n=4, one-way ANOVA) and D) Quantification of dermal area (n=4, one-way ANOVA). Scale bar 50µm. Multiple comparisons for 2B-2D: # = vs. VEH/VEH and † = vs. VEH/CORT (Tukey's test). Significance: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 3 Impact of glucocorticoid excess and 11β-HSD1 inhibition on epidermal barrier function

A) Baseline trans-epidermal water loss (TEWL) in CORT mice and following 7 days CBX treatment (n=8-10, one-way ANOVA), B) Increased barrier integrity (number of tape strips required to disrupt barrier) in CORT mice and reversal by CBX co-treatment (n=8-10, one-way ANOVA) and C) Barrier recovery relative to baseline TEWL (n=7-8, two-way ANOVA). Multiple comparisons for 3A and 3B: # = vs. VEH/VEH and † = vs. VEH/CORT (Tukey's test) and for 3C: * = vs. 2 hour time-point in each treatment group (Sidak's test) and # = vs. VEH/VEH at each time-point. Significance: * = $p<0.05$, *** = $p<0.001$

Figure 4 11β-HSD1 inhibition improves skin wound healing during glucocorticoid excess

A) Representative wound appearance at day 9 (n=8), B) Quantification of wound areas showing cessation of healing from day 4 in CORT mice and improvement of healing by CBX treatment (n=8-24, two-way ANOVA), C) Representative day 9 H+E staining showing advanced granulation tissue (arrows) formation in mice co-treated with CBX compared to CORT alone (n=3). Scale bar A) 13mm, C) 50µm. *=vs. day 4. Multiple comparisons for 4B: * = vs. day 4 in each treatment group (Dunnet's test), # = vs. VEH/VEH at each time-point (Tukey's test). Significance: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 5 Effect of systemic glucocorticoid excess and 11β-HSD1 inhibition on cytokine, extracellular matrix and growth factor gene expression during wound healing

A) IL-1β and B) TNF-α mRNA increased during WH independently of CORT or CBX, C) IL-6 mRNA showed a trend towards induction by CORT at day 2 which was suppressed by CBX, D) KGF mRNA induction at day 4 was suppressed by CORT but not CORT/CBX and induced by CORT but not CORT/CBX at day 9. E) COL-1α1 and F) COL-3α1 mRNA was induced by CORT but not CORT/CBX at day 9. G) MMP-9 mRNA induction by CORT at day 9 and was reversed by CORT/CBX. H) TIMP-1 mRNA induction at day 4 was suppressed by CORT and reversed by CORT/CBX. (n=3-6, two-way

ANOVA). Multiple comparisons for 1B-1D: * = vs. baseline (day 0) in each treatment group (Dunnet's test), # = vs. VEH/VEH at each time-point and † = vs. VEH/CORT at each time-point (Tukey's test). Significance: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

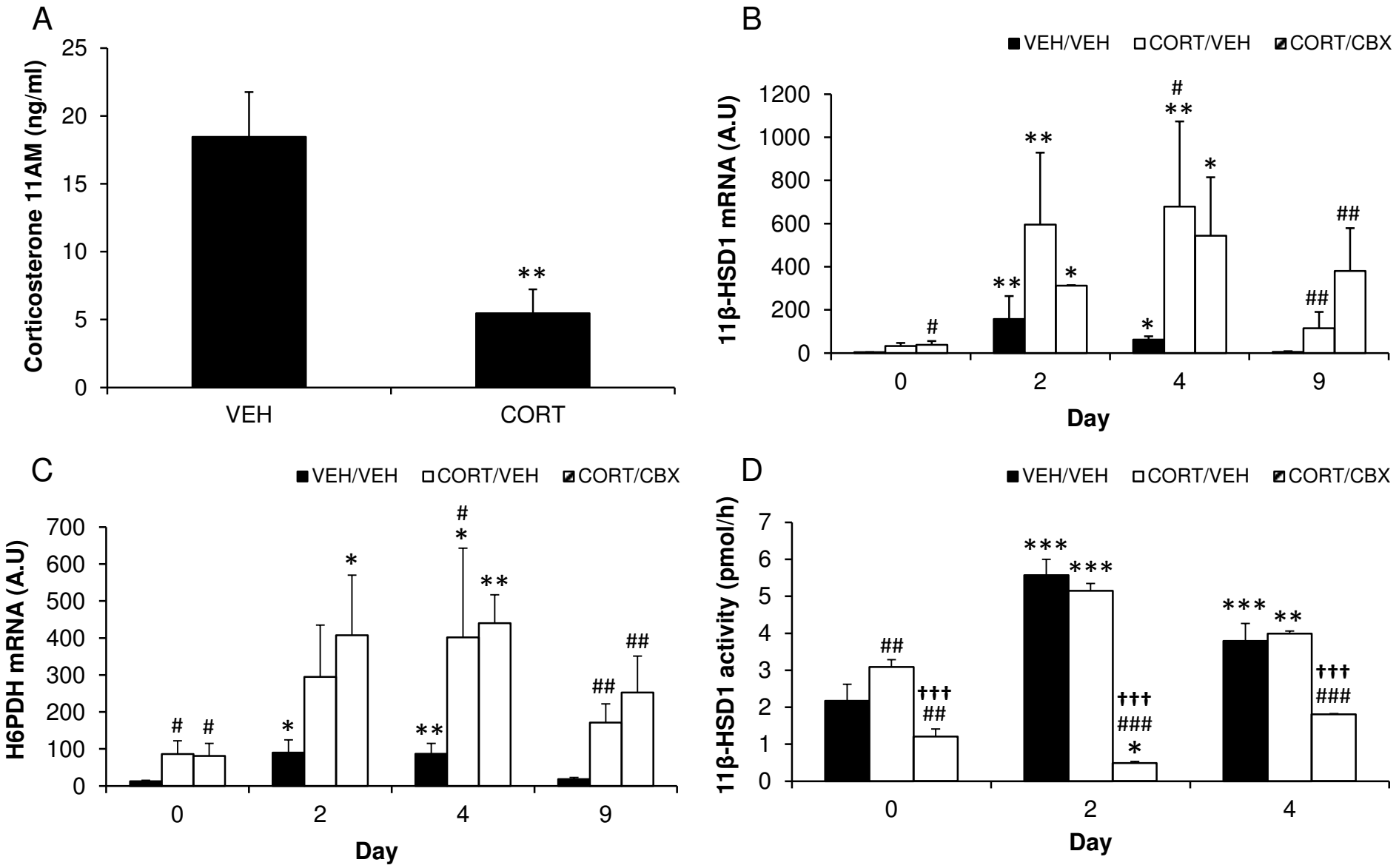

Figure 1

Figure 2

Click here to download Figure Figure 2.pdf

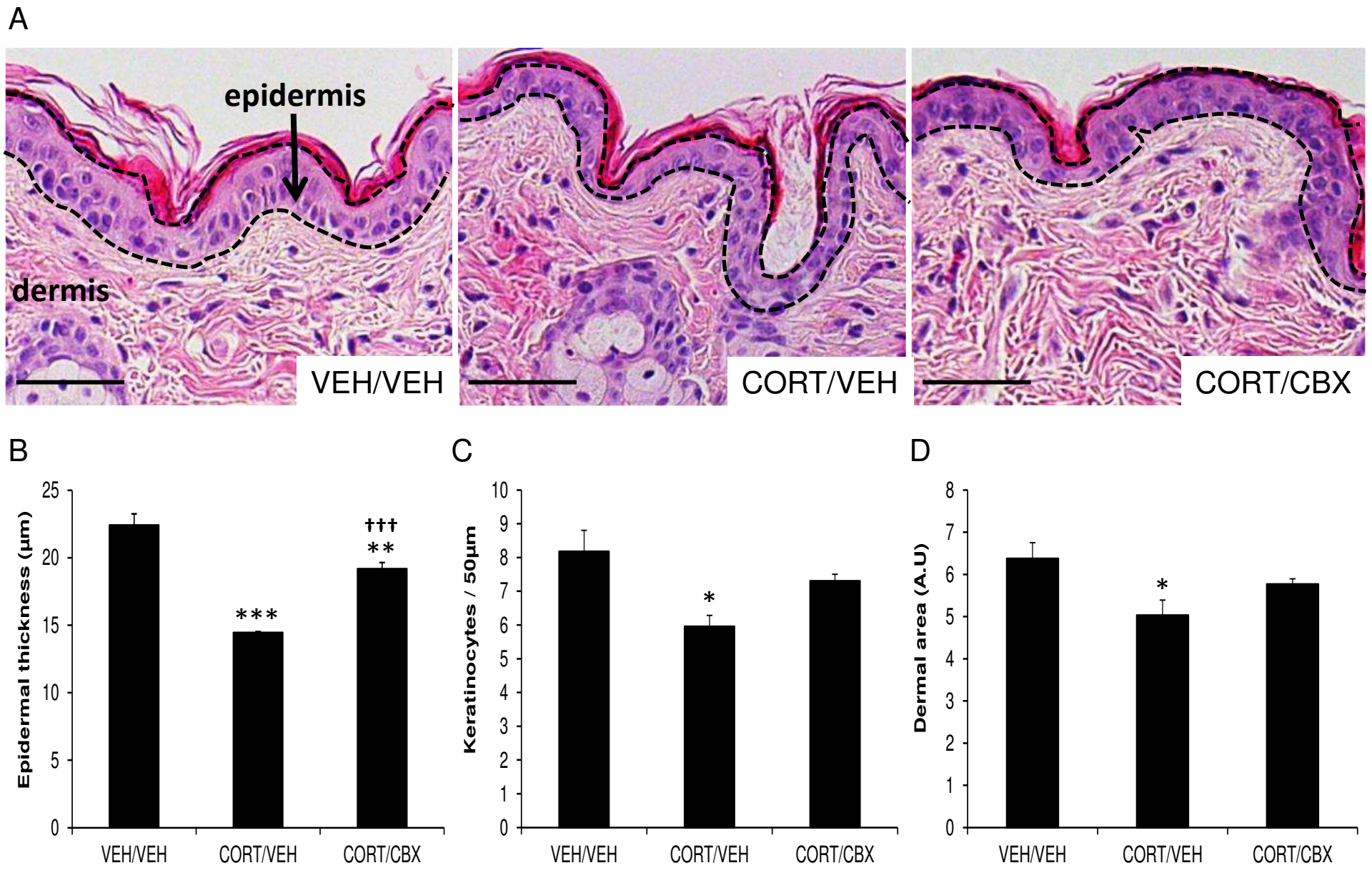

Figure 2

Figure 3

Click here to download Figure Figure 3.pdf

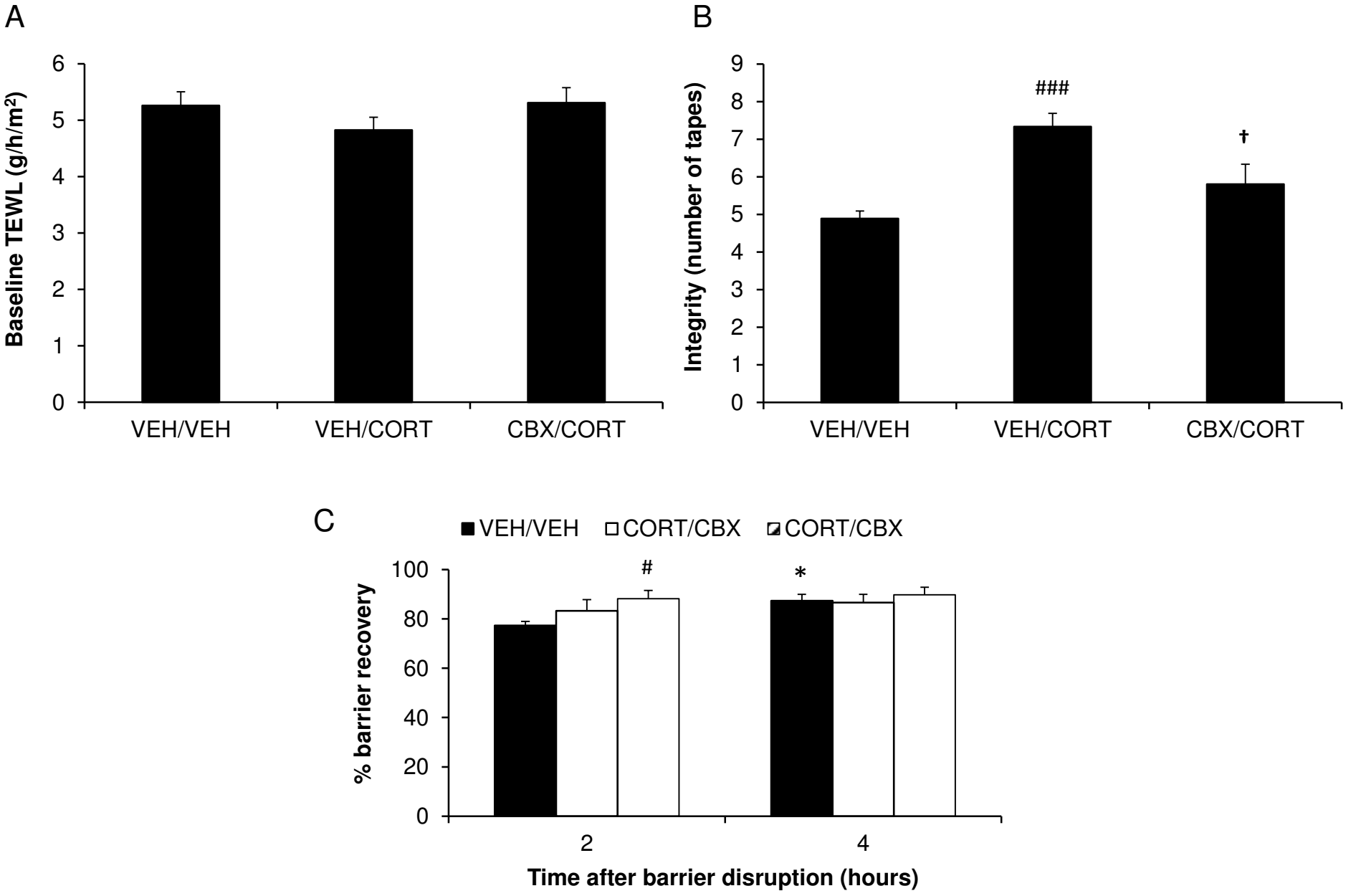

Figure 3

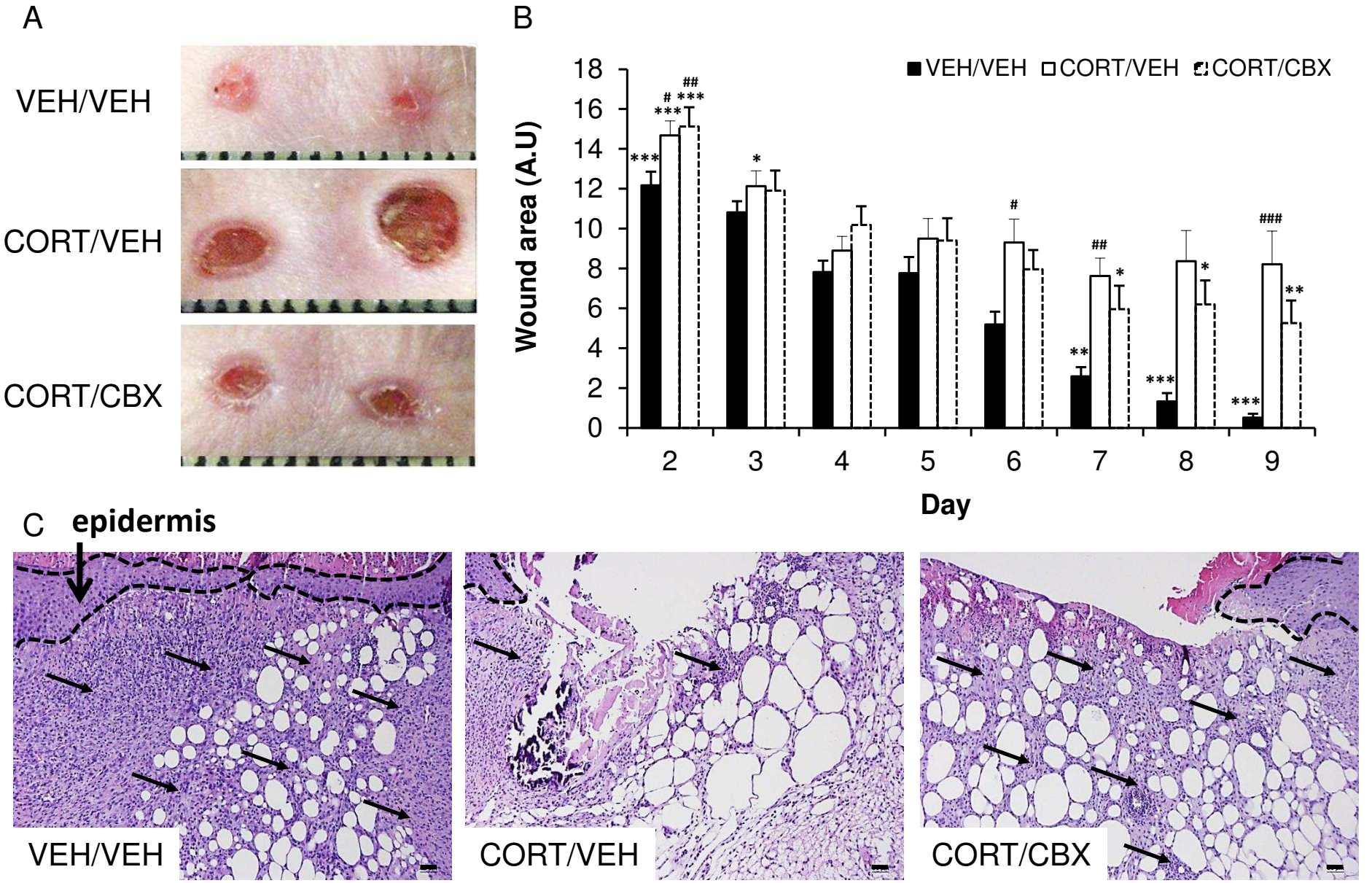

Figure 4

Figure 5

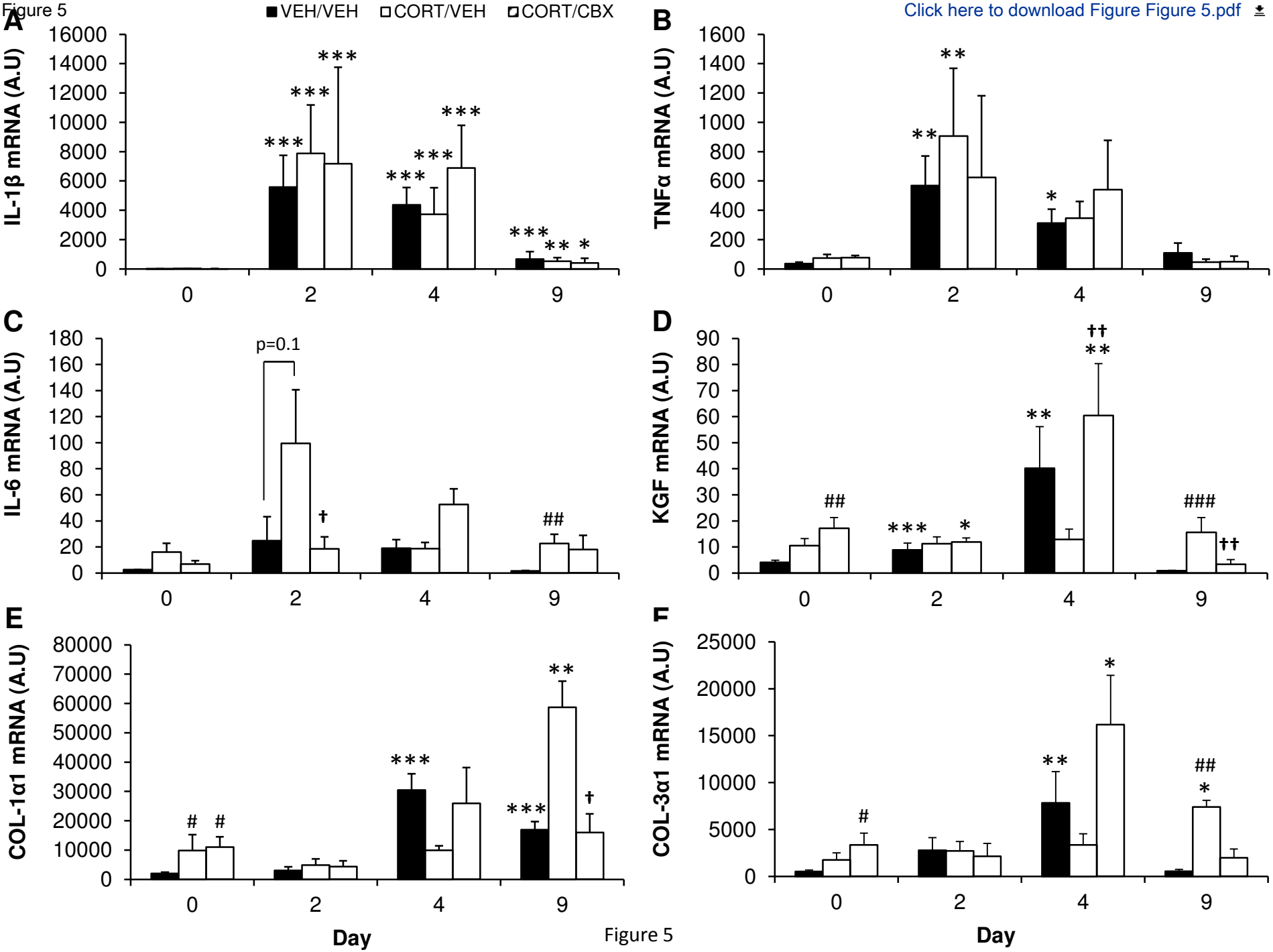

Figure 5

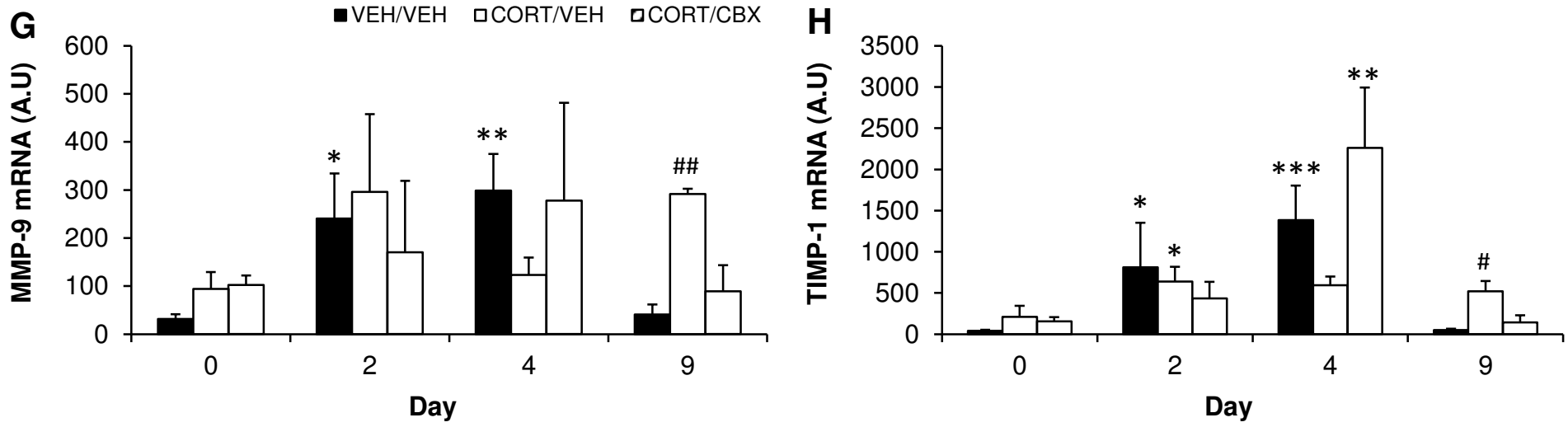
G
VEH/VEH
CORT/VEH
CORT/CBX
MMP-9 mRNA (A.U)
600
500
400
300
200
100
0
*
**
##
0
2
4
9
Day
H
TIMP-1 mRNA (A.U)
3500
3000
2500
2000
1500
1000
500
0
*
*
***
**
#
0
2
4
9
Day

Figure 5