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Vitamin C promotes wound healing through novel pleiotropic mechanisms

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Key words

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Abstract

Vitamin C (VitC) or ascorbic acid (AscA), a cofactor for collagen synthesis and a primary antioxidant, is rapidly consumed post-wounding. Parenteral VitC administration suppresses pro-inflammatory responses while promoting anti-inflammatory and pro-resolution effects in human/murine sepsis. We hypothesised that VitC could promote wound healing by altering the inflammatory, proliferative and remodelling phases of wound healing. Mice unable to synthesise VitC (*Gulo*^{-/-}) were used in this study. VitC was provided in the water (*sufficient*), withheld from another group (*deficient*) and supplemented by daily intra-peritoneal infusion (200 mg/kg, *deficient* + *AscA*) in a third group. Full thickness excisional wounds (6 mm) were created and tissue collected on days 7 and 14 for histology, quantitative polymerase chain reaction (qPCR) and Western blotting. Human neonatal dermal fibroblasts (HnDFs) were used to assess effects of In conclusion, VitC favorably on proliferation. Histological analysis showed improved wound matrix deposition and organisation in *sufficient* and *deficient* + *AscA* mice. Wounds from VitC *sufficient* and *deficient* + *AscA* mice had reduced expression of pro-inflammatory mediators and higher expression of wound healing mediators. Supplementation of HnDF with *AscA* induced the expression of self-renewal genes and promoted fibroblast proliferation. VitC favourably impacts the spatiotemporal expression of transcripts associated with early resolution of inflammation and tissue remodelling.

Introduction

Wound healing is a normal physiological process aimed at restoring the anatomical structure and function of injured skin (1). Many factors have been identified that affect wound healing, such as age, wound location, wound size, nutritional status, immune system status and underlying co-morbidities such as diabetes and obesity (2,3). Delayed wound healing can result in a number of complications such as increased length of hospital stay, amputations and even death (3). There is a 2% prevalence of chronic, non-healing wounds in the general population that is associated with an annual estimated cost of more than \$50 billion and this expenditure is expected to rise in the coming

years (4). Hence there is an unmet need to better identify the mechanisms that delay normal wound healing.

Key Messages

- healing wounds are often characterised by low/inadequate VitC levels
- VitC supplementation may be essential for timely termination of the inflammatory phase of wound healing
- adequate circulating VitC promotes fibroblast migration, matrix deposition and neovascularisation in healing

wounds via modulation of the transcript levels of HO-1, TGF β , CTGF and VEGF

- VitC facilitates wound healing by pleiotropic mechanisms that extend beyond its known role in collagen metabolism
- Gulo^{-/-} mice serve as a useful, humanised model for wound healing that allows for better translation of experimental results to humans

One of the desired goals of wound repair is to adequately restore the physical barrier (wound closure) without a loss of function within a reasonable time frame. Re-establishing the integrity of injured skin necessitates a delicate balance between four sequential yet overlapping stages: haemostasis, inflammation, proliferation and remodelling (maturation) (5). This process involves a highly ordered series of cellular events: platelet activation and fibrin clot formation at the wound site (haemostasis); polymorphonuclear neutrophil (PMN) infiltration to contain invading micro-organisms and clear damaged matrix and tissue debris, followed by macrophage infiltration to engulf and clear apoptotic sated PMNs (inflammation); and fibroblast migration and proliferation (proliferation) to lay down the new matrix collagen that progressively matures, cross-links and organises (remodelling/maturation) (5). These cellular events are orchestrated by chemokines and cytokines such as platelet derived growth factor (PDGF), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), interleukin-6 (IL-6), interleukin-1 β (IL-1 β) and tumour necrosis factor-alpha (TNF- α) (6,7) whose expressions ultimately determine the course and fate of wound healing.

Wounds create an environment of higher catabolic state (8). Following injury, the rate at which micronutrients are metabolised increases significantly often leading to critical deficiencies (8). Indeed, levels of vitamin C (VitC), a small, organic, water-soluble micronutrient with strong antioxidant properties (9,10), fall rapidly during inflammation. Moreover, scorbutic individuals experience delayed healing and decreased rates of collagen synthesis and maturation (11). Along with its strong antioxidant properties, VitC is an essential co-factor for multiple enzymatic reactions and has recently been shown to suppress pro-inflammatory processes by pleiotropic mechanisms while promoting anti-inflammatory and pro-resolution effects in macrophages (12–15). VitC is also intimately involved in collagen metabolism and regulation and therefore many studies have focused on its particular role in wound healing (16–19). Humans lack functional *L-gulonolactone oxidase* (Gulo), the final enzyme for VitC biosynthesis, and hence are dependent upon an external supply (in diet) of VitC (9,10). In contrast, wild-type mice express functional Gulo and maintain high levels of VitC in their tissues. In order to better understand the role of VitC in wound healing we used humanised knock-out mice lacking Gulo (Gulo^{-/-}) in our studies. In these studies, we went beyond the known effects of VitC on collagen synthesis to explore the role of VitC on the spatiotemporal changes in the inflammatory, proliferative and maturation stages of wound healing.

Methods

Animals

All animal studies were performed in accordance with the Virginia Commonwealth University Animal Care and Use Committee's approved protocols. Gulo^{-/-} mice were bred in-house from an established homozygous colony maintained on a C57BL/6J background as previously described (14). Mice were fed ad libitum with regular chow and had free access to water supplemented with AscA (330 mg/l) renewed twice a week to yield *sufficient* mice. Supplements were given in de-ionised water with 20 μ l of 0.5 M EDTA/l to increase the stability of the AscA in solution. At week 10 of age, some mice were rendered VitC *deficient* by reducing VitC supplementation for one week (33 mg/l), followed by complete removal of VitC supplementation for an additional week. This reduced supplementation was shown to result in very low plasma VitC concentration, yet insufficient to result in scurvy (20,21). In this study, mice were divided into three groups: *VitC sufficient*, *VitC deficient* and *VitC deficient + AscA* mice. The third group was given daily parenteral AscA (200 mg/kg intraperitoneal (i.p.) injection) and AscA-supplemented drinking water (330 mg/l) for up to 14 days following wounding.

Surgical procedure

Animals were maintained under isoflurane anaesthesia. In addition, they were also injected subcutaneously with the analgesic buprenorphine (4 μ g/ml) for pain management. Wounds were created using the methods described by Galiano *et al.* (22). Briefly, the mouse hair on the dorsum of the mice thoracic curvature was shaved with an electric clipper followed by an additional treatment with a depilatory cream (Nair), for 3 minutes, to remove any remaining fur. The surgical area was then neutralised with betadine followed by another disinfection step with alcohol swabs. Two full thickness excisional wounds were created on the back of *VitC sufficient/deficient* Gulo^{-/-} mice using a sterile 6-mm biopsy punch. Wounds were covered with non-adherent dressing (TelfaTM, Covidien) Minneapolis, MN and the animals were housed individually with ad libitum water and food access as discussed earlier. At day 7 and day 14 post-wounding, mice were anaesthetised, blood was collected via cardiac puncture (anti-coagulated with sodium citrate 1:10) and kept on ice to be processed for VitC analysis. Mice were then sacrificed and the wound tissue was collected using forceps and scissors. Per mouse, the tissue from one wound was fixed in formalin for 48 hours and processed for histology. The tissue from the second wound was excised, bisected and stored in liquid nitrogen followed by long-term storage at -80°C for subsequent RNA and protein extraction.

Human neonatal dermal fibroblast (HnDF) culture

Primary HnDF cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in high glucose, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum, penicillin (100 μ g/ml) and streptomycin (100 μ g/ml) under a 5%

CO₂ atmosphere at 37°C. All experiments were performed using cells at passages 3–5.

VitC analysis

Blood samples obtained from mice on days 7 and 14 post-wounding were kept on ice, centrifuged and the resultant plasma was deproteinised as previously described (15). Briefly, 100 µl of plasma was deproteinised with 200 µl of cold 20% trichloroacetic acid (TCA), and treated with 200 µl of cold 0.2% dithiothreitol (DTT) to prevent oxidation. The mixture was vortexed intermittently for 2 minutes and centrifuged (10 000g, 4°C, 10 minutes). Supernatants were stored at –80°C for batch analysis using a fluorescence end-point assay (23).

Histological staining and wound assessment

Formalin-fixed paraffin-embedded wound sections (3–4 µm) were cleared in xylene washes, rehydrated in a series of decreasing alcohol concentrations and brought to water. Prepared specimens were stained with Masson's Trichrome according to the manufacturer's protocol (Richard Allan Scientific, Catalogue # KTRA87019, Kalamazoo, MI).

RNA isolation and real-time quantitative PCR (QPCR) analysis

Total RNA isolation and real-time qPCR were performed as described previously (15). Assays were run in triplicate with no template controls and no reverse transcriptase controls. The mRNA expression from a *deficient* mouse or a 'media' well was set to '1.0' and mRNA expression of all other samples was compared relative to this sample and represented as a fold change. To normalise for differences in the amount of total RNA added to each cDNA reaction and possible variation in the reverse transcriptase efficiency among the different cDNA reactions, the housekeeping gene 18S rRNA was used. Automated gene expression analysis was performed using the Comparative Quantitation module of MxPro QPCR Software Santa Clara, CA (Agilent). Both the forward and reverse primers for each target are listed in Table 1.

Western blot analysis

Wound tissue homogenates and HnDF whole cell and nuclear extracts were isolated and used for Western blot analysis as described previously (15). Nuclear extracts were isolated using the NE-PER kit (Pierce Biotechnology, Rockford, IL). Antibodies to hemoxygenase-1 (HO-1) (ADI-SPA-896-D, Enzo life sciences, Farmingdale, NY), OCT-3/4 (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA), lamin B (sc-6216, Santa Cruz Biotechnology), and actin (sc-1616, Santa Cruz Biotechnology) were used in this study. Optical densities of antibody-specific bands were determined using Quantity One acquisition and analysis software (BioRad, Hercules, CA).

VitC uptake by HnDF

HnDFs were grown under normal culture conditions to confluence in 12-well plates. Cells were then exposed to media

Table 1 Murine and human primers used for QPCR

Name	Sequence 5' to 3'
Murine IL-1β forward	CTGAACCTCAACTGTGAAATGCC
Murine IL-1β reverse	CAGGTCAAAGGTTTGAAGC
Murine TNF-α forward	GATGAGAAGTCCCAAATGGC
Murine TNF-α reverse	TTGGTGGTTTGTACGACG
Murine KC forward	CAATGAGCTGCGCTGTCAAGTGCCTGCAG
Murine KC reverse	CTGAACCAAGGGAGCTTCAGGGTC
Murine MPO forward	CTGGATCATGACATCACCTTGACTCC
Murine MPO reverse	GATCTGGTTGCGAATGGTGTGTTGTTCC
Murine HO-1 forward	GGTACACATCCAAGCCGAGAATGCTGAG
Murine HO-1 reverse	CGGTGCAGCTCCTCAGGGAAGTAGAG
Murine VEGF forward	GAGACCCTGGTGGACATC
Murine VEGF reverse	CTTTCTTGGTCTGCATTAC
Murine CTGF forward	CCCAACTATGATGCGAGCC
Murine CTGF reverse	ACAGGCTTGCGATTTTAGG
Murine TGF-β forward	TGACGTCCTGGAGTTGTACGG
Murine TGF-β reverse	CCACGTGGAGTTTGTATCTTTGG
Murine Gal1 forward	CAGCAACCTGAATCTCAAACC
Murine Gal1 reverse	AGTGTAGGCACAGGTTGTTGC
Human IL-6 forward	GGATTCAATGAGGAGACTTGCC
Human IL-6 reverse	TCTGCAGAACTGGATCAGG
Human Nanog forward	AATGTCTTCTGCTGAGATGCC
Human Nanog reverse	GCTGTCTGAATAAGCAGATCC
Human OCT4 forward	CTTGCTGCAGAAGTGGG
Human OCT4 reverse	CACTCGGTTCTCGATACTGG
Human p21 forward	CTGTCTTGTACCCCTTGTTGCC
Human p21 reverse	CCTCTTGGAGAAGATCAGCC
Human p27 forward	TGGACCCAAAGACTGATCC
Human p27 reverse	CATTTCTTCTGTCTGTTGGC

CTGF, connective tissue growth factor; HO1, hemoxygenase-1; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor.

alone or AscA (0.5 and 1 mM) for 3 hours. Media was removed and cells were washed twice with PBS. To each well, 50 µl of tissue culture grade water was added. Cells were lysed by repeated freeze–thaw cycles (×3). Lysates were treated with TCA and DTT and intracellular VitC content was determined as described above.

HnDF proliferation assay

HnDFs were seeded into 96-well plates at an initial density of 3000 cells per well. After overnight incubation, fresh media containing 0, 0.5 or 1 mM AscA was added and incubated for 24 hours. Media was aspirated and wells washed twice with PBS. Proliferation was assessed using the CyQUANT® cell proliferation assay kit (Invitrogen, Carlsbad, CA) according to manufacturer instructions.

Healed skin tensile testing

For these set of experiments, mice were sacrificed at day 14 post-wounding and 20 × 4 mm skin specimens were harvested along the mouse central axis. Specimens were wrapped in PBS-moistened gauze and stored at 4°C overnight prior to testing. Dimensions of each specimen were taken three times using a digital calliper and the average was used to calculate the cross-sectional area. Two samples were harvested per animal

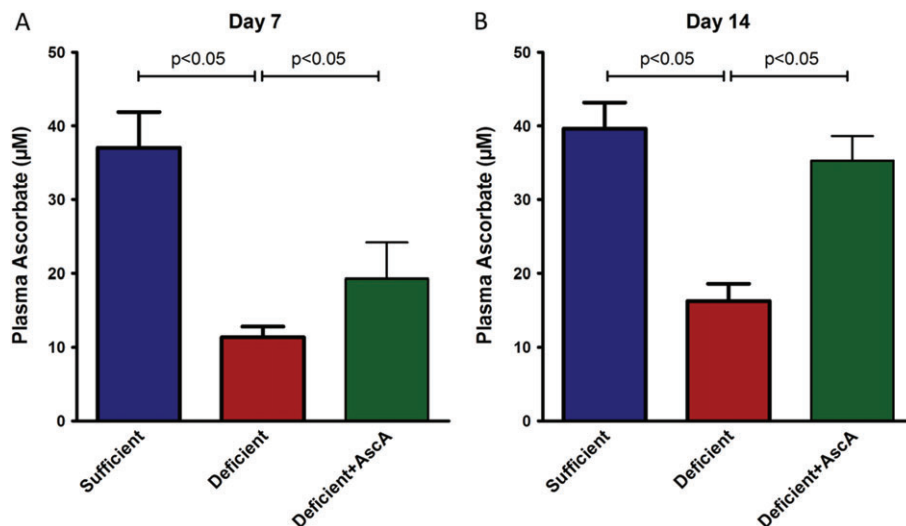


Figure 1 Plasma vitamin C (VitC) levels from VitC *sufficient*, *deficient* and *deficient* + *AscA* *Gulo*^{-/-} mice at day 7 (A) and day 14 (B) post wounding. Daily supplementation of *deficient* mice with *AscA* post-wounding resulted in significantly higher plasma VitC levels in the *deficient* + *AscA* mice compared to *deficient* mice by day 7 post wounding. These levels further rose and were comparable to plasma levels of *sufficient* mice at day 14 ($n = 3-6$ mice per group).

with the healed wound area located mid-substance. Mechanical testing was performed at room temperature using MTS tensile testing machine and Testworks 4.06A software (MTS Systems, Eden Prairie, MN). The starting conditions were a preload of 0.01 Newtons (N), and tensile grip moving rate of 10 mm/minutes. Only samples that failed mid-substance were included in the analysis, otherwise samples were excluded (due to failure at the clamp or samples that slipped from the clamp).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Data are expressed as mean \pm SE. Results were compared using one-way ANOVA. Post-hoc Tukey test was used to carry out multi-comparisons between groups. The level of significance for all statistical tests was $P < 0.05$.

Results

Plasma VitC levels affect the progression of wound healing

To examine the effect of VitC on wound progression, three groups of *Gulo*^{-/-} mice were generated and subjected to the wounding procedure described in the Methods section. As seen in Figure 1A, at day 7 post-wounding, VitC *sufficient* mice had significantly higher circulating plasma VitC when compared to the *deficient* group. The VitC levels in the *deficient* + *AscA* group were also significantly higher than the *deficient* group by day 7. By day 14, circulating plasma VitC levels in the *sufficient* and *deficient* + *AscA* were comparable and significantly higher than the *deficient* group (Figure 1B). Masson's trichrome stained wound sections on day 7 demonstrated dense granulation tissue and higher collagen deposition (blue colour) from *sufficient* and *deficient* + *AscA* mice when compared to the wound sections from *deficient* mice (Figure 2A–C). In the VitC groups, the basal lamina and keratin layer were well developed and present as distinct layers. In contrast, day 7 sections from *deficient* mice demonstrated loose granulation tissue with

reduced collagen deposition. Additionally, in *deficient* mice, the dermis was more cellular (and reticular) and the lamina was not yet fully distinct (Figure 2B). Most of these differences continued to be evident on day 14 post-wounding (Figure 2D–F).

VitC attenuates mediators of inflammation in wound healing

Next we examined the mRNA expression of the pro-inflammatory genes (IL-1 β , KC, TNF- α and MPO) in healing tissue on days 7 and 14 post-wounding. VitC *sufficiency* or treatment of *deficient* mice with *AscA* significantly attenuated transcript levels of these pro-inflammatory genes when compared to wound tissue from VitC *deficient* mice at day 7 (Figure 3). The transcript levels of the pro-inflammatory signalling cytokines (IL-1 β and KC) remained elevated on day 14 in the *deficient* mice compared to the *sufficient* and *deficient* + *AscA* mice (Figure 4). MPO transcripts however were not detectable in all three groups on day 14 (data not shown). TNF- α transcript expression on day 14 showed the same trend as IL-1 β and KC but these differences in expression did not achieve statistical significance. In light of the elevated and persistent expression of pro-inflammatory mediator transcripts in VitC-deficient animals, we examined whether markers of inflammation resolution might be altered. Galectin-1 (Gal-1) expression is associated with generation of pro-resolving lipid mediators and successful resolution of inflammation (24). Therefore, we examined Gal-1 expression in day-7 wounds. Gal-1 transcript levels were significantly higher in VitC *sufficient* mice compared with the *deficient* mice (Figure 5). Although Gal-1 gene expression trended higher in the *deficient* + *AscA* group, it did not reach statistical significance.

VitC induces genes that promote wound healing

We also examined the expression of transcripts levels of several other known modulators of wound healing including hemoxygenase-1 (HO-1), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF) and transforming growth factor-beta (TGF- β). On day 7 post-wounding,

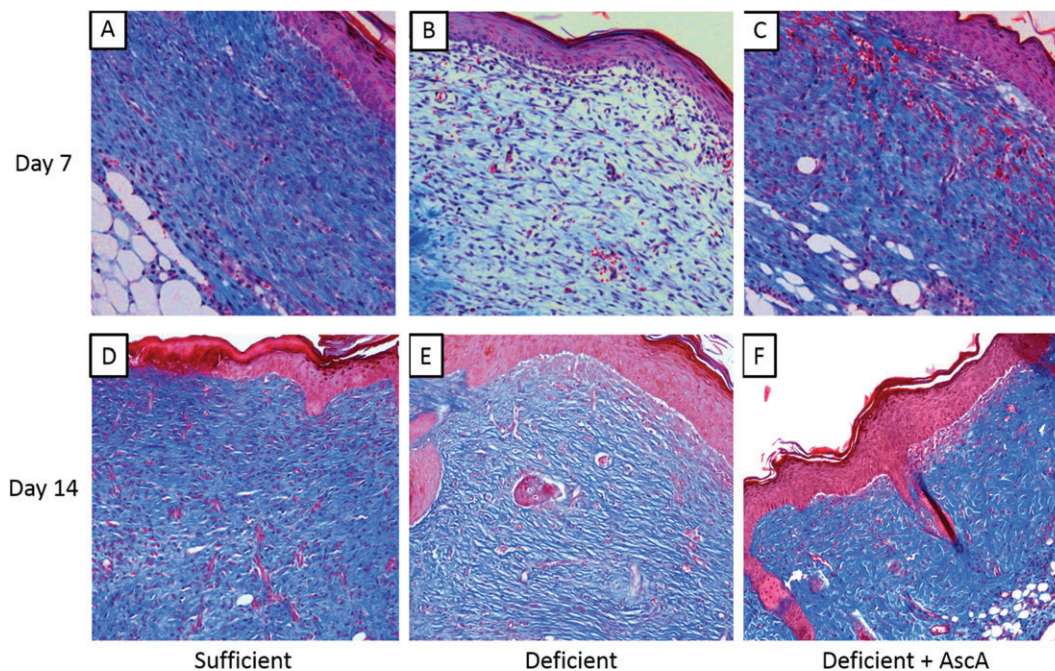


Figure 2 Representative Masson's trichrome stained sections from vitamin C (VitC) *sufficient*, *deficient*, and *deficient + AscA Gulo^{-/-}* mice at day 7 (A–C) and day 14 (D–F) post-wounding. VitC *sufficient* (A) and *deficient + AscA* (C) sections demonstrated more dense granulation tissue with significant collagen deposition (blue colour) and distinct lamina. In contrast, sections from the *deficient* mice were more cellular with loose granulation tissue and reduced collagen deposition (B). Also the lamina remained indistinct. Similar observations were evident from day-14 sections (D–F) with the VitC *deficient* mice group still showing considerably reduced collagen deposition ($n = 3–6$ mice per group).

both VitC *sufficient* and *deficient + AscA Gulo^{-/-}* mice showed significantly higher mRNA expression of HO-1, VEGF and CTGF (Figure 6A–C) in comparison to the *deficient* mice. TGF- β expression was significantly elevated in *sufficient* mice compared to *deficient* mice. TGF- β expression following AscA infusion trended higher in the *deficient + AscA* group but did not reach statistical significance (Figure 6D). Induction at the protein level was confirmed by Western blot analysis for total HO-1 (Figure 6E).

VitC and tensile strength of healed wound

Using the excisional wound model, samples collected at day 14 post-wounding were used to carry out skin tension studies as described in the Methods section. Stress at the healing section was measured using the mid-specimen cross-sectional area (in kilopascals). The healed skin samples from the *sufficient* mice demonstrated a significantly higher stiffness compared to the other groups (Figure 7). However, there were no statistically significant differences between the *deficient* and *deficient + AscA* groups. In addition, we also measured the peak load, a measure of the tensile strength that a material can withstand before breaking. However, no significant differences were observed between the different mice groups (data not shown).

VitC promotes HnDF proliferation

Confluent HnDF cells were incubated for 3 hours with two concentrations of AscA (0.5 and 1 mM). In the absence of exogenous AscA, intracellular VitC concentrations in HnDFs were

extremely low. There was a dose- and time-dependent increase in intracellular VitC concentrations following AscA loading (Figure 8A) suggesting that these cells have the ability to transport VitC intracellularly. Additionally, incubating sub-confluent HnDF cells with the same AscA concentrations (0.5 and 1 mM) for 24 hours resulted in significant dose-dependent increases in HnDF proliferation (24% and 40%, respectively) as seen in Figure 8B.

VitC induces the expression of self-renewal, cell-cycle progression and fibroblast motility genes in HnDF

To further investigate the increased proliferative capacity brought by AscA, HnDF cells were incubated with AscA (0.5 and 1 mM) for 3 hours and mRNA expression of the self-renewal genes Nanog and octamer-binding factor 4 (OCT4) were determined by QPCR. Both Nanog and OCT4 gene expression increased in a dose-dependent fashion following HnDF incubation with AscA (Figure 9A and B). The increased expression was not statistically significant with low level AscA (0.5 mM) but was significant with the higher AscA concentration ($P < 0.05$). The mRNA expression levels of the cyclin-dependent kinase inhibitors and regulators of cell cycle, p21 and p27 were also determined (Figure 9C and D). Transcript levels of p27 were significantly decreased in a dose-dependent fashion by AscA exposure. However p21 expression levels were unchanged. Western blot analysis confirmed the increased nuclear expression of OCT4 seen with QPCR at 24 hours post incubation with AscA (Figure 9E).

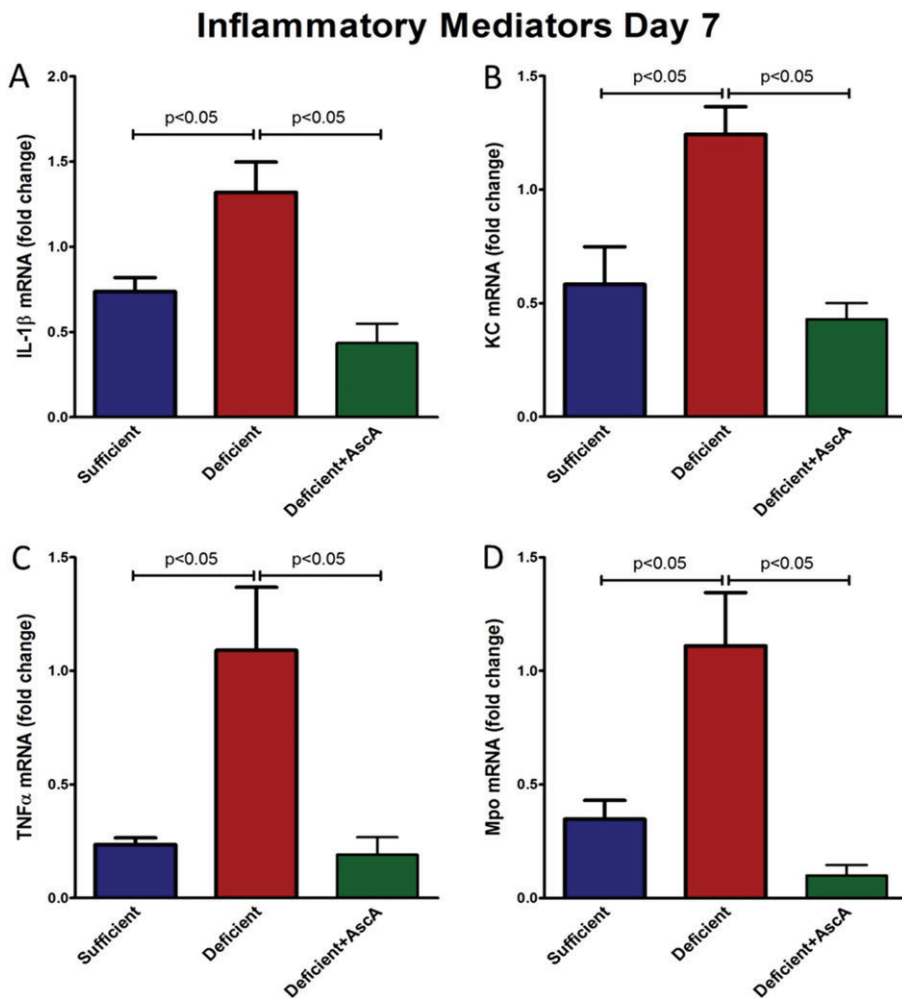


Figure 3 Real time quantitative polymerase chain reaction (qPCR) for IL-1 β (A), KC (B), TNF- α (C) and MPO (D) mRNA from day-7 wounds of vitamin C (VitC) *sufficient*, *deficient*, and *deficient + Asca* *Gulo*^{-/-} mice. Wound tissue from VitC *sufficient* and *deficient + Asca* mice demonstrated an attenuated pro-inflammatory gene expression profile compared to wound tissue from *deficient* mice ($n = 3-6$ for each group).

The pleiotropic cytokine IL-6 has been shown to favourably promote cell motility and matrix remodelling during the inflammatory phase of wound healing (25,26). In addition, IL-6 is a resolution promoting cytokine by virtue of its inhibitory effects on pro-inflammatory signalling via activation of IL-10 and IL-13, and by its ability to enhance polarisation of macrophages at wound healing sites towards an anti-inflammatory/alternatively activated phenotype (27). Therefore we examined IL-6 mRNA expression following exposure of HnDF cells to Asca. As seen in Figure 9F, exposure of HnDF to Asca produced a dose-dependent increase in IL-6 mRNA expression levels; 2.5-fold and fourfold increase with 0.5 and 1 mM Asca, respectively.

Discussion

Wounds and more generally injuries are associated with rapid micronutrient deficiencies (8). Levels of one critical micronutrient, VitC, have been shown to drop significantly (60–70%) at the wound site and not recover completely even after 14 days post-wounding (28,29). These observations may reflect VitC depletion by the plethora of free oxidant radicals generated in the wound microenvironment, and in part by the increased consumption of VitC in different biological processes (e.g. collagen synthesis) that are activated during the repair process. While

higher oral intake is achievable, attaining significant plasma levels are limited by gastric intolerance and also by limited absorption and renal excretion thresholds (30). We and others have shown that parenteral VitC is advantageous in bypassing these limitations to produce and sustain adequate plasma levels (30,31).

In our present study, we were able to show improved wound healing characteristics with VitC sufficiency and also with i.p. VitC repletion of *Gulo*^(-/-) mice. Day-7 wounds from VitC *deficient* mice showed a spatiotemporal gene expression characterised by persistent inflammation combined with delayed resolution and proliferation (Figures 3–6). The pro-inflammatory state persisted throughout day 14 post-wounding in the *deficient* mice (Figure 4) but not in the *sufficient* or *deficient + Asca* groups (Figure 4). In contrast, VitC *sufficient* and supplemented *Gulo*^(-/-) mice showed attenuated expression of pro-inflammatory cytokines, increased expression of favourable biomarkers of wound healing, and better expression of the pro-resolution markers of wound healing (Figures 3–6). However, tangible effects on the skin tensile strength properties at day 14 post-wounding were evident only in the VitC *sufficient* mice. These improvements in wound healing in the VitC *sufficient* and supplemented mice were corroborated by in vitro cultures

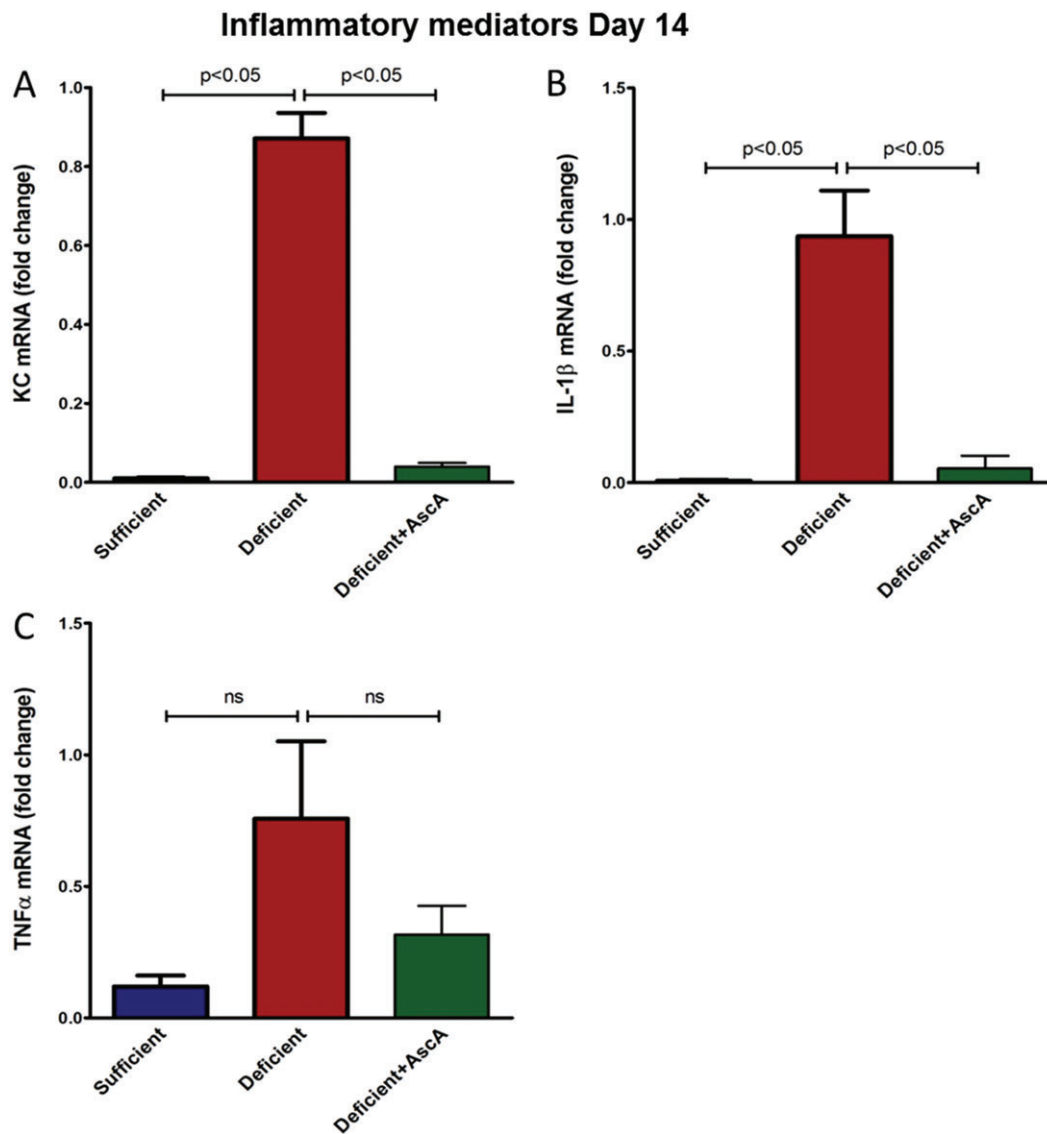


Figure 4 Real time quantitative polymerase chain reaction (qPCR) for IL-1 β (A), KC (B) and TNF- α (C) mRNA from day-14 wounds of vitamin C (VitC) *sufficient*, *deficient*, and *deficient + AscA* *Gulo*^{-/-} mice. Wounds from VitC *deficient* still exhibited a heightened pro-inflammatory gene expression response compared to VitC *sufficient/supplemented* mice ($n = 3-6$ for each group, ns, not significant).

of HnDF which showed that VitC supplementation led to robust uptake and fibroblast proliferation (Figure 8), induction of self-renewal genes (Figure 9) and up-regulation of a pro-resolution cytokine that also plays a role in fibroblast mobility (Figure 9F).

Histological examination of day 7 and day 14 Masson's Trichrome stained sections (Figure 2) showed that wounds from VitC *sufficient* and supplemented mice had better matrix organisation with clear distinction of all skin layers and significant extracellular matrix deposition. In contrast, wounds from VitC *deficient* mice were characterised by a loose dermis that was highly cellular and accompanied by limited collagen deposition. At a molecular level, wounds from *deficient* mice actively expressed the pro-inflammatory cytokines IL-1 β and TNF- α . Additionally, wounds from *deficient* mice demonstrated significantly higher MPO expression compared

with wounds from VitC *sufficient* and AscA infused mice. MPO is an enzyme almost exclusively produced by PMNs (5% of total neutrophil protein) and to some extent in monocytes and is often used as a marker of PMN infiltration (32). High MPO levels have been found to be associated with poor healing wounds (33). A similar pattern was observed with the gene expression of the PMN chemoattractant factor, KC. Murine KC is analogous to human CXCL-1 (a functional homologue of IL-8). We interpret the presence of these pro-inflammatory markers as ongoing/sustained pro-inflammatory cellular infiltration into the wound site and an extended pro-inflammatory phase in wounds from *deficient* mice. In contrast, the attenuated expression of these pro-inflammatory mediators in VitC *sufficient* and AscA infused mice is indicative of termination of pro-inflammatory cell infiltration to the wound site and perhaps

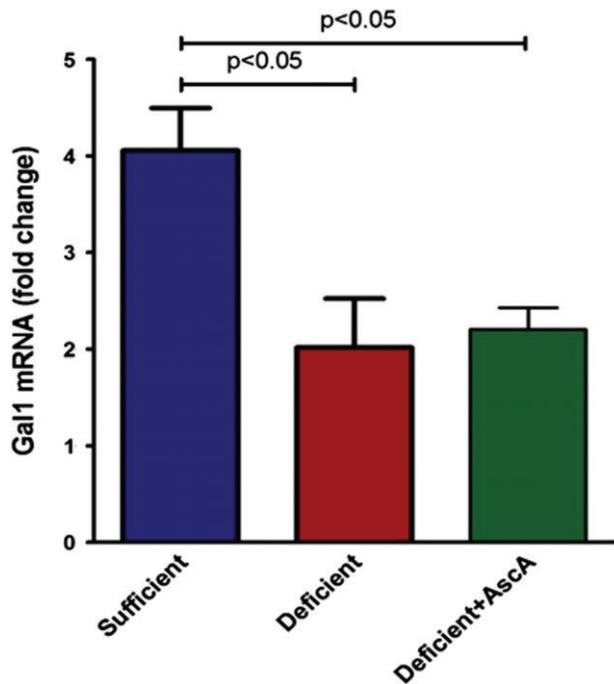


Figure 5 Real time quantitative polymerase chain reaction (qPCR) for Gal-1 from day 7 wounds. Vitamin C (VitC) *sufficiency* is associated with significantly higher Gal-1 expression. VitC *sufficient*, but not *deficient* + AscA, showed significantly higher Gal-1 expression compared to VitC *deficient* wounds at day 7 ($n = 3-6$ for each group).

the end of the pro-inflammatory phase. The expression pattern on day 14 further supports this assumption (Figure 4).

The proliferation stage of wound healing is typically characterised by increased expression of the pro-reparative growth factor cytokines such as TGF- β , CTGF and VEGF (6). TGF- β plays an important role at all stages of wound healing. In the initial stages, it serves as a chemotactic factor for pro-inflammatory cells as well as fibroblasts. In later stages, TGF- β provides a strong mitogenic signal for fibroblasts. Braiman-Wiksmann *et al.* have shown that high levels of TGF- β are most beneficial after epidermal closure is complete (34). In this work, TGF- β expression levels were significantly higher in day-7 wounds from *sufficient* mice. Wounds from mice receiving daily AscA infusion showed TGF- β levels that trended higher compared with *deficient* mice but did not reach statistical significance. Another growth factor in the wound milieu is CTGF whose transcription is regulated mainly by TGF- β . It is believed that it is a downstream mediator of some of the actions of TGF- β (35). However, TGF- β independent regulation of CTGF has also been reported (36). Recently, Alfaro *et al.* (2013) reported that CTGF levels at wound sites increase during the proliferation phase (37). CTGF functions in promoting granulation tissue formation (fibroblast cell division and migration and fibroblast matrix deposition, e.g. collagen type I and fibronectin) and wound remodelling (38). In our study, VitC *sufficiency* and AscA supplementation were associated with significantly higher CTGF expression compared with the *deficient* mice at day-7 post-wounding ($P < 0.05$). Combined with histological evidence, these results indicate an

active cellular and molecular proliferation in the VitC *sufficient* and AscA supplemented mice versus an attenuated/lagging proliferation response in *deficient* mice. Several studies have shown that VEGF significantly promotes angiogenesis and neovascularisation in healing wounds (5). Multiple factors such as pH, reduced oxygen tension, increased lactate as well as pro-inflammatory cytokines have been reported to induce VEGF production at the wound site (5,7). In our study, we observed higher VEGF expression in the VitC *sufficient* and AscA infused mice indicating increased angiogenesis and neovascularisation in the wounds of *sufficient* and AscA supplemented mice. A role for HO-1 in wound healing has only been recently described (39). Grochot-Przeczek *et al.* showed that HO-1 inhibition adversely affects wound healing in diabetic mice while HO-1 over-expression promoted healing (39). Earlier, the same research group showed that HO-1 upregulated VEGF production and therefore could indirectly promote neovascularisation. In our study, HO-1 expression levels were significantly higher in VitC *sufficient* and AscA supplemented mice. In summary, the expression pattern of these growth promoting factors can be interpreted as a robust biological response to wounds in VitC *sufficient* mice when compared to the *deficient* mice. In addition, daily i.p. VitC supplementation appears to restore the growth-promoting response in wounds of VitC *deficient* mice.

Physiological wound healing involves a regulated fibroblast differentiation into myofibroblasts that play an active role in reconstruction of damaged tissue following injury (40). A critical mediator of this process is Gal-1, a lectin produced by various tissues. Recently, Lin *et al.* (2015) showed that Gal-1 induced myofibroblast activation and proliferation. Moreover Gal-1^(-/-) knockout mice experienced reduced myofibroblast migration, which was corrected with topical Gal-1 administration in excisional wound models (41). Elsewhere, Perzelova *et al.* showed that exposure of dermal fibroblasts to Gal-1 stimulated extracellular matrix production that subsequently supported endothelial cell growth (42). In our studies, only VitC *sufficient* mice had significantly higher Gal-1 expression levels as compared with other groups. We have previously shown that both VitC *sufficiency* and VitC supplementation were able to induce early Gal-1 expression in peritoneal macrophages, which correlated with the markers of resolution (15). This suggests that VitC may be required to sustain Gal-1 expression in healing wounds and that the level of supplementation provided to the *deficient* mice is probably insufficient to adequately restore Gal-1 expression in healing wounds.

Much to our surprise, only VitC *sufficient* mice demonstrated significantly higher healed-skin stiffness compared with the other groups despite the observed increased collagen deposition evident by histological analysis in the *sufficient* and *deficient* + AscA mice. Although by day 14, we had achieved plasma VitC levels in the *deficient* + AscA group that were comparable to the VitC *sufficient* mice, it is possible that the VitC levels in the healing wound may not have reached the same level as the *sufficient* mice. While humans have evolved over millennia to up-regulate the VitC transporter SVCT2 at times of deficiency (43) it is unknown whether SVCT2 is up-regulated to the same extent in the healing wounds of these knockout mice. Indeed it has been shown that the knockdown of SVCT2 in

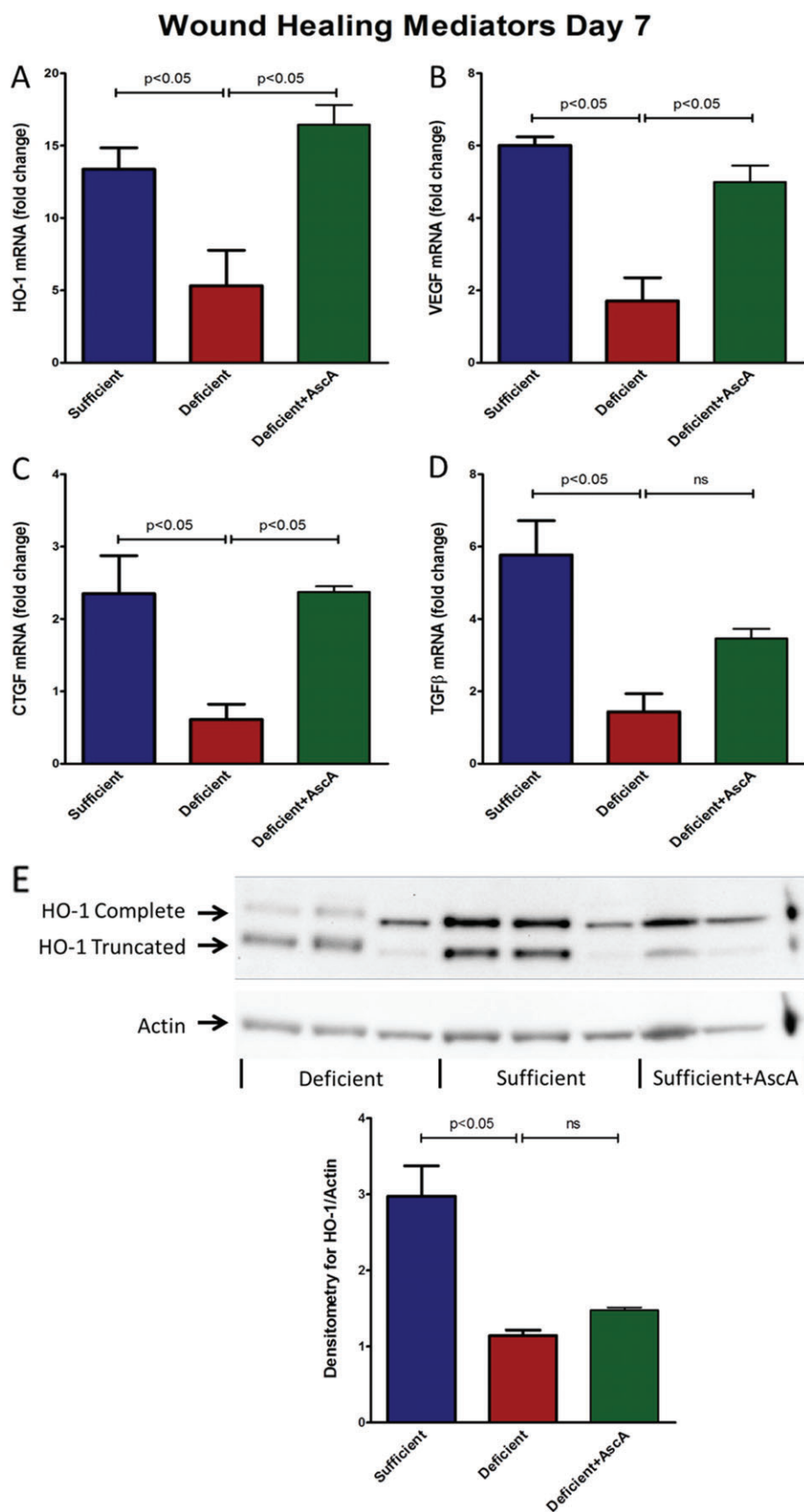


Figure 6 (A–D) Spatiotemporal profiling of HO-1 and growth factors mRNA expression in day 7 wounds. Real time quantitative polymerase chain reaction (qPCR) for HO-1 (A), VEGF (B), CTGF (C) and TGF-β (D) mRNA from day-7 wounds from vitamin C (VitC) *sufficient*, *deficient*, and *deficient + AscA* *Gulo*^{-/-} mice (*n* = 3–6 mice/group, ns, not significant). (E): HO-1 protein expression on day 7 post wounding. Representative Western blot for expression of HO-1 and actin from day 7 wounds of VitC *sufficient*, *deficient*, and *deficient + AscA* *Gulo*^{-/-} mice. The bar chart is a quantitative representation of the Western blot results using densitometry (*n* = 3–6 for each group, ns, not significant).

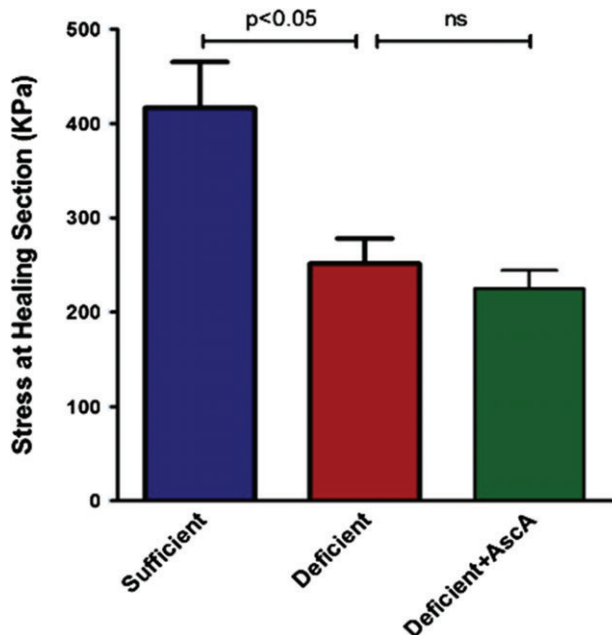


Figure 7 Stress at healing section of wounds collected on day 14 post-wounding. Vitamin C (VitC) *sufficient* mice demonstrated a significantly higher stiffness (calculated at mid specimen) compared with the other groups. There was no statistically significant difference in the wound stiffness between the *deficient* and *deficient + AscA* groups ($n = 6-12$; ns, non-significant).

bone marrow derived stromal cells decreased wound healing, and that supplementing with VitC failed to rescue these cells (44). Therefore, we interpret these results as insufficient uptake of VitC in the healing wound and that perhaps, an additional topical application of VitC at the wound site or a higher dose of i.p. VitC could prove beneficial.

Using HnDF cells, we showed that AscA exposure resulted in a dose-dependent and significant increase in intracellular AscA uptake and fibroblast proliferation (Figure 8). This was associated with a dose-dependent inhibition of p27 expression (Figure 9). The inhibitors p21 and p27 suppress cell-cycle progression by inhibiting cyclin-dependent kinases as well as by down-regulating expression of other genes involved with cell-cycle progression (45). Conversely, AscA treatment was associated with a dose-dependent increase in the expression of pluripotent and self-renewal genes, Nanog and OCT4 (46).

These findings suggest a crucial role for VitC in regulating fibroblast proliferation. Although this is a novel finding in fibroblasts, there is precedence for these actions of VitC in stem cells, and in particular the adipose-derived stem cells wherein VitC has been used to increase the yield and regenerative potential of adipose-derived stem cells (47).

IL-6 is a cytokine with pleiotropic functions that is produced by several cell types in the wound microenvironment including fibroblasts (25,48). It plays an important role in development of Th17 cells and is known to contribute to the inflammatory component of autoimmune diseases (49). However, recent studies suggest a pro-resolution and anti-inflammatory role for IL-6 in wound-healing environments (27). Kuhn *et al.* showed that inhibition of IL-6 resulted in impaired wound healing due to decreased epithelial proliferation (50). IL-6 knockout mice exhibit delayed wound healing (delayed re-epithelialisation, granulation tissue formation and sub-optimal inflammatory response) compared with wild-type mice; this could be reverted with recombinant IL-6 (51,52). Further, Gallucci *et al.* reported IL-6 signalling to promote dermal fibroblast differentiation into myofibroblasts, increase their motility and also promote matrix remodelling (25,53). We found that exposure of HnDF cells to AscA promoted a dose-dependent increase in IL-6 mRNA levels (Figure 9F). These findings suggest a novel mechanism by which VitC could promote fibroblast motility and matrix remodelling. Presumably, insufficient fibroblast VitC levels could impair IL-6 expression and delay the resolution of wound healing.

Thus, on the basis of these *in vitro* studies, we speculate that in the absence of adequate VitC supplementation, the proliferation and maturation phases of wound healing would be delayed or would remain incomplete, thereby resulting in non-healing wounds.

Conclusion

Wound repair is a complex process that requires the co-ordination of various local cellular and biochemical events. To date, the majority of studies have used oral supplementation to augment plasma/tissue levels of VitC. However, recent studies by Padayatty and coworkers and others have shown that oral supplementation does not provide the requisite VitC levels for restoration of plasma VitC levels (21,30). We have recently shown that parenteral routes of VitC administration are safe and effective in critical care situations in both mice and humans

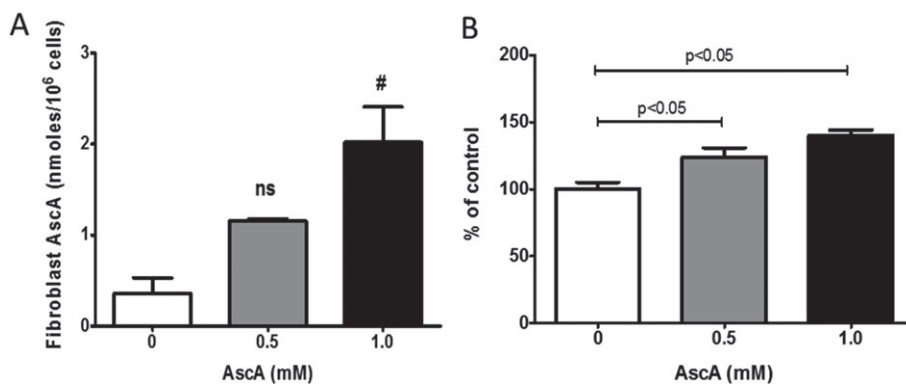


Figure 8 Vitamin C (VitC) (A) uptake and proliferation (B) by HnDF. (A) HnDF VitC concentrations were determined as described in Methods following exposure to AscA for 3 hours (0.5 and 1.0 mM) ($n = 3$ for each group, ns, not significant). (B) HnDF loading with AscA at 0.5 and 1.0 mM for 24 hours resulted in a dose-dependent increase in cell proliferation ($n = 3$ for each group).

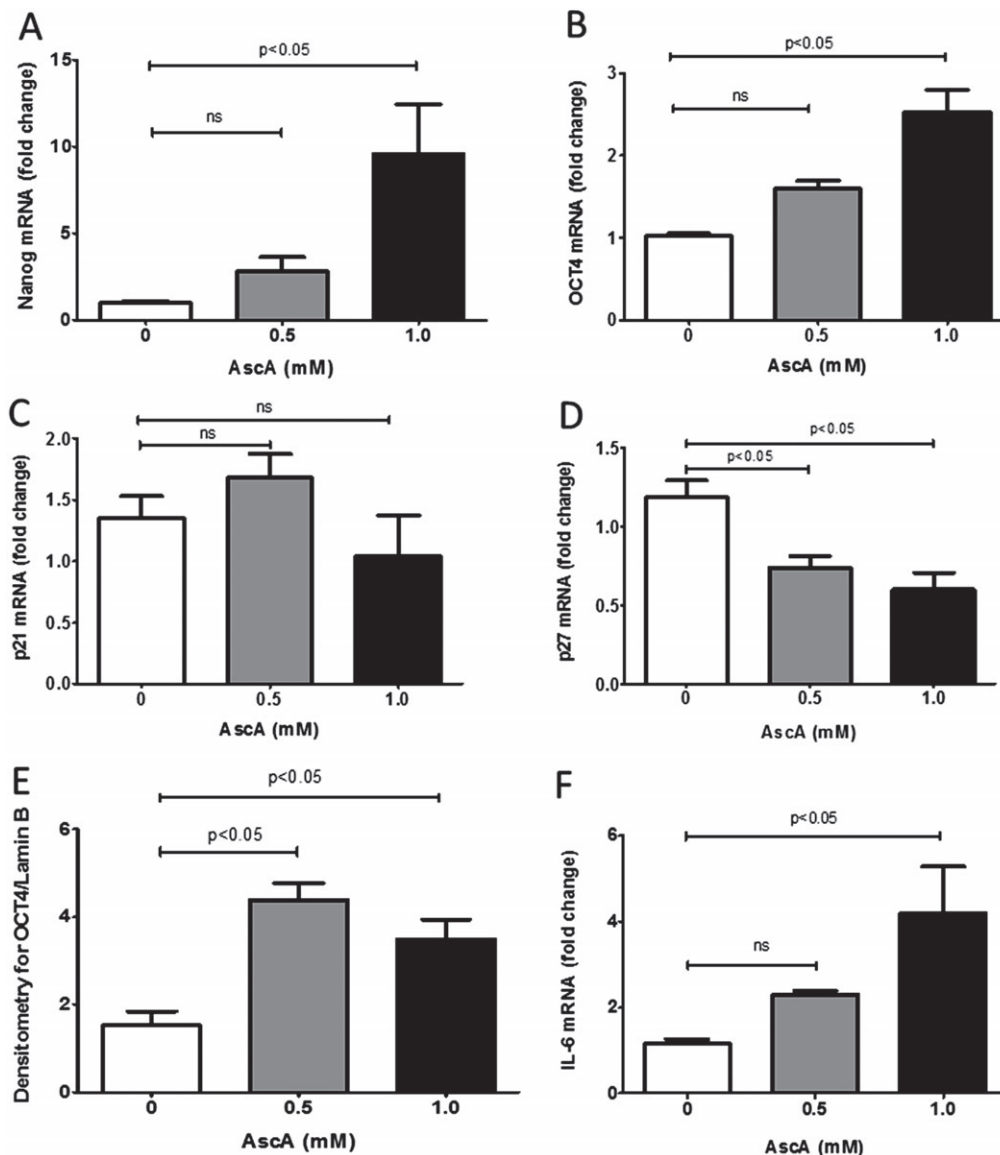


Figure 9 Real-time quantitative polymerase chain reaction (qPCR) for Nanog (A), Oct4 (B), p21 (C), p27 (D) and interleukin-6 (IL-6) (F) from HnDF cells. (A–D and F) HnDF cells were exposed to AscA (0.5 and 1.0 mM) for 3 hours. RNA was harvested and real time QPCR for Nanog, OCT4, p21, p27 and IL-6 was performed as described in Methods ($n = 3$ for each group; ns, not significant). (E) Densitometry for nuclear expression of OCT4 and lamin B from HnDF cells loaded with AscA 0.5 and 1 mM for 24 hours ($n = 3–6$ for each group).

(31). Moreover, these doses of VitC, when administered parenterally, were sufficient to restore circulating plasma VitC levels. The current study presents new evidence that beyond its well characterised role in collagen metabolism, VitC plays a crucial role in orchestrating multiple wound-healing processes. VitC repletion by parenteral infusion has the potential to be a safe and inexpensive therapy for enhancing tissue repair and shortening healing time.

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