



Cellular benefits of single-use negative pressure wound therapy demonstrated in a novel ex vivo human skin wound model

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Abstract

Negative pressure wound therapy is a widely used treatment for chronic, nonhealing wounds. Surprisingly, few studies have systematically evaluated the cellular and molecular effects of negative pressure treatment on human skin. In addition, no study to date has directly compared recently available single-use negative pressure modalities to traditional negative pressure devices in a controlled setting. Here we developed a novel large-scale ex vivo human skin culture system to effectively evaluate the efficacy of two different negative pressure wound therapy modalities. Single-use and traditional negative pressure devices were applied to human ex vivo wounded skin sheets cultured over a period of 48 hours. Cellular tissue response to therapy was evaluated via a combination of histological analysis and transcriptional profiling, in samples collected from the wound edge, skin adjacent to the wound, and an extended skin region. Single-use negative pressure wound therapy caused less damage to wound edge tissue than traditional application, demonstrated by improved skin barrier, reduced dermal-epidermal junction disruption and a dampened damage response. Transcriptional profiling confirmed significantly less activation of multiple pro-inflammatory markers in wound edge skin treated with single-use vs traditional negative pressure therapy. These findings may help to explain the greater efficacy of sNPWT in the clinic, while offering a noninvasive system to develop improved NPWT-based therapies.

1 | INTRODUCTION

Wound healing is a sophisticated and dynamic process that has evolved to close breaches to the skin barrier in a timely fashion. Alterations to any aspect of this normal response can result in chronic

healing impairment.¹ Chronic wounds are a significant physical, psychological and socioeconomic burden,² increasing in prevalence due to the expanding aging population and rising incidence of diabetes.^{3,4} Despite improved understanding of the molecular and cellular basis of wound chronicity,^{5,6} current treatments remain inadequate. This is, in part, due to a lack of appropriate pre-clinical models to effectively evaluate new wound treatments.

Negative pressure wound therapy (NPWT) is recommended for the treatment of hard-to-heal chronic wounds,⁷ as well as burns, skin

Abbreviations: EZ, extended zone; FC, fold change; H&E, hematoxylin and eosin; K6, keratin 6; P, papillary; PW, peri-wound; R, reticular; sNPWT, single-use negative pressure wound therapy; tNPWT, traditional negative pressure wound therapy; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WE, wound edge.

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grafts and surgical wounds.⁸ NPWT provides controlled delivery of subatmospheric pressure to wound tissue, thus improving tissue granulation, increasing tissue perfusion and removing excessive exudate.⁹ Numerous devices are currently available, including traditional, portable, disposable and specialist.³ Traditional NPWT involves the use of a large pump and canister to deliver therapy and collect exudate, and a wound filler (gauze or foam) to aid negative pressure delivery.¹⁰ Traditional NPWT has come under recent scrutiny, with suggestion that wound filler can hinder re-epithelialization¹¹ and cause foreign body reactions,¹² while the large traditional devices are often difficult to use and correctly seal.¹³

Smaller, less cumbersome devices have been developed with the aim to circumvent the limitations described for traditional NPWT.¹⁴ These single-use modalities have shown effectiveness (increased macroscopic wound closure) in recent randomized clinical trials vs both standard dressings^{15,16} and traditional NPWT devices.¹⁷ However, these clinical studies have not addressed the cellular basis for improved single-use NPWT efficacy vs traditional NPWT. The aim of the current study was therefore to directly compare the cellular and molecular changes induced by single-use and traditional NPWT devices applied to a pre-clinical human ex vivo skin wound model.

2 | MATERIALS AND METHODS

2.1 | Devices

A traditional canister-based NPWT device (tNPWT; RENASYS GO, TJ Smith and Nephew Ltd, Hull, UK) was used with wound filler foam. A smaller, more portable and lightweight single-use NPWT (sNPWT; PICO; TJ Smith and Nephew Ltd) device requiring no canister, was used without filler material.

2.2 | Human ex vivo culture system

Commercially available silicone (Silskin, Belfast, UK) was cast to 10 cm × 10 cm and used to model underlying human tissue. A 3 cm × 3 cm × 1 cm (W × H × D) circular “wound” was created in the center of the Silskin, which was mounted in the lid of a 25 cm × 25 cm petri dish. Nutrient supplementation was provided by Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin solution and 1% amphotericin B (all Thermo Fisher Scientific, Paisley, UK) added to the base of the petri dish. Small channels were created in the Silskin to allow media to reach the human skin at the top of the system by capillary action.

Abdominal skin was collected from four human donors (age range: 50–61) undergoing elective surgeries at Castle Hill Hospital (Hull, United Kingdom). Skin was collected with institutional approval and full written, informed patient consent under LREC 17/SC/0220. Freshly collected large skin pieces (approximately 10 cm × 10 cm) were defatted and placed over the Silskin support. The skin was wounded (3 cm diameter) in register with the Silskin “wound”. tNPWT or sNPWT

dressings were applied to the apical surface of the skin, sealed and placed under NPWT (100 and 80 mmHg, respectively). The culture systems were incubated at 37°C and 5% CO₂ for 2 days prior to collection of tissue for histology and transcriptional profiling. A schematic of the human ex vivo skin culture setup is shown in Figure 1.

2.3 | Skin collection

Cultured skin samples were collected from the wound edge (WE), a region approximately 1 cm behind the wound edge and under the middle of the dressing (peri-wound, PW), and a region under, but approximately 1 cm from the edge, of the dressing/drape (extended zone, EZ). Collected skin was cut in half, with one half fixed in neutral buffered formalin for histology, and one half flash frozen in liquid nitrogen for PCR array profiling.

2.4 | Histological analysis

Histology was performed blinded on randomized samples to assess skin structural changes using Hematoxylin and Eosin (H&E), loricrin and picrosirius red, and cellular responses using keratin 6 (K6), Ki67 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Fixed skin was processed and embedded for wax-based histological analysis. Here, samples were embedded so that the side corresponding to the defined region was face-down in the wax mold. Sections were cut at 5 μm thickness, dewaxed in xylene and brought to water down an ethanol gradient (100%–50% ethanol). Hematoxylin and Eosin (H&E) staining was performed using filtered Gill's hematoxylin (Thermo Fisher Scientific) and Eosin B (Sigma-Aldrich, Dorset, UK). Slides were dehydrated to 100% ethanol, cleared in xylene and mounted with Pertex mounting medium (CellPath, Shrewsbury, UK). All brightfield images were assessed on a Nikon E400 microscope with SPOT camera (Image Solutions [UK] Ltd, Preston).

For immunofluorescence, rehydrated sections were blocked in goat serum and incubated overnight at 4°C in rabbit anti-Keratin 6A, rabbit anti-Loricrin (both Biolegend, London, UK) or mouse anti-Ki67 (Leica, Milton Keynes, UK) primary antibodies. Appropriate Alexa Fluor 594-conjugated secondary antibodies were used at 1 μg/mL. Slides were mounted in Mowiol 4-88 (Sigma-Aldrich) containing DAPI at 5 μg/mL (Thermo Fisher Scientific). Fluorescent images were captured on a confocal laser scanning microscope (LSM 710, Zeiss) using 405 nm diode (DAPI), 488 nm argon (TUNEL, below) and 561 nm (K6, Ki67 and loricrin) lasers. Epidermal-dermal junction damage was measured as the percentage of the epidermis not attached to the dermis (from total epidermal length). Barrier integrity was assessed by measuring the percentage coverage of loricrin in the upper epidermis. The percentage of K6 staining in the epidermis demonstrated the level of damage response, while the percentage of Ki67+ve basal epidermal cells showed proliferation effects. All analysis was performed in ImageJ v.1.8 (National Institutes of Health, US) using a conserved threshold across all images.

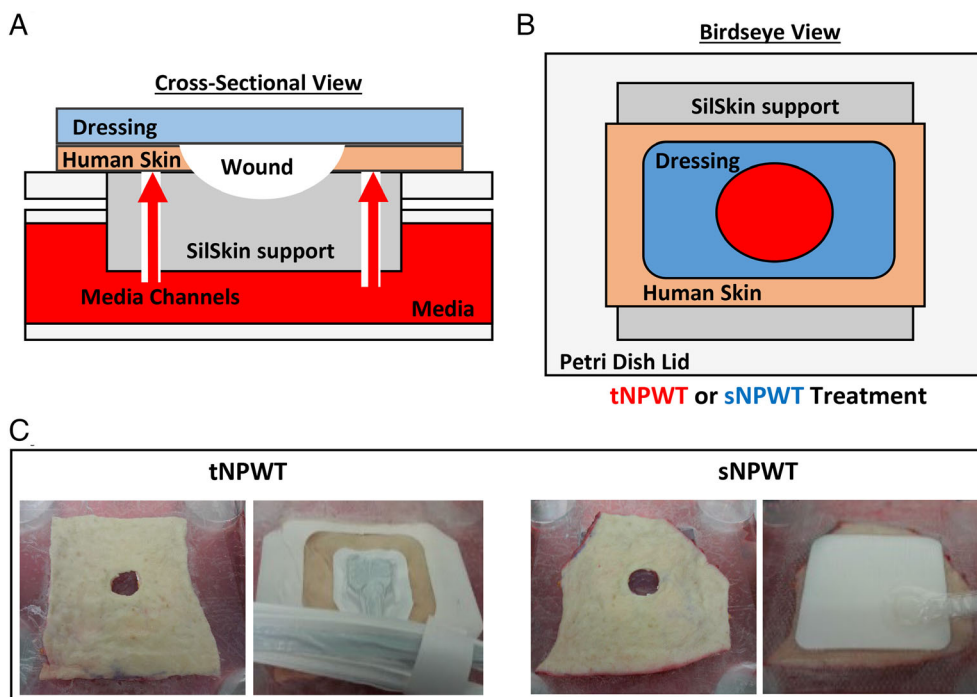


FIGURE 1 The custom-fabricated human ex vivo skin culture system designed for testing negative pressure wound therapy devices. The cross-sectional view (A) shows that dressings are applied topically to human skin supported by artificial dermal material (Silskin) in a large petri dish culture system. To mimic injury, a wound is created in the Silskin and human skin. Channels are also made in the Silskin to allow media to reach the live human skin via capillary action. The birdseye view (B) shows application of traditional negative pressure wound therapy (tNPWT, red) or single-use NPWT (sNPWT, blue). Adhesive dressing tape is used to seal the system to allow negative pressure to form. Photographs (C) again demonstrate tNPWT and sNPWT dressing application to ex vivo cultured human skin [Color figure can be viewed at wileyonlinelibrary.com]

2.5 | Terminal deoxynucleotidyl transferase dUTP nick end labeling

TUNEL staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Sigma-Aldrich) as per manufacturer's instructions. Antigen retrieval was achieved with 20 $\mu\text{g}/\text{mL}$ Proteinase K (Thermo Fisher Scientific). Slides were mounted in Mowiol 4-88 and fluorescent images were taken as described above. TUNEL positive (green) cells were counted as a percentage of total cells (DAPI, blue) to elucidate epidermal and dermal tissue viability.

2.6 | Picrosirius red staining

Sections were stained with picrosirius red to assess dermal extracellular matrix damage.¹⁸ Here, red birefringence indicates mature matrix fibers, while yellow-green birefringence shows immature matrix fiber composition. Percentages of red and yellow-green matrix was determined using the ImageJ Color Deconvolution plugin (which implements stain unmixing following Ruifrok and Johnston's method¹⁹). The percentage of each separated color was then determined via thresholding in ImageJ v.1.8. Papillary and reticular dermis were analyzed separately.

2.7 | RNA isolation

RNA was isolated from snap frozen skin.²⁰ Briefly, skin was homogenized in TRIzol reagent (Thermo Fisher Scientific) and RNA was separated using phenol and chloroform. RNA was purified using the Invitrogen Ambion PureLink RNA Mini Kit (Thermo Fisher Scientific) following manufacturer's instructions.

2.8 | PCR arrays

Prior to cDNA synthesis, RNA was adjusted to a final concentration of 1 μg RNA per μL of RNase free water (Qiagen, Manchester, UK). Each sample was reverse transcribed with Random Primers (Promega, UK) and Bioscript Reverse Transcriptase (Bioline, Nottingham, UK). cDNA was plated into the 96 well RT² Profiler PCR array plates (human wound healing gene panel, Qiagen) with Takyon SYBR mastermix (Eurogentec, Hampshire, UK). Plates were run on a CFX Connect thermal cycler using CFX Manager Software (Biorad, Hertfordshire, UK). R v.3.6.1 was used for *t* test comparisons (tNPWT vs sNPWT), while clustering was based on Euclidian distance and Ward D2's method using the R packages "RColorBrewer" and 'gplots'.²¹

2.9 | Statistical analyses

All histological data are presented as mean + SE of the mean (SEM). Two-way ANOVAs were performed on all histological data, with Tukey *post-hoc* analysis where applicable (GraphPad Prism, San Diego, US).

3 | RESULTS

Single-use negative pressure wound therapy protects against dermal-epidermal junction damage and maintains peri-wound epidermal differentiation. Wounded skin cultured under NPWT for 48 hours was collected for histological and transcriptional assessment (Figure 2A). Histology was used to determine skin structural changes and cellular responses to culture under sNPWT vs tNPWT (Figure 2B). Analysis of H&E stained tissue sections revealed that tNPWT led to a loss of epidermal-dermal adhesion in the wound edge region, a phenotype that was not observed following sNPWT ($P < .001$; Figure 2C,E). Interestingly, a trend toward increased epidermal-dermal damage was also observed in the tNPWT-treated peri-wound and extended zone regions, although this was not statistically significant. Next, the effect of NPWT on localization of loricrin, a key protein required for skin barrier integrity, was assessed (Figure 2D,F). Here, a greater amount and extent of loricrin expression was observed following sNPWT vs tNPWT at both the wound edge ($P < .001$) and peri-wound region ($P < .001$). No differences in loricrin localization between treatment groups was observed in the extended zone region. Collectively, these data reveal specific changes in the peri-wound region of human skin treated with tNPWT, but not sNPWT, which will adversely impact on healing.

Single-use negative pressure treatment leads to a reduced wound edge damage response compared to tNPWT application. Keratin 6 (K6) is an epidermal damage-induced keratin, that is both necessary and functionally important for repair.^{22,23} Significantly less wound edge induction of K6 was observed following sNPWT vs tNPWT ($P < .01$). In fact, the levels observed in sNPWT-treated wound edge skin were only marginally greater than peri-wound and extended zone skin (Figure 3A,C). The improved viability of epidermis under sNPWT was further indicated by increased basal cell proliferation at both wound edge ($P < .001$) and peri-wound regions ($P < .05$) vs tNPWT (Figure 3B,D). Thus, it is clear that sNPWT circumvents the tNPWT-induced epidermal damage response in *ex vivo* cultured human skin.

Single-use negative pressure wound therapy reduces wound edge apoptosis and prevents extracellular matrix degradation associated with traditional application. TUNEL staining was performed to assess levels of cellular apoptosis in human *ex vivo* skin cultured under NPWT (Figure 4A). Wound edge epidermis showed substantially increased cell death under tNPWT compared to sNPWT ($P < .001$; Figure 4B), while epidermis in the peri-wound and extended zone remained largely unaffected by NPWT. Apoptosis in the dermis also displayed a nonsignificant trend toward increased cell death following tNPWT (Figure 4C).

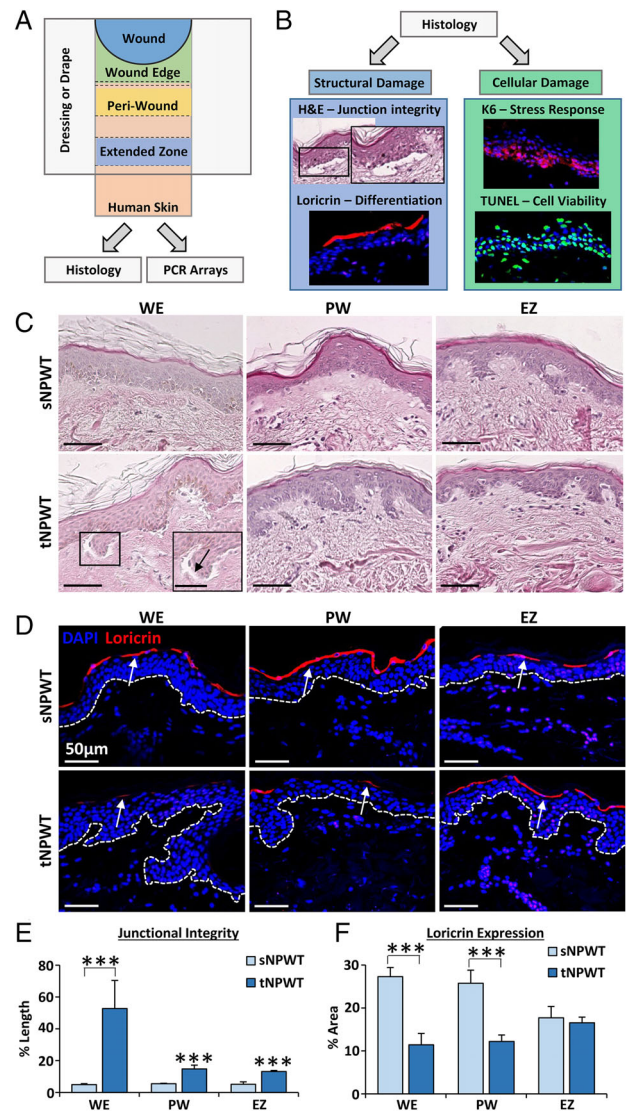


FIGURE 2 Single-use negative pressure wound therapy prevents loss of epidermal-dermal adhesion and protects against damage to the stratum corneum. Schematic indicating skin regions collected for analysis following 2 days of culture under traditional negative pressure wound therapy (tNPWT) or single-use NPWT (sNPWT, A). Wound edge = WE, peri-wound = PW and extended zone = EZ. Histology was performed to determine structural changes (eg, epidermal-dermal adhesion and barrier integrity) and associated cellular damage effects (eg, apoptosis, B). sNPWT protected against WE epidermal-dermal junction separation (arrows, C, E) and loss of barrier integrity (loricrin staining, D, F). Hematoxylin and eosin (H&E) bar = 50 μ m. Inset bar = 25 μ m. Loricrin staining (arrows) = red. DAPI = blue nuclei. White, dotted lines show epidermal-dermal margins. Bar = 50 μ m. Mean \pm SEM. *** $P < .001$. Two-way ANOVA with Tukey *post-hoc* analysis. Asterisks alone vs WE tNPWT [Color figure can be viewed at wileyonlinelibrary.com]

To assess wound edge matrix changes, the dermis was split into upper (papillary) and lower (reticular) regions, where matrix fiber compositions are known to be distinct.²⁴ Picosirus red (PSR) histological staining was imaged under polarized light to differentiate thick (red) and thin (green) fibers. A greater volume of papillary red fibers were

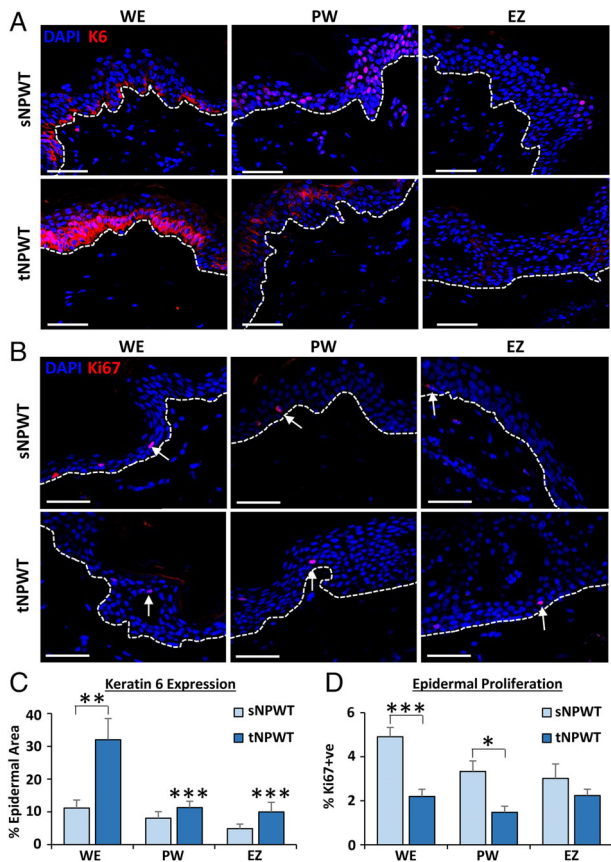


FIGURE 3 Single-use negative pressure wound therapy causes a reduced epidermal damage response. Keratin 6 (K6) expression was significantly lower in wound edge (WE) skin following single-use negative pressure wound therapy (sNPWT), compared to traditional NPWT (tNPWT; A, C). Peri-wound = PW and extended zone = EZ. Cellular proliferation (basal Ki67+ve cells) was also higher in WE skin following sNPWT (B, C). Red staining = K6 and Ki67 (white arrows). DAPI = blue nuclei. White, dotted lines show epidermal-dermal margins. Bar = 50 μ m. Mean + SEM. * $P < .05$, ** $P < .01$, *** $P < .001$. Two-way ANOVA with Tukey *post-hoc* analysis. Asterisks alone vs WE tNPWT [Color figure can be viewed at wileyonlinelibrary.com]

observed in wound edge skin following sNPWT than tNPWT ($P < .05$; Figure 4D,E). Papillary green fibers were unchanged between sNPWT and tNPWT in all skin regions. No significant matrix differences were observed in the reticular dermis (Figure 4F). Collectively, these data show that sNPWT protects against tNPWT-induced epidermal apoptosis and reveals between-treatment differences in dermal papillary matrix fiber thickness.

Damage marker gene expression is co-ordinately upregulated in the wound edge only following traditional negative pressure therapy. PCR arrays were employed to determine transcriptional differences in response to application of sNPWT or tNPWT. Hierarchical clustering analysis revealed clear clustering on treatment modality (sNPWT or tNPWT; Figure 5A). Of note, expression profiles following sNPWT treatment were similar across all three skin regions (wound edge, peri-wound and extended zone). By contrast, the wound edge tNPWT-treated skin was transcriptionally distinct, with unique upregulation of

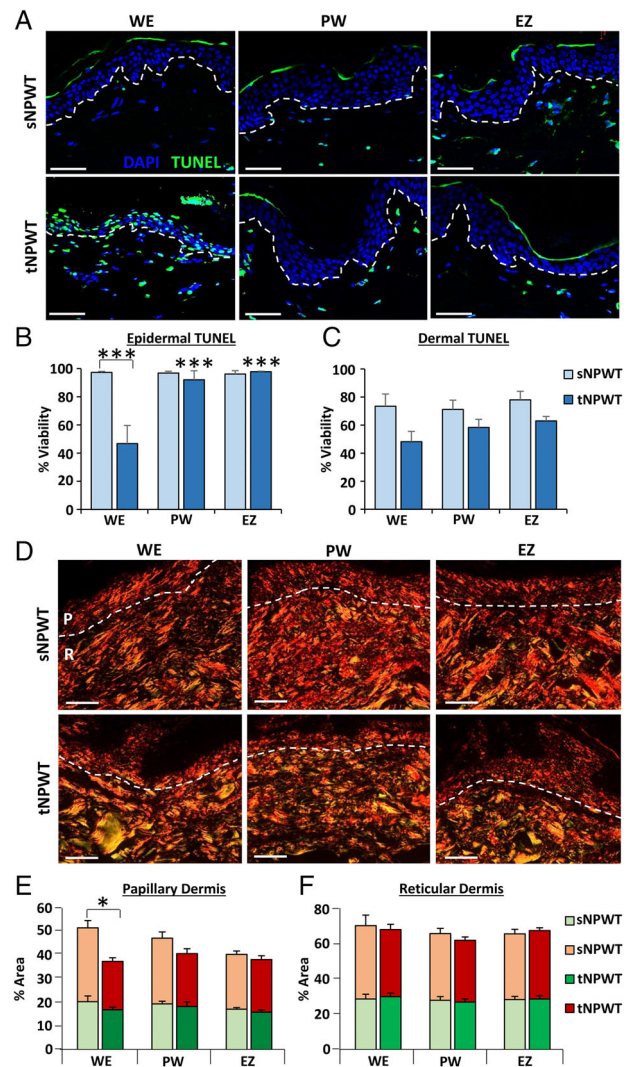


FIGURE 4 Single-use negative pressure wound therapy protects against cellular apoptosis and extracellular matrix degradation. Epidermal and dermal apoptosis were higher in wound edge (WE) skin following traditional negative pressure wound therapy (tNPWT) compared to single-use NPWT (sNPWT; A-C). Peri-wound = PW and extended zone = EZ. White, dotted lines show epidermal-dermal margins. Arrows = TUNEL+ve (apoptotic, green) cells. Picosirius red staining (D) demonstrates increased red fibers in the papillary (P) dermis (E), but not reticular (R) dermis (F), following sNPWT. White, dotted lines separate P and R dermis. Bars = 50 μ m. Mean + SEM. * $P < .05$, *** $P < .001$. Two-way ANOVA and Tukey *post-hoc* analysis. Asterisks alone vs WE tNPWT [Color figure can be viewed at wileyonlinelibrary.com]

44 of the 84 genes (52%) assessed ($P < .05$; Figure 5B). Genes from specific clusters were separately interrogated as fold change between tNPWT and sNPWT (Figure 5C-F). Wound edge skin under tNPWT showed significantly higher levels of a number of pro-inflammatory factors, including the chemokines, *CSF2* ($P < .01$, cluster 2), *CCL7* ($P < .01$, cluster 2) and *CXCL1* ($P < .05$, cluster 5), the cytokines, *IFNG* ($P < .05$, cluster 2), *IL1B* ($P < .05$, cluster 3) and *TNF* ($P < .01$, cluster 2), and the matrix metalloproteinases, *MMP2* ($P < .05$, cluster 5) and

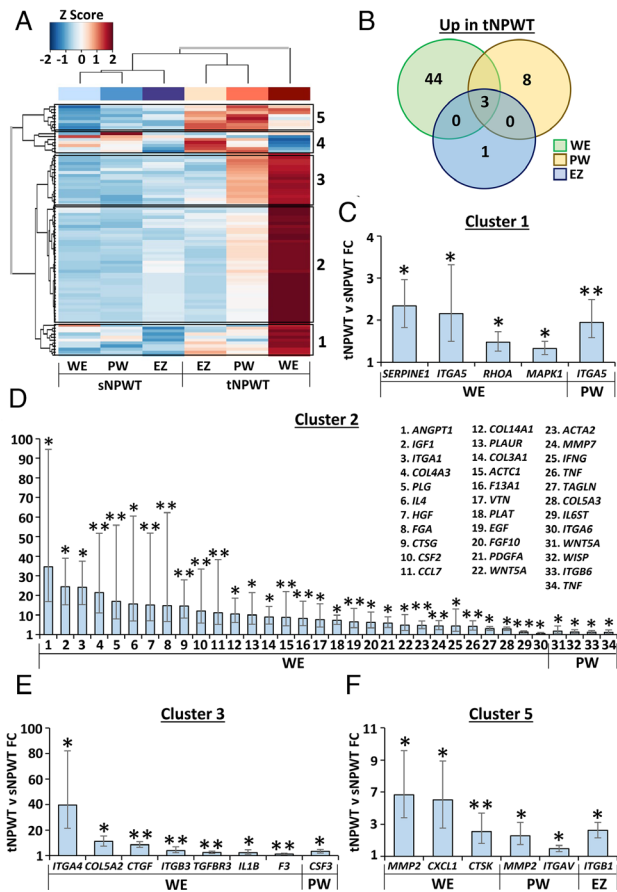


FIGURE 5 Traditional negative pressure wound therapy induces upregulation of damage response genes at the wound edge. Hierarchical clustering analysis was performed on PCR array data, where samples clustered by treatment group (A). Genes formed distinct expression clusters, with chosen clusters labeled 1 to 4. Wound edge = WE, peri-wound = PW and extended zone = EZ. Venn diagram depicting the number of genes upregulated ($P < .05$) under traditional negative pressure wound therapy (tNPWT) vs single-use NPWT (sNPWT) by region (B). PCR-derived fold changes (FC, tNPWT vs sNPWT) in individual genes from each cluster (C–F). Mean \pm compound SEM. * $P < .05$, ** $P < .01$. $n = 3$ independent donors. Independent two-tailed student's t tests were performed [Color figure can be viewed at wileyonlinelibrary.com]

MMP7 ($P < .01$, cluster 2). The peri-wound region also displayed higher levels of *CSF3* ($P < .05$, cluster 4), *MMP2* ($P < .05$, cluster 5) and *TNF* ($P < .05$, cluster 2) following tNPWT, compared to sNPWT. These findings confirm an increased damage response in the tNPWT-treated wound edge skin that is not observed following sNPWT treatment.

4 | DISCUSSION

Traditional NPWT was first used in the 1990s, improving granulation tissue formation in pigs and in the clinic.^{25,26} NPWT is now a widespread wound care treatment, not only used to treat chronic wounds, but also in surgical incisions,²⁷ burns²⁸ and trauma injuries.²⁹ New,

portable single-use (sNPWT) modalities are now available that deliver negative pressure using a multi-layered, canister-free technology,³⁰ that is more economically viable than traditional modalities.³¹ Here we developed and implemented a pre-clinical human ex vivo skin culture system to directly compare the biological effects of sNPWT and tNPWT devices. We show for the first time that sNPWT induces substantially less epidermal damage, apoptosis and inflammation than tNPWT in human skin ex vivo.

The observation that tNPWT can heighten inflammation is supported previously.^{32,33} However, these reports have largely focused on the effects of sustained compression and damage at the wound bed: foam filler interface. Retention of wound filler can also increase inflammatory cell influx and promote foreign body reactions.^{12,34,35} Our data now suggest the importance of considering wound-edge epidermal-derived inflammatory mediators. Indeed, heightened inflammation is a key contributing factor in pathological healing.³⁶

In the present study, tNPWT was shown to induce a heightened damage response in the epidermis, upregulating keratin 6 and increasing the expression of pro-inflammatory markers at the wound edge. While short-term inflammation is required to initiate the wound healing cascade, prevent infection and trigger keratinocyte migration,³⁷ sustained release of cytokines drives excessive wound inflammation.³⁸ Further, tNPWT-induced inflammation led to elevated epidermal apoptosis and epidermal-dermal junction break down, not dissimilar to that observed following pressure injury.³⁹ Matrix metalloproteinase levels were also higher following tNPWT, as shown in our previous in vivo study,³⁵ suggesting the potential to contribute to delayed healing via excessive proteolysis of matrix components.⁴⁰ Collectively, our ex vivo findings suggest that sNPWT beneficially alters multiple aspects of wound edge physiology vs tNPWT, and this may confer improved clinical efficacy. We note that a limitation of the current study is the difference in absolute negative pressure delivered by the two devices.

Ex vivo human wound models provide an ethical alternative to nonhuman in vivo studies and reduced risk vs invasive clinical studies.⁴¹ However, existing ex vivo culture models use small skin explants (up to 1 cm width) and are therefore incompatible with the application of NPWT devices. Our study provides the first report of a considerably upscaled (10 cm width), viable ex vivo human skin culture model suitable for testing NPWT and other wound care devices. It is important to acknowledge that ex vivo human wound models cannot fully recapitulate the complexities of the in vivo healing environment.^{41,42} For example, the model used in this study is not suitable to assess granulation tissue formation or adipose contribution to healing, and lacks system factors (eg, hormones) blood flow or active lymphatics. The key advantages of the reported model are that it retains native human skin structure and bypasses cross-species differences that can hinder interpretation of nonhuman in vivo wound studies. Overall this study has provided crucial new insight into the relative effects of two existing wound care devices (sNPWT and tNPWT) on wound edge skin biology. Further in-depth studies are now needed to fully elucidate sNPWT's cellular mode(s) of action.

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CONFLICT OF INTEREST

Varuni R. Brownhill is an employee of TJ Smith and Nephew Ltd. The authors have no other competing financial interests.

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