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A Pilot Study of Negative Pressure Therapy with Autologous Skin Cell Suspensions in a Porcine Model

Bonnie C. Carney, PhD,[a,b,e] *Lauren T. Moffatt, PhD,*[a,b,e] *Taryn E. Travis, MD,*[b,c,e] *Saira Nisar, MBBS, MS,*[b] *John W. Keyloun, MD,*[b,d] *Nicholas J. Prindeze, MD,*[b,d] *Mary A. Oliver, BS,*[b] *Liam D. Kirkpatrick, BA,*[b] *and Jeffrey W. Shupp, MD*[a,b,c,e,*]

[a] *Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC*

[b] *Firefighters' Burn and Surgical Research Laboratory, MedStar Health Research Institute, Washington, DC*

[c] *The Burn Center, Department of Surgery, MedStar Washington Hospital Center, Washington, DC*

[d] *Department of Surgery, MedStar Washington Hospital Center and MedStar Georgetown University Hospital, Washington, DC*

[e] *Department of Surgery, Georgetown University School of Medicine, Washington, DC*

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ABSTRACT

Background: Negative pressure wound therapy (NPWT) is an option for securing meshed split thickness skin grafts (mSTSGs) after burn excision to optimize skin graft adherence. Recently, the use of autologous skin cell suspension (ASCS) has been approved for use in the treatment of burn injuries in conjunction with mSTSGs.To date, limited data exists regarding the impact of NPWT on healing outcomes when the cellular suspension is utilized. It was hypothesized that NPWT would not negatively impact wound healing of ASCS+mSTSG.

Materials and Methods: A burn, excision, mSTSG, ASCS ± NPWT model was used. Two Duroc pigs were utilized in this experiment, each with 2 sets of paired burns. Four wounds received mSTSG+ASCS+NPWT through post-operative day 3, and 4 wounds received mSTSG+ACSC+ traditional ASCS dressings. Cellular viability was characterized prior to spraying. Percent re-epithelialization, graft-adherence, pigmentation, elasticity, and blood perfusion and blood vessel density were assessed at multiple time points through 2 weeks.

Results: All wounds healed within 14 days with minimal scar pathology and no significant differences in percent re-epithelialization between NPWT, and non-NPWT wounds were observed. Additionally, no differences were detected for pigmentation, perfusion, or blood ves-

* Corresponding author. The Burn Center, 110 Irving Street, NW, Suite 3B-55, Washington, DC 20010, Tel.: 202.877.7302
E-mail address: jeffrey.w.shupp@medstar.net (J.W. Shupp).

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sel density. NPWT treated wounds had less graft loss and improved elasticity, with elasticity being statistically different.

Conclusions: These data suggest the positive attributes of the cellular suspension delivered are retained following the application of negative pressure. Re-epithelialization, revascularization, and repigmentation are not adversely impacted. The use of NPWT may be considered as an option when using ASCS with mSTSGs for the treatment of full-thickness burns.

Introduction

The standard of care for the treatment of full-thickness burns is excision and autografting with meshed split thickness skin grafts (mSTSG)[1-3]. When large total body surface area (TBSA) burns are sustained, donor sites from which to harvest STSGs are limited. Additionally, reducing the amount of donor skin harvested can be of value for all patients regardless of burn size, as donor sites can be a source of significant pain and morbidity.

The RECELL System enables a clinician at the point-of-care to prepare an autologous skin cell suspension (ASCS) containing keratinocytes, melanocytes, and fibroblasts from a small piece of the patient's skin[4]. Typical equipment for meshing STSG only allows for expansion of up to six times the original size. With the RECELL System, an expansion of up to 80 times is reported, hence reducing donor site requirements. ASCS has been used to treat donor site wounds[5], deep partial-thickness (DPT) burns[6,7] and mixed-depth burns inclusive of full-thickness injury. For deeper burns without continuous dermis, ASCS is used as an adjunct to a widely meshed STSG. Recently, Holmes et. al demonstrated that the use of the RECELL System resulted in comparable healing outcomes compared to less widely mSTSG-treated full thickness burn wounds without ASCS in a cohort of 30 patients, with a reduction in required donor site size.[8]

In many burn centers, the standard of care fixation method for mSTSG is the application of negative pressure wound therapy (NPWT) devices over autografted skin during the immediate postoperative period. Negative pressure wound therapy is helpful for successful graft adhesion, working by minimizing disruption by shear forces and promoting the continual removal of the wound bed drainage often associated with hematoma or seroma formation.

Negative pressure wound therapy is also thought to cause changes in the cytoskeletal conformation in cells, stimulate granulation tissue formation, and reduce the local inflammatory response[9]. There is also evidence of increased blood flow to the wound, reduced edema and exudate, and reduction of bacterial loads[9]. These advantages are ultimately helpful in reducing time to healing.

In a 2016 review published in *Burns*, there were 47 studies reporting the positive effect of NPWT when used as a bolster dressing on autografted sites[10]. Total and partial graft-loss, need for re-grafting, time to complete wound healing, and hospital length of stay were all reduced with NPWT over autograft. The reviewed studies included several randomized, controlled clinical trials. The review also investigated several other uses for NPWT, and of all the potential indications, the use of NPWT over fresh mSTSG sites had the best supportive data and was recommended by the authors for use in clinical practice.

Although it is generally accepted that NPWT contributes to better healing, there are few studies investigating the cellular mechanisms behind this treatment[11]. A recent paper described the mechano-signaling transduction potential of cells within mSTSGs when ex-vivo grafts were treated with NPWT[12]. There was differential expression of genes related to fibroblast proliferation and cytoskeletal conformational changes, genes related to angiogenesis, and a myriad of keratins and keratin-associated proteins were also increased after NPWT.

In each of the completed clinical trials assessing the effectiveness of the RECELL System as an adjunct to autografting, NPWT has not been evaluated as an immediate post-operative dressing. The lack of knowledge as to how applied ASCS would be affected by NPWT may cause some practitioners to avoid using ASCS in combination with NPWT. It is unknown whether the ASCS could be damaged by or drawn into the vacuum sponge component of an NPWT device. Conversely, it is likewise unknown if ASCS may be positively impacted by NPWT and successfully contribute to increased healing rates and reduced donor site requirements.

The purpose of this study is to evaluate the impact on healing when NPWT is used as a bolster dressing over a full-thickness defect treated with ASCS+mSTSG. Cell counts and viability, time to re-epithelialization, graft-adherence, pigmentation, elasticity, and blood vessel density and perfusion were evaluated. The pig was used as a model system due to its structural similarity to human skin and the plethora of papers that report the pig to be the most useful animal model for human wound healing studies[13,14].

Materials and Methods

Animal model

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee prior to beginning the experiments, and animals were housed and cared for in compliance with all relevant regulations governing animal use in research. These regulations include a 12-hour light/dark cycle, temperatures between 18-27°C with an average of 22 °C,

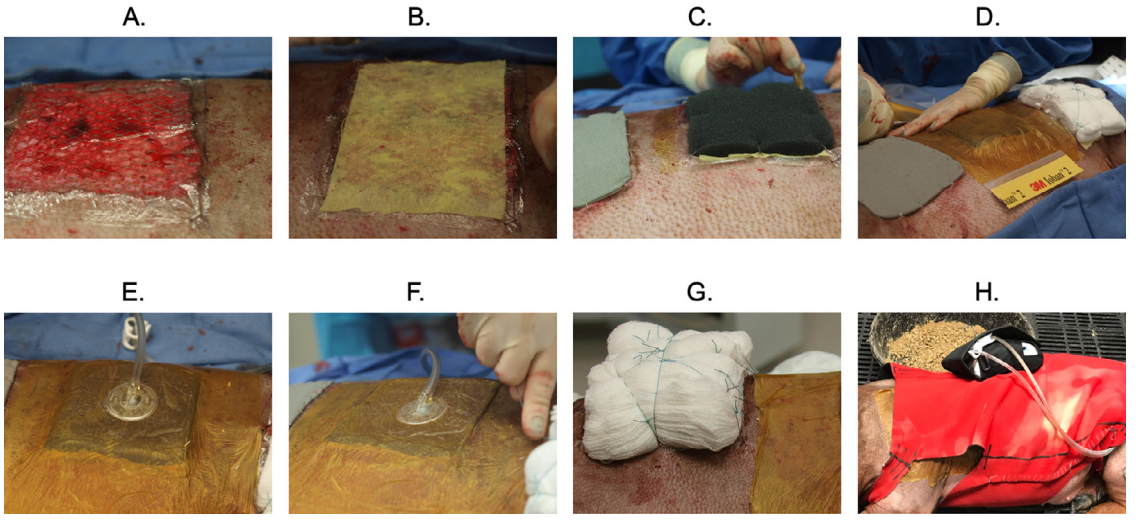

Fig. 1 – *mSTSGs were treated with either a NPWT device or a bolster dressing*
Telfa clear dressing was applied after RES spray as the primary dressing in all wounds regardless of treatment (A). Xeroform dressing was applied as the secondary dressing in all wounds regardless of treatment (B). A granulofoam sponge and benzoin were applied in the NPWT-treated wounds (C). Ioban was applied (D). The tubing was attached (E). A seal was created (F). A bolster dressing was applied to the non-NPWT group (G). The animal had a small NPWT device on its back during the 3-day treatment period (H).

humidity between 30%-70%, water *ad libitum*, a standard lab diet of 2%-3% of body weight/day (Envigo, Teklad), and two forms of enrichment. A burn, excision, mSTSG, ASCS model was used as described in the literature, with modifications[15]. Two juvenile (41.1, 44.1 kg), castrated, male Duroc pigs were used in this experiment, each with 2 sets of 2 burns created on each flank for a total of 4 wounds per pig (n=8 total wounds for treatment). Animals were fasted for at least 12 hours prior to anesthesia induction. Animals were anesthetized with an intramuscular injection of ketamine (15-30 mg/kg) and xylazine (1-4 mg/kg) and were subsequently intubated and remained on isoflurane anesthesia (3%-5%) for the remainder of the procedure as previously described[16]. Animals were monitored, with vitals signs recorded throughout the procedure and recovery period, and with anesthesia adjusted appropriately based on these metrics. On Day -2 and Day 0, peripheral IV access was established for administration of maintenance fluids during the procedure.

On Day -2, hair was removed to obtain a smooth surface for burning. Digital images and baseline 3 mm punch biopsies of uninjured skin were then taken from a site remote to the intended wounding area and were stored in AllProtect reagent for molecular assays (Qiagen, Valencia, CA) or formalin for histologic assays. Full-thickness burn wounds were created using a 4-inch by 4-inch aluminum branding iron heated to 150°C, applied for 15 seconds. A border of 2 inches was left between the two burns due to the intended treatment with a NPWT device which requires a seal achieved by adherence to uninjured skin. Punch biopsies (3 mm) were taken to confirm the depth of injury. The wounds were then dressed with Mepilex Ag (Molnlycke, Vastergotland, Sweden). A custom-made neoprene vest was applied over the animal to protect the wound sites[17]. A fentanyl patch was placed on a shaved portion of the skin for ongoing pain control.

On Day 0, dressings were removed and digital images and punch biopsies were taken of the burned tissues and stored in AllProtect as described above. The uninjured skin areas were shaved and surgically prepped. The burns were excised to the level of viable, bleeding tissue using a goulian knife and hemostasis was achieved with topical pressure. All eight wounds required excision down to subcutaneous fat with very little viable bleeding dermis observed. In one wound where the burn wound bed was not homogenous and contained small areas of uninjured skin, wounds were still excised down to fat as described above to limit the possibility that viable dermal appendages could contribute positively towards wound healing in one wound and not others. STSG was harvested from the upper portion of the lower extremity using a Zimmer dermatome at a depth of 0.012"[15]. A 6 cm^2 portion of the STSG was soaked in saline for subsequent processing using the RECELL System. The remainder of the STSG was meshed 4:1 (Brennan, Molnlycke) and applied to the prepared wound beds. The donor sites were dressed with Mepilex Ag (Molnlycke).

The unmeshed 6 cm^2 STSG was processed using the RECELL System to obtain ASCS according to the manufacturer's instructions (AVITA Medical, Valencia, CA). The same individual prepared the ASCS suspension in both animals to exclude inter-processor differences in preparation. The 6 mL of prepared ASCS was split into 4 different syringes and 1.5 mL of solution was sprayed on the mSTSG using a ratio of 1 to 80 as described by the manufacturer (Video 1). An aliquot of ASCS (20 μL) was retained for characterization as described below. On all wound beds, Telfa Clear dressing (Covidien, Dublin, Ireland) was applied directly after spraying cell suspension for

Table 1 – Wound treatments.

Animal	Wound	Treatment
1	Right (cranial)	Non-NPWT (bolster)
	Right (caudal)	NPWT
	Left (cranial)	NPWT
	Left (caudal)	Non-NPWT (bolster)
2	Right (cranial)	NPWT
	Right (caudal)	Non-NPWT (bolster)
	Left (cranial)	Non-NPWT (bolster)
	Left (caudal)	NPWT

primary dressing (Fig. 1A). Telfa Clear dressing was followed by Xeroform dressing (Covidien) (Fig. 1B).

In 1 of the 2 wounds per flank, granulofoam sponge, benzoin, and adhesive (Ioban, 3M Science, St. Paul MN) was applied (Fig. 1C and D), followed by an ACTIV.A.C. NPWT device (KCI, San Antonio, TX) (Fig. 1E). A medium intensity continuous setting of -125 mmHg suction was used with visible sponge compression and the absence of air leak confirmed via the NPWT device (Fig. 1F). The skin graft not treated with NPWT was secured using a tie over bolster created with gauze as the tertiary dressing over the Xeroform (Fig. 1G). The animal was then turned, and the procedure was repeated on the contralateral flank. On each animal, the sites that were treated with NPWT vs. bolster were switched (Table 1) to control for the differential healing potential of wounds created at different points on the cranial-caudal axes. The NPWT device was put into the carrying case provided by the manufacturer and was sutured to a custom-made neoprene vest that kept the animal's dressings in place (Fig. 1H). A one-time dose of Buprenorphine was given after extubation and during recovery, as soon as gross purposeful movement was observed. A second fentanyl patch was placed on a shaved portion of the skin for ongoing pain control.

The NPWT remained in place for 3 days. Every 10 hours, the NPWT device was paused, the tubing was clamped, and the NPWT was removed from the carrying case and was replaced with a fully charged device to ensure battery life. The tubing was then un-clamped and the device was turned back on. A total of 7 NPWT device changes occurred throughout the 72-hour period. Minor air leaks were detected immediately through the utilization of the alarm setting of the device. Minor air leaks that developed over the 72 hours were controlled with application of additional adhesive and resulted in a total time without suction of less than 2 hours.

On experimental Day 3, the NPWT was removed along with all secondary dressings, leaving the Telfa Clear in place. Punch biopsies were taken from the autografted sites and donor sites and were stored in AllProtect as described above. Telfa Clear is a transparent, thin dressing, and as such, punch biopsies were able to be taken through the dressing. On the autografted sites, xeroform dressing was replaced, and Mepilex Ag was added as a tertiary dressing for additional padding. A neoprene vest was applied over all dressings. The donor sites were dressed with Mepilex Ag alone.

On Day 5, wounds were assessed. All dressings were removed, including the Telfa Clear which was carefully removed as not to destroy the newly formed epidermis (Video 2). Digital photographs of wounds were taken to assess re-epithelialization. STSG adherence was assessed using a subjective scale as described below[18]. Laser doppler images were taken to assess perfusion. Biopsies of the autografted sites were taken from the areas of the grafted wound that contained mSTSG specifically and not the interstices. Biopsies of the donor sites were taken as described above. Xeroform was applied directly to the autografted sites as a primary dressing and Mepilex Ag was used to provide additional padding as a secondary dressing. Mepilex Ag was used to dress the donor sites. A neoprene vest was applied.

On Days 7, 12, and 14, a wound assessment took place as described above with dressing removal, wound cleasing, and the acquisition of digital and Laser doppler images, and biopsies. The autografted sites were gently cleansed with soap and water, and the rest of the surgical field was prepped with chlorhexidine. In addition, skin probes were used to non-invasively measure skin color and elasticity (Delfin Technologies, Stamford, CT). Skin color was measured with a "Skin color catch" probe that measures melanin. The probe uses 3 white LEDs corresponding to daylight, and illuminates a skin area of 0.3 cm^2. The light reflecting back from the skin is detected with an RGB sensor and different color space coordinates are calculated to provide a "melanin index" for each measurement that does not have units. Elasticity was measured using an "Elastimeter" probe which measures instant skin elasticity measured in N/m by pressing the probe against a skin surface. The indenter within the probe imposes a constant deformation when it is in contact with the skin, and the skin resists the deformation similarly to a spring. The experimental time-course is outlined in Fig. 2.

ASCS Characterization

Immediately after creation of the ASCS and before application to the wound bed, a small aliquot of ASCS was transferred to a tube and this aliquot was used to conduct cell counts and percent viability assays using standard techniques. The time between viability assessment and application to the wound bed was within 60 minutes. A trypan blue dye exclusion test was performed for all samples. Subsequently, Molecular Probes LIVE/DEAD Viability/Cytotoxicity Kit, for Mammalian Cells (Invitrogen, L3224) was used according to the manufacturer's protocol using the following procedure. The two components of the assay, calcein AM (4mM) and ethidium homodimer-1 (2

Fig. 2 – *A burn, excision, mSTSG, RES model was used to study the application of NPWT*
The experimental time course began at Day -2 when burns were created. On Day 0, the burns were excised, autografted, had ASCS sprayed, and were assigned to the NPWT or non-NPWT group treated for a period of 3 days. On Day 3, the NPWT was removed and samples were taken and data was acquired. On Days 5, 7, 12, and 14, samples were taken and data was acquired.

mM), were diluted in lactated Ringer's solution to concentrations that achieved distinct labeling of cells. Working concentrations of 40 μM calcein AM and 5 μM ethidium homodimer-1 were determined to be most effective. Upon incubation completion, 10 μL of the cells were evaluated using fluorescence on an Axio Imager Microscope (Zeiss, Jena, Germany). Cell numbers (live and dead) were used to tabulate total number of cells and percent viability for each sample. Cells were counted using ImageJ software (NIH, USA). The same individual performed the characterization of the ASCS and counted the cells in the two different animals allowing for exclusion of inter-individual error in counting methods.

Re-epithelialization

Re-epithelialization was measured using two different techniques. In the first technique, digital pictures were used to grade percent re-epithelialization based on a previously published method[19]. Two individual blinded reviewers graded digital pictures from Days 5, 7, 12, and 14 for re-epithelialization and each picture was given a score from 0 to 5. 0=100%, 1=76%-99%, 2=50%-75%, 3=25-49%, 4=1-24%, 5=0%. Each reviewer scored each image twice. The scores were averaged and NPWT and non-NPWT groups were compared.

In the second technique, digital pictures were used to measure re-epithelialization using digital planimetry and Image J to calculate open wound area at Days 5, 7, 12, and 14) (Supplementary Fig. 1). Each interstice of the meshed graft was carefully traced using a high resolution image of the wound bed. The nature of the photographs did not allow for the use of a macro to calculate this metric. The open wound area was then compared to the total wound area at each time point to obtain a percentage.

Graft adherence

Graft-loss was assessed by a blinded assessor on Day 5 after the Telfa Clear dressing was removed. The following previously published questionnaire was used to assess the degree of graft loss and a grade was assigned (Table 2)[18].

1. Is there any graft loss?
2. If yes, assign percentage
3. If yes, assign likely cause (shear, infection)
4. Assign Grade

Laser doppler imaging

LDI was used to assess wound perfusion as previously described[20]. Images were taken using the same scan area dimensions at a consistent distance from the wound surface. Using Moor LDI image processing software version 5.3 (Moor Instruments Ltd, Axminster, UK), flux images of each wound were analyzed for mean perfusion units within 3 separate regions of interest. The perfusion units for each wound were averaged and NPWT and non-NPWT groups were compared.

Histology

Formalin-fixed punch biopsies were embedded in paraffin (FFPE) and were sectioned at 6 μm and floated on glass slides. Sections were stained with hematoxylin and eosin (H&E) and imaged under brightfield microscopy to provide representative histologic structure of the healing wounds.

Sections from Day 3 were stained with alpha smooth muscle actin (α-SMA) (Abcam, ab5694) antibody at a dilution of 1:250 to assess blood vessel density in the treated wounds. Immunofluorescence was carried out as previously described[21]. Two images per wound were taken of the papillary dermis at 10X magnification. Blood vessels were counted by one investigator using Image J and normalized to the area of the high-powered field.

Statistics

Two-way ANOVAs with multiple comparisons and Sidak's correction for multiple comparisons were used to compare

Table 2 – Graft-loss grading scale.

Grade	Area lost	Topical wound care required	Surgery indicated
1	Minimal	-	-
2	< 50%	+ / -	-
3	< 50%	+	+ / -
4	> 50%	+	+

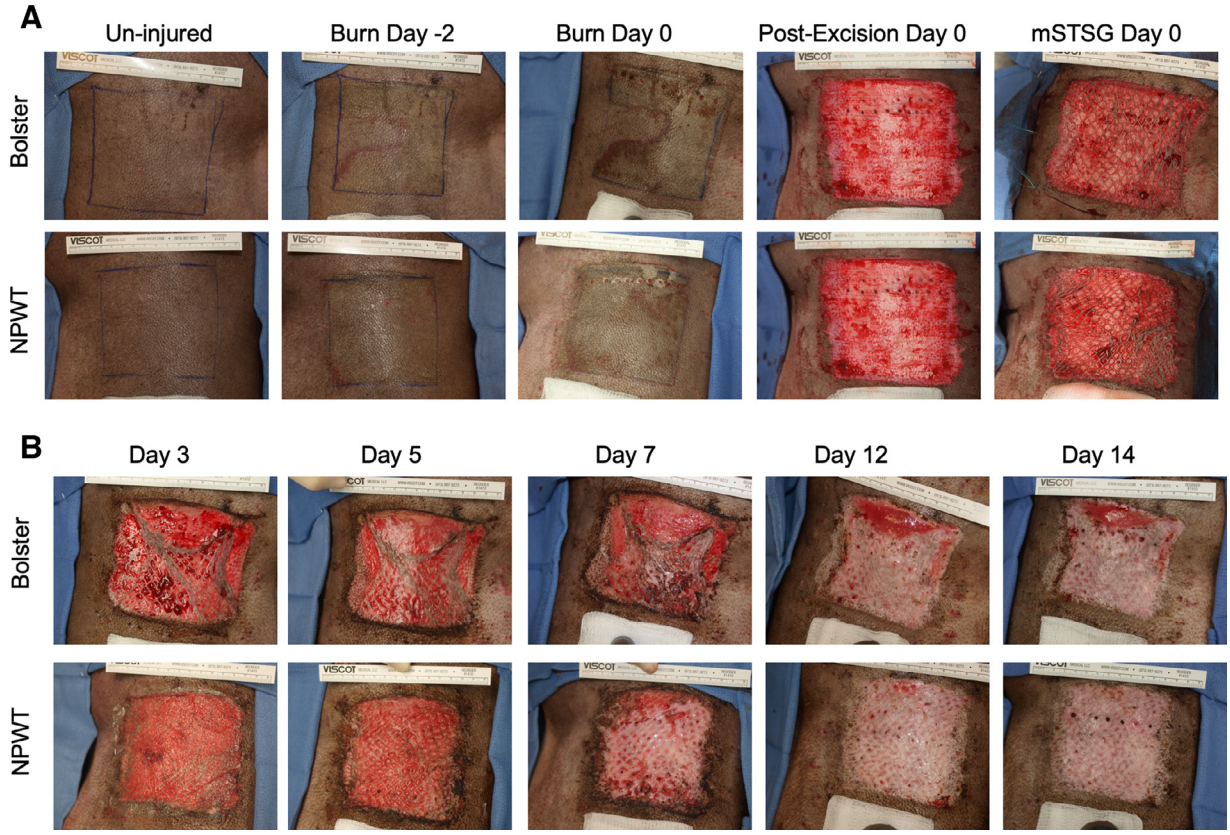

Fig. 3 – *In Animal 1 in the most cranial wounds, mSTSGs treated with NPWT healed faster compared to non-NPWT* Digital pictures were taken at each time point before injury, after burning on Day -2, Day 0 before and after excision, after mSTSG, and at Days 3, 5, 7, 12, and 14.

groups when analyzing % re-epithelialization, pigmentation, elasticity, and perfusion with n=4 wounds in each treatment group. Blood vessel counts were compared using an unpaired t-test. A power calculation was not performed, and this analysis is considered pilot data. This pilot data may inform future studies that are more well-powered to come to a definitive, statistically-powered conclusion as to whether NPWT has an effect on wound healing of full thickness wounds with mSTSG and the application of ASCS.

Results

Full-thickness burns were created that progressed in severity

Burn depth was characterized using H&E staining of baseline, uninjured skin, burned tissue collected immediately post-injury (Day -2), and burned tissue at Post-burn Day 2, or "Day 0" prior to excision as described in previous literature (Supplemental Fig. 2)[22-27]. Baseline uninjured skin had an intact epidermis with rete ridges, intact dermal structures, and normal collagen structure and orientation (Supplemental Figure 2C). After burn wound creation on Day -2, thermal injury is observable by the alteration of the epidermis with loss of rete ridges and appearance of elongated string bean nuclei. The dermis contained altered and discolored collagen[28-32], occluded blood vessels[32-37], and damaged dermal appendages down to full-thickness (Supplemental Figure 2B)[32-34]. At Day 0, the severity of injury increased with detachment of the epidermis, severe collagen discoloration, and severe damage to dermal appendages down to full-thickness (Supplemental Figure 2A).

The application of NPWT to a mSTSG that was over-sprayed with ASCS does not negatively affect wound healing:

Autologous skin cell suspension characterization

Trypan blue staining revealed that the ASCS contained approximately 50% viable cells, and a total of approximately 2-3E6 cells/cm^2 were sprayed onto the mSTSG (Supplemental

Table 3 – ASCS cell viability and cell counts.

Animal Number	Assay	RES Viability (%)	Total Cell Count (cells/cm)
1	Trypan Blue	58.78 ± 3.04	3.20E6 ± 9.38E4
	Fluorescent	67.55 ± 7.87	2.40E6 ± 1.67E5
2	Trypan Blue	49.90 ± 4.29	2.05E6 ± 7.72E4
	Fluorescent	45.92 ± 2.49	1.65E6 ± 2.96E5

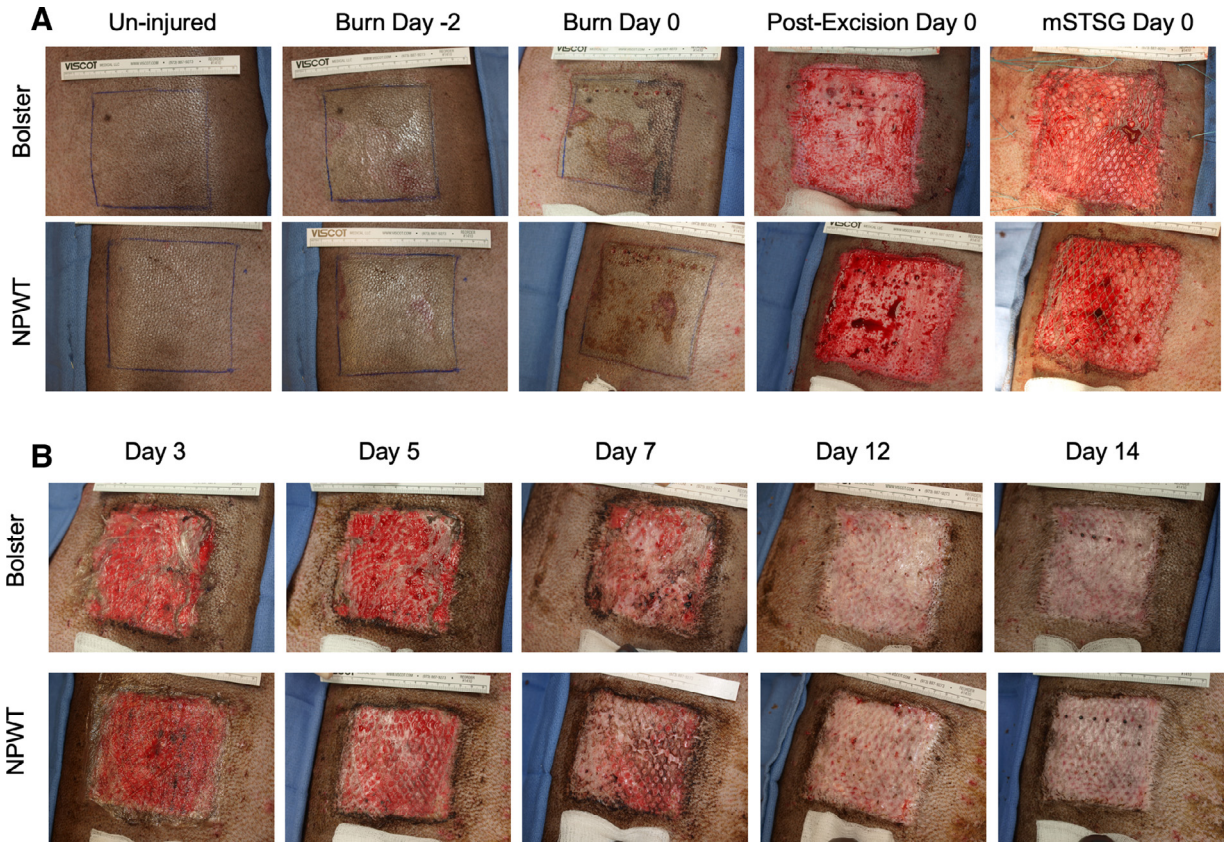

Fig. 4 – *In Animal 1 in the most caudal wounds, mSTSGs treated with NPWT healed in a similar manner to non-NPWT* Digital pictures were taken at each time point before injury, after burning on Day -2, Day 0 before and after excision, after mSTSG, and on Days 3, 5, 7, 12, and 14.

Fig. 3, Table 3). This was largely in agreement with the fluorescent staining, which confirmed that the ASCS was approximately 50% viable, and a total of 1.5-2.5E6 cells/cm^2 were sprayed onto the mSTSG (Supplemental Fig. 3, Table 3). This staining is likely representative of the viability of the cells that were actually applied to the wound bed because the suspension was applied within 1 hour after visualizing viability via staining of cells.

Digital picture assessment

In Animal 1, the most cranial wound on the right flank, which was treated with a bolster dressing, had some graft-loss (˜30%) due to a technical failure of the dressing and sutures to keep the autograft in place. The contralateral wound that was also most cranial that was treated with NPWT did not have this technical failure of the dressing (Fig. 3). This graft-loss led to open wound granulation tissue through Day 14. Aside from the portion of the wound that was lost, the remainder of the graft was well-adhered. This wound was included in all subsequent analysis to include data that indicates that the use of NPWT may yield more well-adhered mSTSGs. The most caudal wounds had similar graft adherence with no significant losses (Fig. 4). They healed in a parallel time-course and were 100% re-epithelialized by Day 12. In Animal 2, the cranial and caudal wounds healed in a manner that corresponded to the caudal wounds of Animal 1 regardless of dressing method and were 100% re-epithelialized by Day 12. All donor site wounds in both animals healed well and were re-epithelialized by Day 12 (Supplemental Fig. 4).

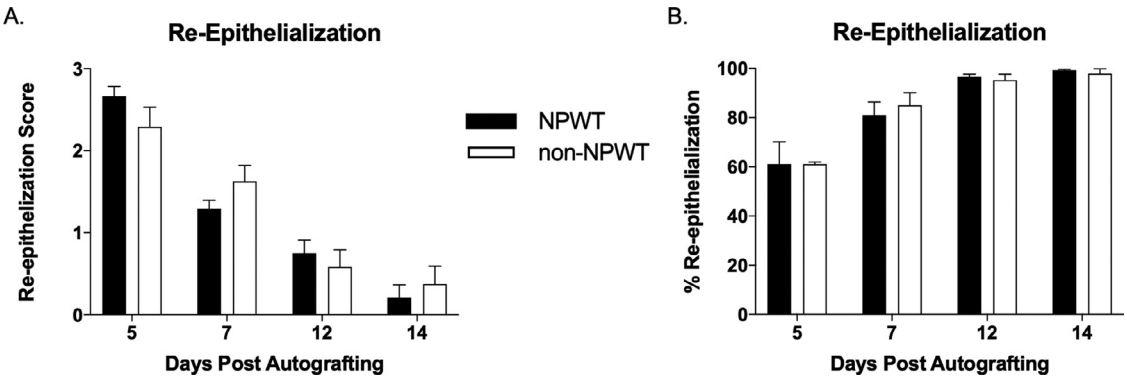

Fig. 5 – *Re-epithelialization was not altered by NPWT treatment*
Percent re-epithelialization was scored using a scale from 0-5, with 0=100% re-epithelialization and 5=0% re-epithelialization. Digital images of pictures were graded by 3 independent blinded assessors. Average ± SEM is presented with *n*=4 wounds per group.

Re-epithelialization

There were no significant differences in the percent re-epithelialization between NPWT and non-NPWT groups (Fig. 5). At Day 5, the sites were given a score of approximately 2.5, indicating approximately 50% re-epithelialization (Fig. 5A), and actual measurements revealed 61.09 ± 9.01 and 61.15 ± 0.82 % re-epithelialization in NPWT and non-NPWT groups respectively at the same timepoint (Fig. 5B). By Day 7, the sites were given a score of approximately 1.5, indicating a score of 76%-99% re-epithelialization, and actual measurements revealed 81.02 ± 5.37 and 85.00 ± 5.11 % re-epithelialization in NPWT and non-NPWT groups respectively at the same timepoint. By Days 12 and 14, the sites were given a score of approximately 0-0.5, indicating approximately 100% re-epithelialization, which was also in agreement with actual measurements made by digital planimetry.

Histological architecture

Representative H&E staining of Animal 1 cranial (NPWT vs. non-NPWT: Fig. 6) and caudal (NPWT vs. non-NPWT: Fig. 7) wounds are shown. H&E staining indicated no differences in histomorphometric structure of grafted sites between groups. In both groups at Day 3 there was very little dermal tissue, as is expected due to the 0.012" thickness of the mSTSG that was placed. There was an inflammatory cell infiltrate in both groups and all wounds, and a scab could be visualized. At Day 5, the epidermis was more well-defined and was thicker than normal skin epidermis. The granulation tissue continued to thicken, and inflammatory cell infiltrate remained. At Day 7, a thick epidermis was apparent and the dermal component became more well defined. At Days 12 and 14, the epidermis thickness decreased and the dermal component continued to demonstrate a large inflammatory cell infiltrate, as well as hypercellularity of fibroblast cells. The dermal component was significantly thicker at Days 12 and 14 compared to Day 3.

Graft loss grading scale

On Day 5 when the Telfa Clear dressing was removed, there was very little graft-loss on either of the animals, regardless of treatment, as graded by a blinded assessor. In Animal 1, the most cranial wound on the right flank was given a Grade 3, indicating <50% graft loss requiring topical wound care with no surgery (Table 3). In Animal 2, the most caudal wound on the right flank was given a Grade 1 score indicating minimal graft-loss with no topical wound care or surgery required. These two wounds were both in the non-NPWT group. The remaining 2 wounds in that group were given a Grade 0 score indicating no graft-loss. Likewise, all NPWT-treated mSTSGs were given a Grade 0 score (Table 4). Overall, the non-NPWT group had a score of 1.0 ± 1.41 vs. 0 ± 0 in the NPWT group.

Pigmentation is not significantly affected by treatment with NPWT

Overall, ASCS-treated mSTSG resulted in hypo-pigmentation compared to uninjured skin observed at Day 7, and Day 12, (Uninjured= 743.92 ± 35.15 vs. NPWT=720.67 ± 29.82 and non-NPWT=723.33 ± 30.81, n=4, P>0.05, Day 7) (Uninjured=791.33 ± 14.188 vs. NPWT=656 ± 15.65 and non-NPWT=676.17 ± 24.11, n=4, P<0.05, Day 12). Despite these changes in pigmentation over time associated with uninjured skin vs. grafted wounds, there were no differences between the NPWT and non-NPWT groups (Fig. 8).

Elasticity is significantly affected by treatment with NPWT

Overall, the mSTSG sites were less elastic and hence, stiffer, compared to uninjured skin at Day 7. At Day 7, there were no differences between NPWT and non-NPWT groups. At Day 12, the non-NPWT group was less elastic and stiffer compared to NPWT and uninjured groups (Uninjured= 65.8 ±10.4 vs. NPWT=109.5 ± 21.23 vs. non-NPWT=177.5 ± 35.4, P<0.05) (Fig. 9).

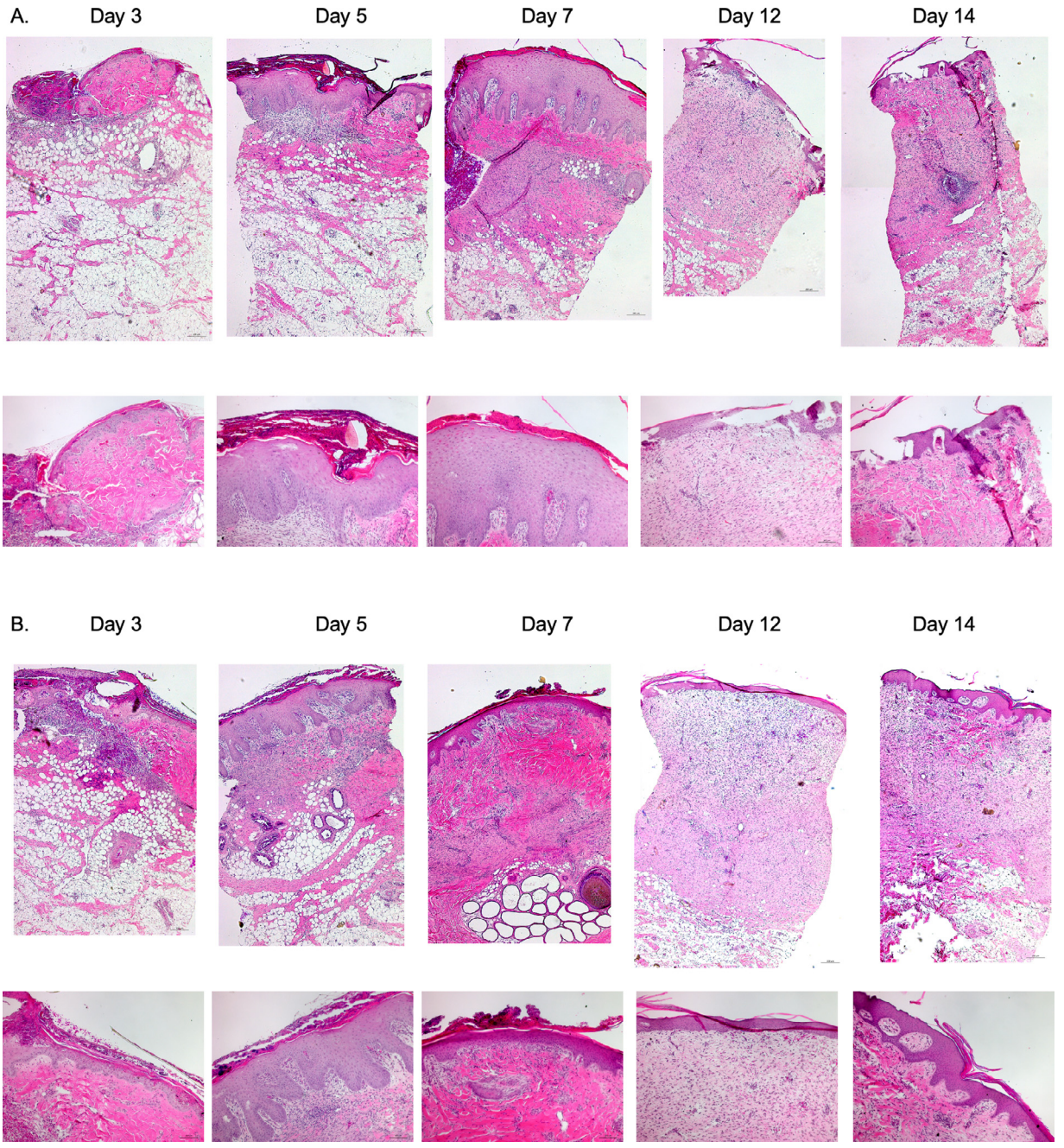

Fig. 6 – *In Animal 1 in the most cranial wounds, H&E staining revealed similar histo-architecture between non-NPWT and NPWT-treated mSTSGs*
Biopsies were taken at Days 3, 5, 7, 12, and 14 and were formalin fixed and paraffin embedded. Sections were then stained for H&E and images were captured for the non-NPWT group (A) and NPWT group (B) at 5X magnification (top) and 10X magnification (bottom).

Perfusion and Blood Vessel Density are not Significantly Affected by Treatment with NPWT

LDI imaging

There were no significant differences in perfusion between the two treatment groups (Fig. 10A). Overall, perfusion was highest at Day 5 (approximately 500 PU) and then decreased over time (approximately 350 at Day 9, ˜230 at Day 14 and approximately 200 at Day 14) (Fig. 10B).

α-SMA Immunofluorescence

There was no significant difference in perfusion detected at Days 5, 7, 12, or 14 by LDI. As such, it was not expected that there would be a difference in blood vessel density at these timepoints either. A time point more proximal to NPWT application was chosen for the investigation of blood ves-

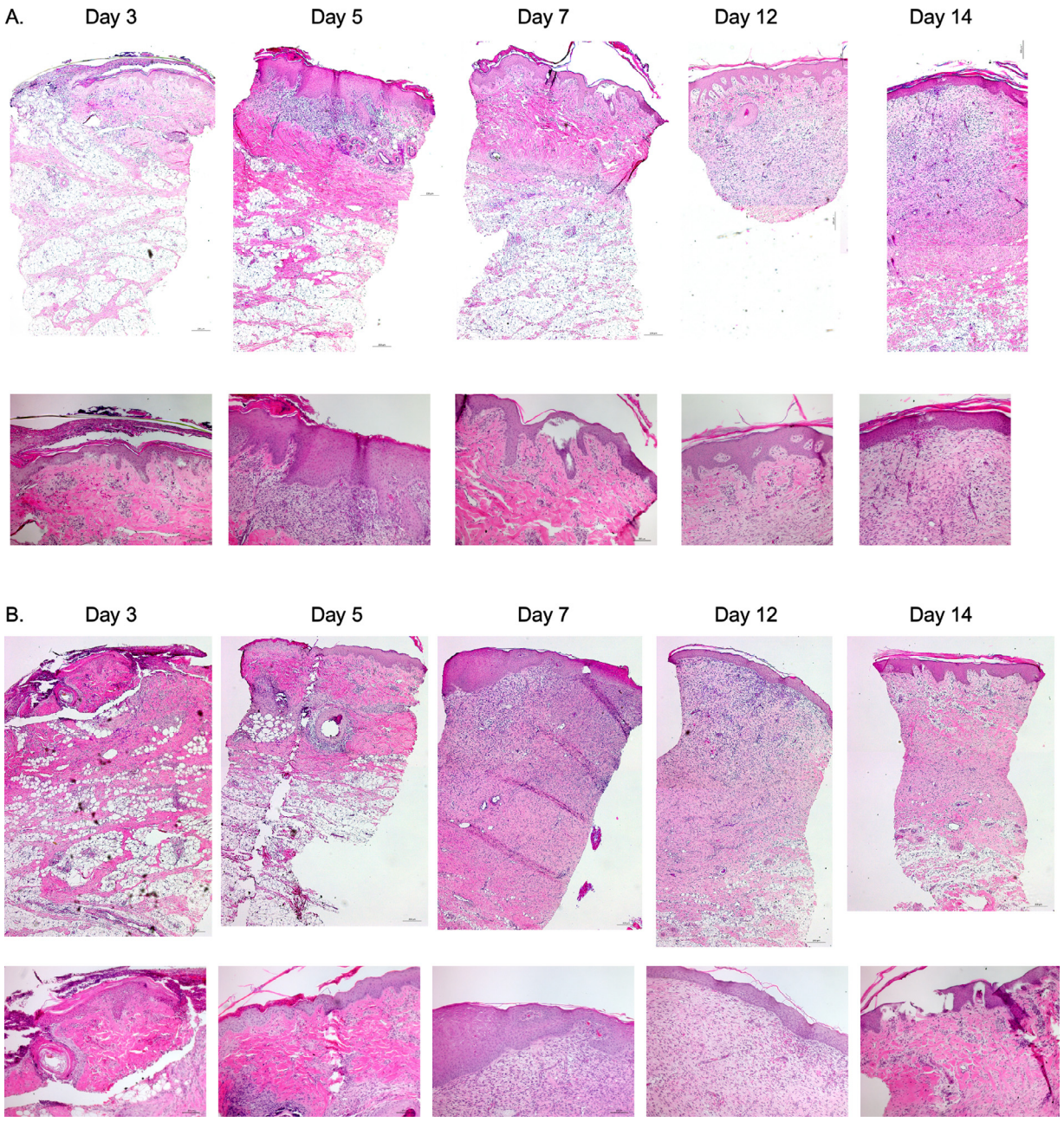

Fig. 7 – *In Animal 1 in the most caudal wounds, H&E staining revealed similar histo-architecture between non-NPWT and NPWT-treated mSTSGs*
Biopsies were taken at Days 3, 5, 7, 12, and 14 and were formalin fixed and paraffin embedded. Sections were then stained for H&E and images were captured for the non-NPWT group (A) and NPWT group (B) at 5X magnification (top) and 10X magnification (bottom)

sel density because the effect of the device may be more clearly elucidated closer to its application. Punch biopsies from Day 3, were stained for α-SMA and baseline skin was used as a comparison between uninjured tissue and mSTSG tissue.

Grafted wounds at Day 3 had a similar blood vessel density to normal skin (uninjured=41.53 vessels/mm^2 vs. non-NPWT and NPWT=44.62 vessels/mm^2) (Supplemental Fig. 5). There was no difference in blood vessel density between the two treatment groups (NPWT= 43.99 ± 4.24 vs. non-NPWT= 45.27 ± 2.26 vessels/mm^2) (Fig. 11).

Discussion

The purpose of this pilot study was to investigate the potential effect of a NPWT device on the healing of burn wounds treated with mSTSG over-sprayed with ASCS. It was hypothesized that wounds treated with NPWT would heal in a sim-

Table 4 – Graft-loss grading of NPWT and non-NPWT-treated wounds.

Animal	Wound	Treatment	Grade
1	Right (cranial)	non-NPWT (bolster)	3
	Right (caudal)	NPWT	0
	Left (cranial)	NPWT	0
	Left (caudal)	non-NPWT (bolster)	0
2	Right (cranial)	NPWT	0
	Right (caudal)	non-NPWT (bolster)	1
	Left (cranial)	non-NPWT (bolster)	0
	Left (caudal)	NPWT	0

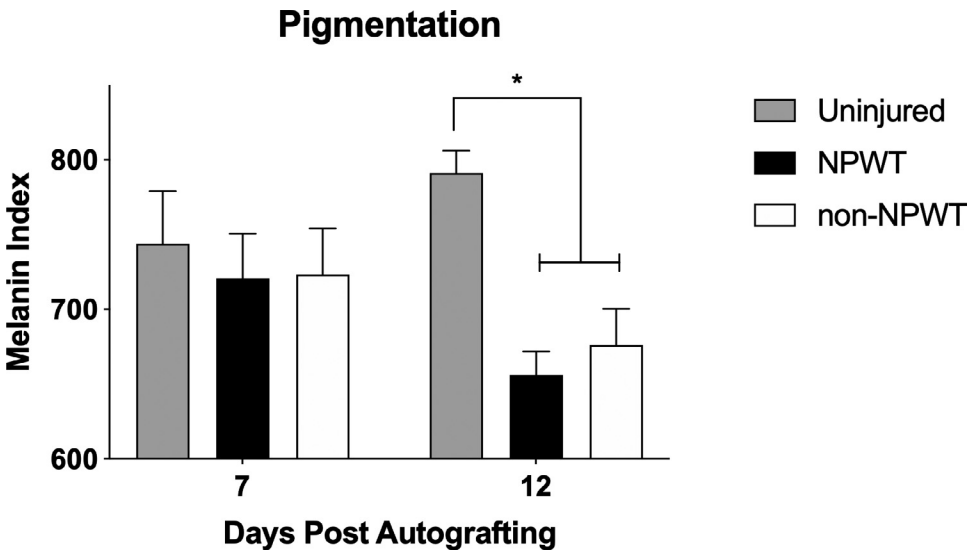

Fig. 8 – *Pigmentation was not different between NPWT and non-NPWT groups*
A Skin Color Catch non-invasive skin probe was used to evaluate melanin in uninjured skin, NPWT, and non-NPWT groups over time. Average ± SEM is presented with *n*=4 wounds per group.

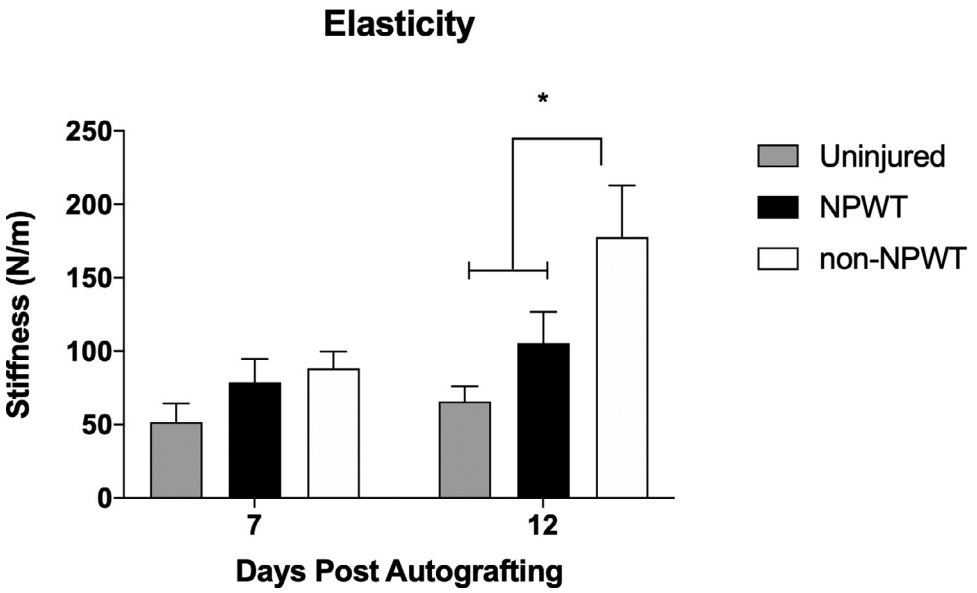

Fig. 9 – *Elasticity was increased in the NPWT group compared to non-NPWT*
An elasticity non-invasive skin probe was used to evaluate elasticity in uninjured skin, NPWT, and non-NPWT groups over time. Average ± SEM is presented with *n*=4 wounds per group.

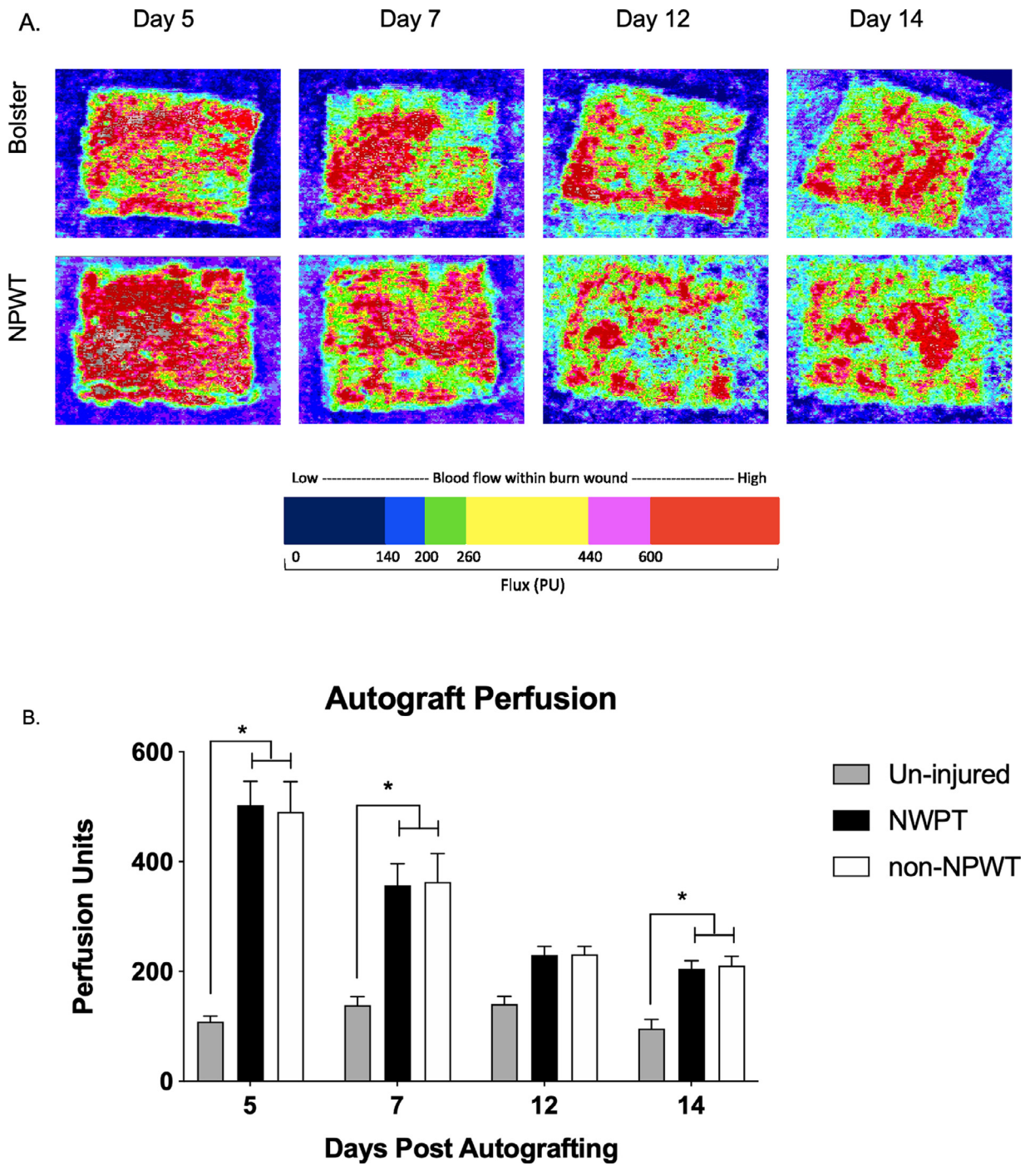

Fig. 10 – *Perfusion in mSTSG sites was not different between treatment groups*
LDI images were taken at Days 5, 7, 12, and 14 to measure perfusion in uninjured skin, NPWT, and non-NPWT groups over time (A). 3 regions of interest were used to obtain average perfusion units for each image (B). Average ± SEM is presented with *n*=4 wounds per group.

ilar manner to wounds treated with mSTSG and ASCS without NPWT (non-NPWT). Without evidence demonstrating the clinical impacts of NPWT on ASCS-treated wounds, one may have avoided using this approach. This avoidance could be due to concerns over the NPWT damaging or removing cells from the wound bed. However, when ASCS is used, the most commonly used primary dressing is Telfa^{TM} Clear, which acts as a layer protecting the newly applied cells. It was hypothesized in the present study that this primary dressing may be effective in protecting these cells with the application of NPWT.

In this model, wound closure was used as the primary metric to evaluate the hypothesis. As indicated by photographs, grading and measurements of percent re-epithelialization by

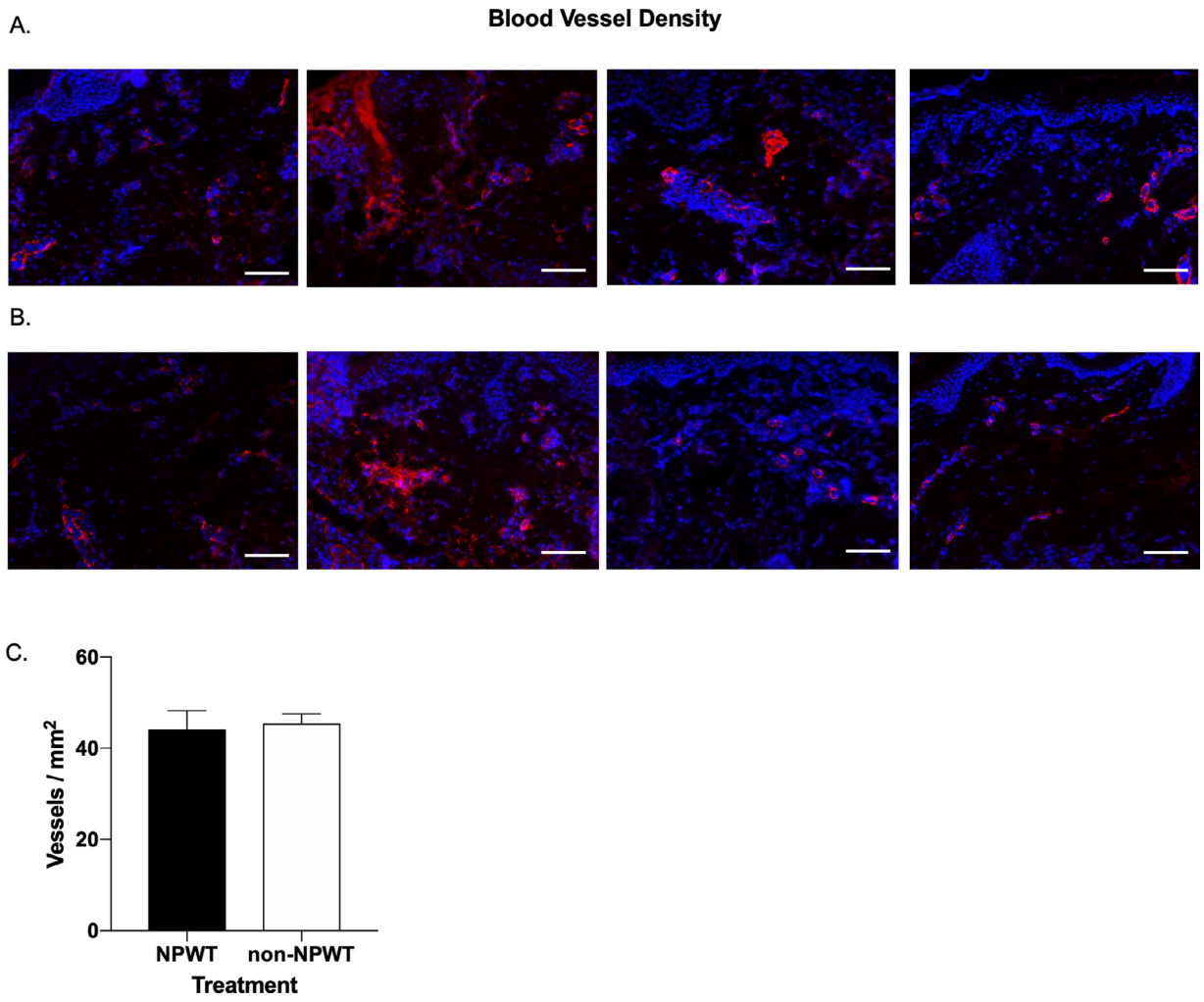

Fig. 11 – *Blood vessel density was not different between treatment groups*
Punch biopsies were taken of non-NPWT grafted sites (*n*=4 different wounds) (A) and NPWT grafted sites (4 different wounds) (B) at Day 3. Biopsies were FFPE, sectioned, and immunofluorescently stained with α-SMA to identify blood vessels. Blood vessels were counted and normalized to area. Red=α-SMA, Blue=DAPI, Scale bar= 100μm. Average ± SEM is presented with *n*=4 wounds per group. (Color version of figure is available online)

blinded assessors, and a graft loss grading scale, there was no difference in healing outcomes. All excised full-thickness burns wounds treated with ASCS+mSTSG were fully re-epithelialized within 14 days. It is known that wounds that heal within 21 days have a lower chance of developing into hypertrophic scar (~30%), while those that heal in excess of 21 days have a ~70% chance of developing scar[38-41]. Therefore, regardless of mSTSG fixation technique, these wounds closed within an appropriate time-period and had minimal scar pathology. This trajectory of re-epithelialization was likely aided with application of ASCS which were either 67% or 45% viable by fluorescent staining in Animals 1 and 2, respectively. This difference in percent viability is not due to inter-individual ASCS preparation technique because the same investigator perfomed the processing in both animals. Furthermore, anecdotally, the observed viability is within the normal range for ASCS preparation (40-80%; data not published). Given that each animal contained wounds that were treated with both treatments (non-NPWT and NPWT), but with the same ASCS, the difference in viability between the animals did not impact re-epithelialization and is controlled for within the experimental design. When re-epithelialization was analyzed by Animal (Animal 1 with 67% ASCS viability vs. Animal 2 with 45% ASCS viability), and not by treatment group (non-NPWT vs. NPWT), the % re-epithelialization trended higher in Animal 1 at Day 5 (72.62 +/- 4.52 vs. 54.06 +/- 4.79, p=0.1167) and Day 7 (89.78+/- 2.57 vs. 79.54 +/- 4.47, p=0.3622). These data indicate that the application of higher viability ASCS increased the rate of % re-epithelialization. In addition, the previously published grading scale for % re-epithelialization[19] was validated here by comparing re-epithelialization scores from blinded assessors with actual measurements of % re-epithelialization made by digital planimetry. This comparison, which showed strong agreement between the two different measurements, indicates that scoring can be used as an option in lieu of actual measurements of % re-epithelialization in future studies.

Clinically, NPWT has been demonstrated to improve graft adherence. In this experiment, there was some graft loss in the non-NPWT group, while the NPWT group had no graft loss. This loss was primarily due to graft slippage and movement of the animal which can be further optimized in future work by including additional redundant sutures to the tie over bolster technique. NPWT is a particularly useful technique to employ on areas prone to shear and movement and works to prevent shifting of the autograft after placement. Therefore, as NPWT does not negatively impact healing outcomes with ASCS, it may be beneficial to implement into clinical practice to prevent graft slippage.

NPWT is also employed because it is thought to improve perfusion, which was measured by LDI and blood vessel staining in this experiment. While there was no difference in perfusion between the non-NPWT and NPWT groups, it is possible that a time point more proximal to NPWT application needs to be investigated to study this nuanced difference.

NPWT is thought to induce cytoskeletal conformational changes and changes to collagen or ECM structures. The only metric that was different between the two groups was elasticity, where the NPWT group was more elastic and less stiff. This data could indicate that NPWT contributes positively towards increased pliability of healing wounds. Another possible explanation for the increased elasticity in the NPWT group was that one of the wounds in the non-NPWT group experienced graft-loss, resulting in slower wound healing that may have resulted in less elastic healed skin at the Day 12 timepoint where a difference was observed, or skin on a slower trajectory towards normal elasticity in this group. The reasoning behind the difference in elasticity is difficult to detect with a small sample size, but here, graft-loss may provide a possible explanation of these results. Biopsies that were preserved for molecular analysis or histological analysis could be used in the future to investigate ECM components such as elastin and collagen.

Normal Duroc pig skin contains melanocytes in an active state of pigment production, as well as pigmented keratinocytes[42]. As such, the ASCS would contain these cells, and it was hypothesized that wounds treated with mSTSG and ASCS would be normally pigmented. If the ASCS were negatively impacted by NPWT, it would be hypothesized that this group would be hypopigmented compared to the non-NPWT group and normal skin. Interestingly, neither hypothesis was correct. Both the non-NPWT and NPWT groups were hypopigmented at Day 12 compared to normally pigmented skin, and they were hypopigmented to the same degree. Melanocytes are a cell population that is easily damaged, and melanogenesis is affected by states of inflammation and oxidative damage[43]. When damage rates are high, melanogenesis can be shut down and result in hypopigmentation. It is possible that these wounds would have returned to normal pigmentation after the resolution of the inflammatory phase and progression into proliferation and remodeling. Thus, wound pigmentation warrents further investigation at later timepoints. In addition, anecdotally with the application of ACSC in patients, it often takes months before pigmentation returns, indicating consistence between the animal model patient wound healing.

Conclusions

The use of NPWT over a mSTSG that is over-sprayed with ASCS did not impact the ASCS positive contributions to wound healing, as re-epithelialization, revascularization, and repigmentation were not adversely impacted compared to the standard dressing regimen. Results from this pilot study suggest that NPWT may be a viable option as a bolstering technique when ASCS is used with mSTSGs in the treatment of full-thickness burns.

Author Contributions

BCC conducted the animal research, conducted the lab assays, analyzed the data, and wrote the manuscript. LTM and TET conducted the animal research and contributed to editing the manuscript. SN, JWK, and NJP conducted the animal research and analyzed the data. MAO and LDK conducted the lab assays and analyzed the data. JWS conceived of the project, contributed to analysis and interpretation of data, and edited and revised the manuscript.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jss.2021.05.010.

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