Enhanced Survival and Accelerated Perfusion of Skin Flap to Recipient Site Following Administration of Human α 1-Antitrypsin in Murine Models

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Objective: Skin flaps are routinely used in reconstructive surgery yet remain susceptible to ischemia and necrosis. Distant flaps require lengthy time to detach causing patient discomfort. Human α 1-antitrypsin (hAAT) is a clinically available serum glycoprotein. hAAT was shown to support mature vessel formation and enhance tissue survival following ischemia-reperfusion injuries. The purpose of the presented study was to examine the effect of hAAT on skin flap survival and distant “tube” flap perfusion through its recipient site.

Approach: Random-pattern skin flaps were performed on mice treated with clinical-grade hAAT using three unique routes of administration (transgenic, i.p. and s.c. infiltration); necrotic area and tissue perfusion were assessed. Blockade of vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) were used to explore aspects of mechanism of action. A distant tube flap model was performed to examine time to perfusion.

Results: hAAT-treated mice displayed approximately two-fold smaller necrotic flap areas versus controls across all hAAT administration routes. Flaps displayed greater perfusion as early as 3 days postsurgery ($64.6\% \pm 4.0\%$ vs. $43.7\% \pm 1.7\%$ in controls; $p=0.007$). hAAT-mediated flap survival was prominently NOS dependent, but only partially VEGF dependent. Finally, distant flaps treated with hAAT displayed significantly earlier perfusion versus controls (mean 9.6 ± 1.6 vs. 13.1 ± 1.0 days; $p=0.0005$).

Innovation: The established safety record of hAAT renders it an attractive candidate toward improving skin flap surgery outcomes, particularly during VEGF blockade.

Conclusions: hAAT treatment enhances survival and accelerates perfusion of skin flaps in animal models in a NOS-dependent manner, partially circumventing VEGF blockade. Further mechanistic studies are required.

Keywords: wound healing, VEGF, nitric oxide synthase, revascularization, inflammation

INTRODUCTION

SKIN FLAPS ARE routinely applied in reconstructive surgery to cover defects that arise from trauma, resections, or necrotizing skin infec-

tions. Maintenance of blood supply is crucial for preventing ischemia and subsequent flap necrosis.¹ Although local flaps are most common, some defects require distant flap applica-

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tion in a staged reconstruction. Such procedures rely on optimal wound healing and reliable vascular attachment at the recipient site before the pedicle can be detached.² This process might span several weeks, during which the patient often suffers from a functional disability and aesthetic disfigurement.³ Thus, therapeutic approaches aimed at accelerated flap revascularization and enhanced flap survival, represent an important need in present-day reconstructive surgery. Transient ischemic postconditioning (“training”) has been shown to successfully shorten the duration of time required to achieve vascular attachment.⁴ However, the procedure may cause intense discomfort to the patient, resulting in poor compliance. Several recent studies describe the use of new molecules for the reduction of ischemia–reperfusion injury in the skin.^{5–7} However, there remains the need for agents that promote vascularization and may represent a positive combinatory approach for expediting distant flap detachment, particularly in patients susceptible to compromised wound healing.

Human alpha1-antitrypsin (hAAT) is an endogenous circulating anti-inflammatory glycoprotein that rises in its levels during acute-phase responses, specifically in response to inflammatory and hypoxic triggers. A rare genetic deficiency in hAAT most commonly manifests as nonsmoking emphysema.⁸ The pathophysiology is presumed to involve failure of the lungs to counter proteolytic insults due to insufficient hAAT levels. Another suggested mechanism is an inability to support the vascular bed of the alveolar system, as the hallmarks of human emphysema have been accurately reproduced in animals by blocking vascular endothelial growth factor (VEGF).⁹ Subsequently, hAAT was shown to upregulate VEGF production under ischemic conditions.¹⁰ Several animal studies demonstrate a protective effect for hAAT therapy in the context of ischemia–reperfusion injuries, in models of heart,¹¹ lung,¹² kidney,¹³ and brain¹⁴ injuries, and several transplant models.¹⁵

Considering the outstanding safety record of lifelong hAAT therapy, several clinical trials conducted with non-hAAT-deficient patients, have been recently undertaken to explore relevant therapeutic outcomes in related conditions. These include, for example, cardiac tissue recovery in patients with acute myocardial infarction.¹⁶ hAAT therapy presents as a sound candidate for promoting vascularization alongside flap training, with a potential for synergy in which flap training is minimized. In the present study, we explore the effects of hAAT and flap training on skin flap revascularization and survival using state-of-the-art animal models.

CLINICAL PROBLEM ADDRESSED

Multiple studies describe various approaches for enhancing skin flap survival, ranging from gene delivery,¹⁷ stem cell-based therapies,¹⁸ and drug repurposing.¹⁹ Still, an effective and widely accepted method ensuring skin flap survival has not emerged. This leaves patients with an underlying susceptibility to develop flap failure. In this study, we demonstrate that hAAT-treated mice display significantly improved flap survival and accelerated perfusion of distant flaps through the recipient site. Considering the extensive safety record of clinical-grade hAAT therapy in nondeficient individuals,^{20,21} we anticipate it can be effectively translated into the clinic as an approach to promote tissue repair, with the prospect of shortening the debilitating time required for distant flaps to undergo pedicle detachment. For example, hAAT therapy may be applicable as an approach to shorten pedicle detachment in paramedian forehead flap procedures during nasal reconstruction. In addition, hAAT therapy could benefit procedures, such as cross-leg flap or groin flap for hand reconstruction when microsurgery is impossible or has failed.

MATERIALS AND METHODS

Animals

Animal experiments were performed using 6–8-week old C57BL/6 mice (Envigo+ Laboratories, Inc., Rehovot, Israel) or mice transgenic for human hAAT on a lung surfactant promoter (hAAT^{+/+} mice; circulating levels of hAAT <250 ng/mL; backcrossed to C57BL/6).²² Mice were housed at a vivarium facility, and experiments were approved by the Institutional Animal Care and Use Committee (Approval #48-09-2016). During all procedures, mice were anesthetized with isoflurane inhalation (5% for induction, 2.5% maintenance), hair removed using an electrical razor, followed by application of hair depilation cream for 30 s. Skin was disinfected with 0.5% chlorhexidine gluconate in 70% ethanol solution. Table 1 lists the model, modalities, and treatments of all experiments presented.

Random vascular pattern skin flap model

Caudally based random 3×1 cm skin and panniculus carnosus flap was raised on mouse dorsum and sutured back in place with 4-0 silk suture (Fig. 1A). Mice were treated with either 60 mg/kg clinical-grade hAAT (*n* = 10; Glassia, Kamada Ltd., Israel), an equimolar amount of clinical-grade human albumin (*n* = 9; 75 mg/kg; Bio Products Laboratory Ltd., Elstree, United Kingdom), or

Table 1. Summary of experimental design

In Vivo Model	Treatment Group			Modulation	Figures
	PBS	Albumin	hAAT		
Random-pattern flap	N (<i>n</i> =7)	N (<i>n</i> =9)	N (<i>n</i> =10)		Figs. 2, 3
		F (<i>n</i> =7)	F (<i>n</i> =7)		
		N	N	Bevacizumab (<i>n</i> =5)	Fig. 4
		N	N	L-NAME (<i>n</i> =6)	Fig. 4
Distant tube flap		N	N	L-NIL (<i>n</i> =6)	Fig. 4
		F (<i>n</i> =8)	F (<i>n</i> =8)	Training/sham	Fig. 5

N, necrotic area measurement; F, fluorescein uptake measurement; hAAT, human α 1-antitrypsin; PBS, phosphate-buffered saline.

phosphate-buffered saline (*n*=7; PBS; Biological Industries, Beit Haemek, Israel) administered i.p. or, conversely, by subcutaneous infiltration along flap length, immediately after surgery. VEGF inhibition: Bevacizumab (*n*=5; 5 mg/kg; Avastin[®]; Roche, Basel, Switzerland) was administered i.p. immediately after skin flap surgery. NOS inhibition: L-NAME (*n*=6; 10 mg/kg/day) or L-NIL (*n*=6; 3 mg/kg/day) (both from Cayman Chemical Company, Ann Arbor, MI) was administered i.p. for 3 days following surgery; control mice

received an equal volume of PBS. Flaps were photographed on days 0 and 7 using a digital camera (iPhone 8) on a fixed standoff. Necrosis area was measured by two independent and blinded observers. Percent necrosis was calculated out of total flap area at day 7.

Histological analysis

Animals were sacrificed on day 7 (*n*=4 per treatment group). Samples for histology were immediately immersed in 10% neutral-buffered formalin (Sigma-Aldrich, Rehovot, Israel). Five-micrometer histological sections of paraffin-embedded samples were stained by Hematoxylin and Eosin (H&E; Jackson ImmunoResearch, West Grove, PA).

Fluorescein uptake

In-vivo fluorescent imaging was performed using *in vivo* imaging system (IVIS) Lumina (PerkinElmer, Waltham, MA) and fluorescein sodium (*n*=7 per treatment group; 4 mg/kg, i.p.; HUB Pharmaceuticals, Rancho Cucamonga, CA) according to the manufacturer's instructions. Uptake was calculated as percent fluorescent counts in flap area relative to fluorescent counts in flap area on day 0.

Distant "tube" skin flap model

An axial flap ($\sim 0.7 \times 2.5$ cm) based on the lateral thoracic artery was designed, as shown in Fig. 1B (*n*=8 per treatment group). Flap base was positioned over the scapulae and its axis diagonal caudally toward the dorsum midline. The flap was then raised, distal to proximal. After making the distal cut, the skin, subcutaneous tissue, and panniculus carnosus muscle were elevated and the lateral thoracic artery could be seen from beneath the skin to verify its position before cutting the medial and lateral flap incisions. After the distal cut was made, the position of the lateral thoracic artery was verified before cutting the medial and lateral flap incisions. The flap is completely raised and then folded with the medial side of the flap sutured to the lateral side to create a tube except for the most distal 5 mm. The donor site is closed in the same manner, with the most distal part of the flap sutured back to the most distal part of the donor site.

Transient ischemic conditioning

Transient ischemic conditioning ("Training") was performed by placing a microvascular clamp at the flap pedicle for three 10-min cycles, separated by 5-min intervals. Flap perfusion by recipient site was assessed by fluorescein uptake while the pedicle was clamped. Flap viability was then confirmed by de-clamping of the pedicle to allow flap blood flow.

Figure 1. Illustration of the two flap models used in the study. Design and orientation of flap models. Dashed lines, sutures. (A) Random flap, (B) distant tube flap. The clamp is positioned around the lateral thoracic pedicle during transient ischemic conditioning. Color images are available online.

Image analysis

Image analysis was performed using ImageJ (NIH, Bethesda, MD) and Digimizer software (MedCalc Software, Ostend, Belgium). Histological images were prepared by overlap stitching of three microscope view fields ($\times 10$ magnification), ImageJ pairwise stitching plugin.²³ IVIS images were analyzed with Living image software (Perkin-Elmer).

Statistical analysis

Nonparametric Mann–Whitney or Kaplan–Meier logrank tests were used to assess statistical significance with 95% confidence interval, as indicated. Results are shown as mean $\pm$ SD or mean $\pm$ SEM, as indicated. $p < 0.05$ was considered signifi-

cant. Statistical processing was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

RESULTS

Enhanced survival of random-pattern skin flaps under hAAT therapy

Three forms of hAAT delivery were utilized to evaluate the effect of hAAT on skin flap repair: transgenic expression of hAAT, i.p. administration of clinical-grade hAAT, and local infiltration of clinical-grade hAAT. A 3×1 cm caudally based random-pattern skin flap was raised, sutured back to place (Fig. 1A). As shown in Fig. 2A, the area of necrotic tissue observed 7 days after surgery was

Figure 2. Random-pattern skin flap survival in the presence of hAAT. Caudally based random-pattern skin flap was performed, and flap survival was assessed 7 days after surgery. Comparison of three hAAT administration routes and sources. **(A)** *Transgenic hAAT expression.* WT mice ($n=10$); hAAT^{+/+}, hAAT-overexpressing transgenic mice ($n=10$). Percent necrotic area. Data are representative of two independent experiments. **(B)** *Systemic hAAT administration.* Single injection of human serum albumin (75 mg/kg, $n=7$) or hAAT (60 mg/kg, $n=10$); Percent necrotic area. Data are representative of three independent experiments. **(C)** *Local hAAT administration.* Subcutaneous infiltration along flap borders of PBS (100 μ L, $n=7$), human serum albumin (125 μ g/100 μ L PBS, $n=9$), or hAAT (100 μ g/100 μ L PBS, $n=9$). Data pooled out of three independent experiments. All graphs show Mean $\pm$ SD, *** $p < 0.001$, **** $p < 0.0001$ between indicated groups by nonparametric Mann–Whitney test. **(D)** *Macroscopic and histological analysis.* Representative images of s.c.-treated flaps. White, demarcation line for histological sectioning; red, approximated necrotic area borders. Right, paired representative histological sections. H&E staining, $\times 10$ magnification; stitched images rendered from three microscope view fields per sample. hAAT, human $\alpha 1$ -antitrypsin; PBS, phosphate-buffered saline; H&E, Hematoxylin and Eosin; WT, wild-type. Color images are available online.

lower in transgenic mice expressing hAAT (hAAT^{+/+}) versus wild-type (WT) controls ($27.6\% \pm 12.2\%$ and $49.2\% \pm 8.2\%$, respectively; $p=0.002$). A single i.p. injection of hAAT (60 mg/kg) at the time of surgery resulted in a similar reduction compared with human serum albumin (Fig. 2B, $32.8\% \pm 13.8\%$ vs. $59.54\% \pm 9.7\%$; $p=0.0004$). Finally, local s.c. administration of hAAT ($100 \mu\text{g}/100 \mu\text{L}$ PBS) along flap length was found equally effective, significantly reducing flap necrosis area to $28.9\% \pm 5.9\%$, versus mice treated with either albumin ($125 \mu\text{g}/100 \mu\text{L}$ PBS, $59.8\% \pm 7.0\%$, $p<0.0001$) or PBS ($62.03\% \pm 3.4\%$, $p<0.0001$) (Fig. 2C).

Histological evaluation of skin sections from albumin or hAAT-treated flaps demonstrated an increased degree of inflammation and damage toward the distal end of the flap, with a higher degree of epidermal proliferation and progression in hAAT-treated mice (Fig. 2D).

hAAT-treated flaps demonstrate preserved perfusion *in vivo*

As blood supply plays an integral part in facilitating flap survival, we sought to evaluate the extent of flap perfusion following hAAT treatment (Fig. 3). As shown, hAAT-treated mice displayed increased fluorescein uptake into flap area three days (64.6% $\pm$ 4.0% vs. 43.7% $\pm$ 1.7%; $p=0.007$) and 5 days after flap surgery (60.6% $\pm$ 3.7% vs. 40.4% $\pm$ 1.8%; $p=0.0099$), versus human serum albumin treatment. Importantly, while no macroscopic necrosis was observed in either treatment groups at the time of fluorescein uptake measurement, the area of the flap that displayed negative fluorescent signal correlated with the subsequent area of ne-

crosis. These observations suggest that flaps of hAAT-treated mice display superior perfusion across a greater portion of the flap area versus flaps of albumin-treated mice.

hAAT-mediated flap survival partially persists under VEGF inhibition

Considering the positive regulation of VEGF production by hAAT, we sought to challenge hAAT-mediated flap survival using a clinically applicable VEGF-neutralizing antibody (Bevacizumab, 5 mg/kg). To simplify dosing and minimize excessive i.p. injections to the animals, hAAT^{+/+} mice were employed without exogenously added hAAT, relying on their transgenic hAAT levels. As shown (Fig. 4A), 7 days after surgery, CT hAAT^{+/+} mice displayed significantly lower necrotic area values than CT WT mice ($50.5\% \pm 6.7\%$ vs. $25.3\% \pm 11.4\%$, respectively; $p=0.0012$ between these two CT groups). Adding inhibition of VEGF resulted in an increase of the measured necrotic area in both groups: WT mice elevated the necrotic area to $62.9\% \pm 9.8\%$, and hAAT^{+/+} mice elevated the necrotic area to $39.8\% \pm 9.1\%$. Interestingly, Bevacizumab-treated hAAT^{+/+} mice displayed necrotic flap area that was similar to or lower than CT WT mice that had an *intact* VEGF pathway ($39.8\% \pm 9.1\%$), by this suggesting that intact VEGF activity is only partially required for improved hAAT-mediated flap procedure outcomes.

Inhibition of nitric oxide synthase counters hAAT-mediated flap survival

As VEGF blockade only partially interfered with hAAT-mediated flap survival, we sought to examine another inflammation-triggered pathway required for vascularization: nitric oxide synthase (NOS).

Figure 3. *In vivo* perfusion assessment of random-pattern flap. A caudally based random-pattern skin flap was performed, and flap perfusion was assessed 3 days after surgery. Comparison between s.c. human serum albumin ($125 \mu\text{g}/100 \mu\text{L}$ PBS, $n=7$) and hAAT ($100 \mu\text{g}/100 \mu\text{L}$ PBS, $n=7$) administered at the time of surgery. Fluorescein uptake (4 mg/kg , i.p.), IVIS imaging. (A) Relative flap area fluorescent counts, day 0 set at 100%. Mean $\pm$ SEM, $**p<0.01$ between treatment groups per time point by nonparametric Mann–Whitney test. (B) Representative flap images. Fluorescent image superimposed on photograph. Dashed line, flap area. IVIS, *in vivo* imaging system; VEGF, vascular endothelial growth factor. Color images are available online.

Figure 4. Effect of hAAT on flap survival during VEGF inhibitor treatment. A caudally based random-pattern skin flap was raised, and flap survival was assessed 7 days after surgery. Comparison between WT and hAAT^{+/+} mice, **(A)** Mice treated with either a single dose of anti-VEGF antibody immediately after surgery (Bevacizumab, 5 mg/kg, $n=5$ per group) or with PBS (CT). Percent necrotic area. Mean $\pm$ SD, * $p<0.05$, ** $p<0.01$ between indicated groups by nonparametric Mann–Whitney test. Data are representative of three independent experiments. **(B)** Mice treated with either of NOS inhibitor, L-NAME (10 mg/kg/day; $n=6$); or NOS inhibitor, L-NIL (3 mg/kg/day; $n=6$); or PBS (CT). Percent necrotic area. Mean $\pm$ SD, * $p<0.05$, ** $p<0.01$ between indicated groups using nonparametric Mann–Whitney test. Data are representative of three independent experiments.

Random flap survival was examined in hAAT^{+/+} and WT mice that were treated with either an eNOS inhibitor (L-NAME, 10 mg/kg/day for 3 days), iNOS inhibitor (L-NIL, 3 mg/kg/day for 3 days), or PBS (CT). As shown in Fig. 4B, 7 days from flap surgery, necrosis area was reduced in hAAT^{+/+} mice versus WT mice ($52.2\% \pm 6.1\%$ vs. $26.3\% \pm 9.3\%$, respectively; $p=0.0022$ between these two CT groups). Treatment with L-NAME resulted in no significant change of flap necrosis area in WT mice ($49.7\% \pm 6.6\%$). However, flap necrosis area was increased in hAAT^{+/+} mice treated with L-NAME versus CT hAAT^{+/+} mice ($58.8\% \pm 2.9\%$ vs. $26.3\% \pm 9.3\%$, respectively; $p=0.0021$). Similarly, hAAT^{+/+} mice treated with L-NIL displayed an increase in area of flap necrosis versus CT hAAT^{+/+} mice ($60.2\% \pm 2.2\%$ vs. $26.3\% \pm 9.3\%$, respectively; $p=0.0023$). hAAT^{+/+} mice treated with either of the two NOS inhibitors demonstrated a small but significant increase in flap necrosis versus their respective WT counterparts, suggesting that NOS inhibition precludes hAAT-mediated flap survival.

Distant tube flaps heal and display faster perfusion by recipient site under hAAT treatment

To directly assess the effect of hAAT treatment on the establishment of intact vascular connections, an axial tube flap model was employed (Fig. 1B). In this model, perfusion of distal flap tip was assessed by pedicle occlusion, followed by fluorescein injection; only an *intact vascular connection* will allow the distal tip to show a positive fluorescent signal. Axial flaps were raised, and the distal end treated with s.c. infiltration of either hAAT (100 μ g/100 μ L PBS) or albumin (125 μ g/100 μ L PBS). In addition, flaps were either “trained” or left untrained (receiving a sham procedure instead).

The mean day of observed flap perfusion by recipient site was *earlier* in hAAT-treated flaps versus albumin-treated flaps, in both trained flaps (2.6 ± 0.7 days versus 4.43 ± 1.0 days, $p=0.0033$) and untrained flaps (9.6 ± 1.6 days vs. 13.1 ± 1.0 days, $p=0.0005$) (Fig. 5).

It appears that adequate perfusion of the distal flap tip is significantly accelerated under treatment with hAAT, presumably allowing the pedicle of hAAT-treated flaps to be detached at a significantly earlier time versus albumin-treated controls.

DISCUSSION

This study examines hAAT therapy as a novel therapeutic approach for improving flap surgery outcomes. Several modes of hAAT administration were explored, all representing important aspects of hAAT therapy, and all showing significant enhancement of random-pattern skin flap survival. An initial mechanistic dissection of hAAT-mediated flap survival revealed a requirement for intact NOS pathway, but an unexpected capacity to partially maintain outcomes in the presence of effective VEGF blockade. Using a distant “tube” flap model, accelerated flap donor-site revascularization by hAAT therapy was confirmed. On the whole, our data suggest that hAAT may be considered for use as an adjunct treatment for supporting skin flap healing, particularly that of a distant flap, thereby allowing for significantly earlier pedicle detachment. Such an outcome holds an immensely positive impact for patients that undergo staged flap surgery.

Successful revascularization of any injured tissue requires an inflammatory trigger, coupled with its timely resolution.²⁴ Unresolved inflammation readily translates to impaired tissue repair. This study focuses on a single dose of hAAT, administered at the time of injury. Exogenous hAAT pres-

Figure 5. Perfusion of flap by recipient site in a distant “tube” flap model. A “tube” flap was performed and treated with distal tip s.c. human serum albumin (125 μ g/100 μ L PBS, $n=8$) and hAAT (100 μ g/100 μ L PBS, $n=8$) administered at the time of surgery. Flaps were either “trained” by daily applications of a microvascular clamp to flap pedicle (10-min ischemia time, 5-min reperfusion time; $\times 3$) or not trained. **(A)** Time to perfusion through distal tip. Flap perfusion was defined as positive fluorescein signal obtained from flap while pedicle was clamped. Scatter box-plot of measured perfusion day. Min to Max box plot, middle line states Mean $\pm$ SD, ** $p < 0.01$, *** $p < 0.001$ using nonparametric Mann–Whitney test between indicated groups. **(B)** Representative images, non-trained day 8 distant flaps, with and without pedicle clamping. Fluorescent image merged with photograph. Color images are available online.

ents a half-life of 3–4 days in humans and 1–2 days in mice. Combined with the physiological increase of hAAT levels upon inflammatory triggers, one can suggest that the inflammatory response is modulated, rather than blocked under hAAT-enriched conditions. This, in turn, could facilitate proactive resolution and support tissue repair. While the extent and mechanisms of hAAT-mediated anti-inflammatory effects are reviewed elsewhere,²⁵ the present study does surface a need for further examination of inflammation dynamics in hAAT-treated skin during injury.

One approach for advancing distant flap revascularization involves transient ischemic post-conditioning (“training”). While incorporating no exogenous medical therapy, the painful occlusion places patient compliance as a bottleneck parameter for the success of this approach. In this study, we hypothesized that added hAAT may accelerate the outcome of flap training, providing an incentive for patients to agree to the intervention. Indeed, our data show that “training” benefits from added hAAT, and that hAAT benefits from “training.” Whether this represents an additive or synergistic effect remains unclear. Further studies should examine whether downmodifying “training” protocol alongside added hAAT could establish a novel combinatory approach.

A crucial cellular activity during tissue repair, migration, is required from both infiltrating im-

mune cells and other cell types (e.g., epithelial cells, fibroblasts, and endothelial cells). Interestingly, despite it being a neutrophil elastase inhibitor and an IL-8-binding protein,²⁶ hAAT allows neutrophil to migrate, in part due to their capacity to directly neutralize hAAT in a paracrine manner.²⁷ Concurrently, neutrophils appear to survive damaging environments when added hAAT, providing a partial explanation for the bacterial-burden-reducing attribute of hAAT.²⁸ Under hAAT-enriched conditions, stimulated dendritic cells adopt a semimature, anti-inflammatory profile; appearing to maintain inducible expression of a key chemokine receptor, CCR7, preserving their migration capacity.²⁹

Regarding nonimmune cells, a recent study reports enhanced migration of a lung epithelial cell line under hAAT treatment.³⁰ Another study demonstrates improved fibroblast monolayer gap repair, a process which relies on both cell migration and cell proliferation.³¹ Similarly, hAAT was shown to facilitate smooth muscle myocyte migration and proliferation.³² Finally, our group has reported that endothelial cells respond to hAAT by rapid tube formation, alongside increased expression of VEGF, and superior vessel sprouting.¹⁰ These rather distinctive examples all share an underlying trigger for cellular migration–inflammation. It appears that although anti-inflammatory, hAAT is able to preserve or

enhance context-dependent inflammation-driven migration of relevant cells at the injured site. Enhanced cell migration might not be specific to vessel formation, representing a systemic encouragement to restore an injured tissue. It is important that further studies examine migratory responses under hAAT-enriched conditions, particularly during skin repair.

Our findings depict hastened and improved flap perfusion in the presence of hAAT. Knowingly, assessment of flap perfusion is limited in the form of macroscopic IVIS imager data acquisition. The rationale for the system involved the possibility of repeated, relatively noninvasive examinations, especially considering that excessive stress might affect the process of wound healing. However, as demonstrated using the distant flap model, no fluorescent signal was found in control clamped flaps, while hAAT-treated flaps were able to collect fluorescent serum from their newly connected peripheral blood supply. This confirmed our notion that fluorescein uptake occurs predominantly in perfused skin, providing a reliable assessment of flap perfusion. A similar outcome was observed when we applied Indocyanine green stain and measured its fluorescence using the same IVIS platform (not shown). Further studies of flap perfusion under hAAT therapy, complemented with microperfusion measuring techniques, such as laser speckle microangiography, are warranted.³³

The use of hAAT^{+/+} mice subsists in recent literature.^{22,34} It represents a *steady* release of elevated hAAT into circulation, much closer in resemblance to a physiological rise, than the pulse-like inoculation of exogenous hAAT. It also categorically excludes possible involvement of human plasma-derived moieties found in clinical-grade preparations. Interestingly, flap survival in hAAT^{+/+} mice persisted in the presence of VEGF blockade. hAAT has been shown to increase VEGF expression,³⁵ as well as prevent protease-dependent inactivation of secreted VEGF.³⁶ It is possible that our chosen Bevacizumab dose was countered by excess VEGF produced in hAAT^{+/+} mice, a vignette worth further examination. Conversely, the many functions of hAAT may offer benefits that are unrelated to VEGF. Our findings suggest that hAAT may potentially allow Bevacizumab-treated patients to maintain their treatment regimen while still undergoing required reconstructive procedures.³⁷

In contrast to VEGF blockade, NOS inhibition dramatically countered hAAT-mediated flap survival. While hAAT was shown to reduce LPS-induced NO release from cultured monocytes

(reviewed by Guttman *et al.*²⁵), its impact on NO levels in a complex tissue containing multiple cell types, such as the injured skin, warrants further study. The role of NO in ischemia–reperfusion injury and normal circulation of skin has been previously described.³⁸ A recent study reported that similar to hAAT, the protective effect of metformin on skin flap survival is negated by L-NAME.³⁹ Flap survival has been shown to require *early* iNOS activity,⁴⁰ opening a possibility for exploring the optimized timing of hAAT therapy.

In conclusion, the present study demonstrates enhanced random-pattern skin flap in hAAT-treated mice and accelerated perfusion of distant flaps by its recipient site. This suggests that hAAT therapy may benefit various procedures, where flap transfer must be delayed. In addition, it is possible that hAAT-treated distant flap pedicle may be detached at an earlier time, sparing esthetic and functional impairment of patients. Finally, it is plausible that the enhanced survival and preserved perfusion of hAAT-treated flaps may lead to an improved cosmetic outcome of successful flap procedures. While further mechanistic studies are warranted, the excellent safety record of hAAT, as well as the availability of clinical-grade hAAT preparations, render it a highly translatable therapeutic approach.

INNOVATION

There is no widely available and effective therapeutic approach which accelerates tissue repair and enhances skin survival following traumatic or surgical injury. To the best of our knowledge, this is the first work which describes the direct impact of hAAT therapy on skin repair outside the context of genetic hAAT deficiency. In addition, we demonstrate the use of a distant flap model, transferable and relevant to the clinical setting. hAAT presents as a promising candidate for effective prevention of complications associated with skin flap surgery, as well as reducing the healing time from such procedures.

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Ronen Schuster, PhD, has recently completed his PhD at the Department of Clinical Biochemistry and Pharmacology at the Ben-Gurion University of the Negev under the Supervision of **Eli Lewis, PhD**. His research deals with the role and mechanism of α 1-antitrypsin in the tissue repair process. **Eldad Silberstein, MD** is the interim director of the Department of Plastic Surgery at Soroka Medical Center. **Or Bar-Nathan, B.Med.Sc**, is a 5th-year medical student at the Ben-Gurion University of the Negev School of Medicine, and **Alon Tiosano, B.Med.Sc**, is a medical intern at the same school. **Profs. Lewis and Silberstein** coordinate the activity of the Faculty of Health

KEY FINDINGS

- Perfusion of distant flap although its recipient site is accelerated in hAAT-treated animals.
- Human α 1-antitrypsin enhances random pattern skin flap survival and preserves perfusion.
- Effects of human α 1-antitrypsin on flap survival partially persist under VEGF blockade but are reversed during NOS inhibition.
- The established safety and availability of hAAT places it as a highly translational and promising approach for supporting the healing of skin flaps.

Sciences “wound healing research hub,” bringing together basic science and clinical investigators around the multidisciplinary field of wound healing research.

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Abbreviations and Acronyms

hAAT = human α 1-antitrypsin
H&E = Hematoxylin and Eosin
IVIS = *in vivo* imaging system
NOS = nitric oxide synthase
PBS = phosphate-buffered saline
VEGF = vascular endothelial growth factor
WT = wild-type