Mechanisms of Action of External Volume Expansion Devices

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Background: External volume expansion by suction has been proposed to improve the survival of fat grafting by preparing the recipient site. In previous experimental work, external volume expansion demonstrated the capacity to stimulate cell proliferation, vessel remodeling, and adipogenesis. This study investigated possible mechanisms underlying these observed changes.
Methods: A miniaturized external volume expansion device was applied to the dorsum of mice for 2 hours. Hypoxia during stimulation was assessed with pimonidazole hydrochloride, and tissue perfusion was measured for up to 2 days using hyperspectral imaging. Treated tissues were evaluated by microscopy for edema, inflammation, and the effects on cell proliferation and vessel remodeling.
Results: External volume expansion–treated tissues were grossly expanded with 2 hours of stimulation, developing a macroscopic swelling that regressed slowly over the course of hours following stimulus cessation. This gross swelling was reflective of histologic signs of intense edema, persistent for at least 1 hour after external volume expansion. Tissues were hypoxic during stimulation, and hyperspectral imaging demonstrated decreased tissue content of both oxygenated and deoxygenated hemoglobin in the first hour after external volume expansion release. The onset of inflammation was already apparent by the end of stimulation and remained elevated through 2 days after external volume expansion. At this time point, epidermal and dermal cell proliferation and vascular density were significantly increased.
Conclusion: External volume expansion sets in motion various mechanisms, including mechanical stimulation, edema, ischemia, and inflammation, that over distinct time periods maintain an environment conducive to cell proliferation and angiogenesis, which can be elicited even by a single 2-hour external volume expansion cycle. (*Plast. Reconstr. Surg.* 132: 569, 2013.)

Fat grafting has great potential to facilitate a regenerative response through simple injections. In breast surgery, there is the potential to achieve a natural-appearing breast reconstruction and/or augmentation without the comorbidity or risk of complications of flaps and implants, which makes it particularly appealing for reconstructive cases and young patients. In its current state,

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A Video Discussion by Geoffrey C. Gurtner, M.D., accompanies this article. Go to PRSJournal.com and click on "Video Discussions" in the "Videos" tab to watch.

however, fat grafting remains limited by partial efficiency.[1] Fat grafts have been reported to survive in an unpredictable fashion (30 to 80 percent take), with an inverse correlation of survival to injection volume.[2–4] This results in the requirement for multiple sessions to achieve satisfactory volumes.[5,6] To date, significant research has focused on manipulation of the fat itself to improve engraftment. Although some discoveries have been promising, few have translated into clinical success.

Experience with skin grafts has shown that an improved recipient site will potentiate graft take.[7] Similar to skin grafts, grafted fat relies initially on diffusion and later on new vessels sprouting from the recipient site. Poor vessel density–to–grafted volume ratio and high subcutaneous compartment pressures after fat injection are likely factors that negatively affect fat engraftment. Khouri et al. initially proposed that an external volume expansion system (Brava System; Brava, LLC, Miami, Fla.) could be used for nonsurgical breast augmentation by transferring to the breast the principles of tissue expansion.[8] External volume expansion devices mechanically stretch and stimulate tissues by suction in a noninvasive manner. Stretch releases the skin[9] and by direct mechanical action on single cells,[10,11] induction of ischemia,[12,13] inflammation, and soluble mediators[14] stimulates cell proliferation and, most importantly, vascular remodeling. The latter was the conceptual basis on which a decade later the same system was used empirically for preoperative site preparation for fat grafting.[15,16] With this method, Del Vecchio and Bucky reported a 60 to 200 percent increase of human breast volume by quantitative magnetic resonance imaging after autologous fat injection, with a long-term consistent increase of 64 ± 13 percent in volume.[17] More recently, Khouri et al. reported an 82 ± 18 percent fat survival at 6 to 12 months in a series of 81 external volume expansion–treated patients enrolled in a multicenter trial that they compared to an average 55 ± 18 percent in a recently published series of non–external volume expansion–treated fat grafted breasts.[18]

To contribute to the understanding and demonstration of the effects of external volume expansion devices, we previously developed a miniaturized murine external volume expansion model and were able to show that 28 days of continuous treatment with external volume expansion induces cell proliferation and vascular remodeling and has neoadipogenic effects on the hypodermis.[19] We now apply a similar model to investigate the early tissue response to external volume expansion treatment.

MATERIALS AND METHODS

Animal Model

A dome-shaped rubber device with a diameter of 1 cm and an internal volume of 1 ml was fabricated, and connected to a suction pump (VAC Instill; Kinetic Concepts, Inc., San Antonio, Texas) at a pressure of –25 mmHg according to our previously published method.[19] The device was applied to the dorsal skin of mice 5 cm cephalad to the tail and 3 cm lateral to the midline spine without fixatives. The skin around the device was stabilized by a semirigid rubber doughnut-shaped frame, with an internal diameter of 2.5 cm. A total of 24 male, 8-week-old, SKH1-E hairless mice (Charles River Laboratories, Wilmington, Mass.) were used in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility and in accordance with our institutional animal care and use committee guidelines under an approved protocol. Eighteen mice were treated for 2 hours with the external volume expansion device set at a pressure of –25 mmHg; six were used as untreated controls. At the end of treatment, mice were killed immediately (2-hour group, $n = 6$), 1 hour (2-hour plus 1-hour group, $n = 6$), or 2 days (2-hour plus 48-hour group, $n = 6$) after removal of the device. The time points for follow-up after external volume expansion were chosen on the basis of the type of biological responses investigated and on the previous experiences of our group with in vivo mechanobiological models,[12,14,20,21] and the observations of other authors in ischemia-reperfusion models and on tissue responses after skin stimulation. Tissues were harvested with a 10-mm biopsy punch. Samples were fixed in 10% neutral-buffered formaldehyde for 24 hours and stored in 70% ethanol at 4°C.

Hypoxia

Pimonidazole hydrochloride staining was used to identify hypoxic cells. Pimonidazole selectively binds to thiol-containing proteins in cells with oxygen tension less than 10 mmHg and was injected intraperitoneally (70 mg/kg) in the 2-hour external volume expansion and untreated groups 30 to 60 minutes before the animals were killed.

Hyperspectral Imaging

Spatial maps of tissue perfusion and oxygenation were generated using a medical hyperspectral imaging system (OxyVu-2; HyperMed, Inc., Greenwich, Conn.). The optical properties of this device have been described.[22] Briefly, this device uses optical hardware to collect images of a sample

over a 20-second period at select wavelengths between 500 and 660 nm. Diffuse reflectance tissue spectra were determined for each pixel within this collection of images using proprietary algorithms. Mean oxyhemoglobin and deoxyhemoglobin values were obtained by decomposition from a 79-pixel-diameter region of the images corresponding to the stimulated area using standard spectra for oxyhemoglobin and deoxyhemoglobin. Perfusion was measured as total hemoglobin, calculated as the sum of oxyhemoglobin and deoxyhemoglobin. Tissue oxygenation was calculated as oxyhemoglobin divided by total hemoglobin. Hemoglobin values are reported in arbitrary units that have previously been shown to correlate with in vivo molar concentrations.[23]

Before imaging, the system was calibrated to a standard pixel reflectance. Imaging was performed with mice under anesthesia, at standard room temperature, and respiratory motion artifact was corrected with the use of a fiducial target. A baseline scan was obtained from all animals immediately before external volume expansion on day 0. Treated areas in mice of the 2-hour plus 1-hour group and the 2-hour plus 48-hour group were scanned at multiple time points for 1 hour after removal of the external volume expansion device. Mice of the 2-hour plus 48-hour group were further scanned 4 hours, 1 day, and 2 days after the end of stimulation.

Histology and Immunohistochemistry

Samples were embedded in paraffin and cut into 5-μm sections. Hematoxylin and eosin staining was performed according to standard protocols. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded ethanol series. Antigen retrieval for proliferating cell nuclear antigen was accomplished by microwaving in 10 mM sodium citrate (pH 6.0). Sections for endothelial cell marker platelet endothelial cell adhesion molecule 1 and pan-leukocyte marker CD45 were treated with 40 μg/ml proteinase K (Roche Diagnostics Corp., Indianapolis, Ind.) for 30 minutes at 37°C. Platelet endothelial cell adhesion molecule 1, CD45, and proliferating cell nuclear antigen primary antibodies (Table 1) were incubated at 4°C overnight. Signal was intensified using the tyramide amplification system (Perkin-Elmer, Boston, Mass.), and positive staining was detected with 3,3′-diaminobenzidine (Dako North America, Inc., Carpinteria, Calif.). Slides were counterstained with hematoxylin. Images were acquired using a Nikon E200 microscope (Nikon Corp., Tokyo, Japan). Materials are listed in Table 1.

Cell proliferation in the epidermis and in the deep dermis was assessed by cell counting in 40× fields of treated areas with Adobe Photoshop (Adobe Systems, Inc., San Jose, Calif.) software and expressed as the ratio of proliferating cell nuclear antigen–positive nuclei to total nuclei. Epithelial appendages were excluded from counting. The same method was used to quantify the ratio of inflammatory cells to total cells in the deep dermis. Blood vessel density was quantified as the number of platelet endothelial cell adhesion molecule 1–stained vessels identified in 10× fields. For each slide, three fields per staining were evaluated by three independent observers with experience in skin histology and trained in the specific methods used in this study.

Statistical Analysis

Results are expressed as the mean ± SD in text and figures. Differences in hyperspectral imaging features between time points were evaluated by general mixed linear models[24] using Fisher's least significant difference multiple comparisons procedure without multiplicity adjustments (Proc Mixed procedure of SAS Statistical Software Version 9.2; SAS Institute, Inc., Cary, N.C.) given repeated measures were made. Time and subject were identified as fixed and random effects, respectively, for these models. Compliance with the distributional assumption of normality was evaluated using the Kolmogorov-Smirnov one-sample goodness-of-fit test for normality[25] applied to model residuals. For quantitative immunohistochemistry, one-way analysis of variance (WinSTAT; R. Fitch Software) with Tukey post hoc correction was used to determine the significance

Table 1. Primary and Secondary Antibodies

Antibody	Manufacturer	Product Code	Dilution
PCNA	Thermo Scientific	RM-9106-S	1:200
CD31	BD Pharmingen	553370	1:100
CD45	BD Pharmingen	550539	1:100
Hypoxyprobe1 MAB1	HPI, Inc.	HP1 200 Kit	1:250
Biotinylated anti-rat IgG	Vector Laboratories	BA-4001	1:100
Biotinylated anti-rabbit	Thermo Scientific	TR-125-BN	Ready to use

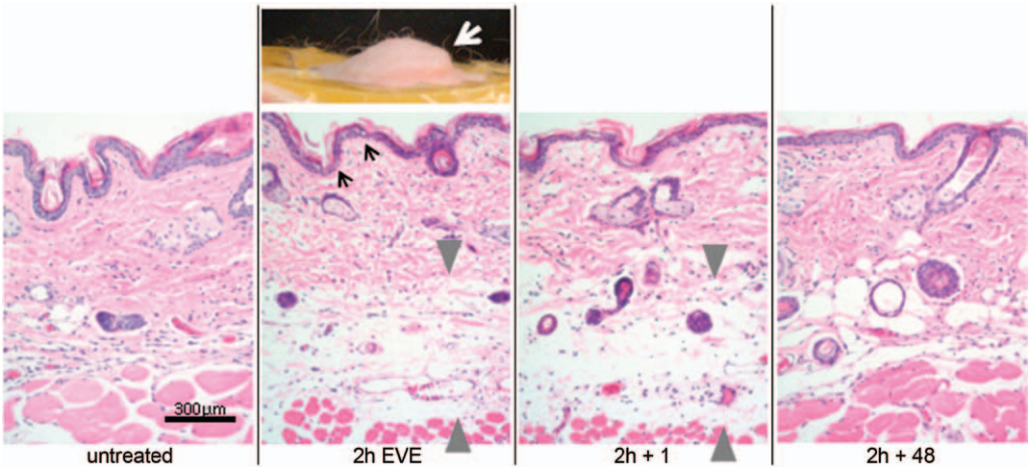

Fig. 1. At the removal of the external volume expansion device, after 2 hours of stimulation, a macroscopically evident tissue swelling (*white arrow*) has developed in the treated area (*second from left*). Microscopically, this corresponds to accumulation of intense edema in the deep dermis and hypodermis (between *gray arrowheads*) not present before stimulation, and still present 1 hour after the end of stimulation (*second from left* and *second from right*). Isolated nuclear vacuolization is seen in some epithelial cells at the end of treatment (*black arrows*). Normal microscopic anatomy is restored 2 days after external volume expansion treatment (*right*) (hematoxylin and eosin; original magnification, × 10). *EVE*, external volume expansion.

of differences. A value of $p < 0.05$ was considered statistically significant.

RESULTS

At the end of the external volume expansion cycle, the treated area developed local swelling (Fig. 1, *left*). Swelling was reduced but persistent at 1 and 4 hours after stimulation. On histologic examination, gross edema was evident in the deep dermis/hypodermis and was unvaried between the end of treatment and 1 hour thereafter; the superficial dermis was less affected. Epidermal cells showed focal vacuolization. By 2 days after stimulation, normal histologic architecture had been restored (Fig. 1, *second from left*).

Tissue Hypoxia

The staining for pimonidazole hydrochloride showed diffuse positivity of the majority of cells in the dermis and epidermis of external volume expansion–treated skin compared with rare positive cells in untreated skin, demonstrating the presence of hypoxia in external volume expansion–treated tissues (Fig. 2).

Hyperspectral Imaging

Oxyhemoglobin levels in stimulated samples were similar to pretreatment baseline levels between 5 seconds and 3 minutes 30 seconds after removal of the external volume expansion device. By 6 minutes after stimulus cessation, oxyhemoglobin levels were significantly decreased and

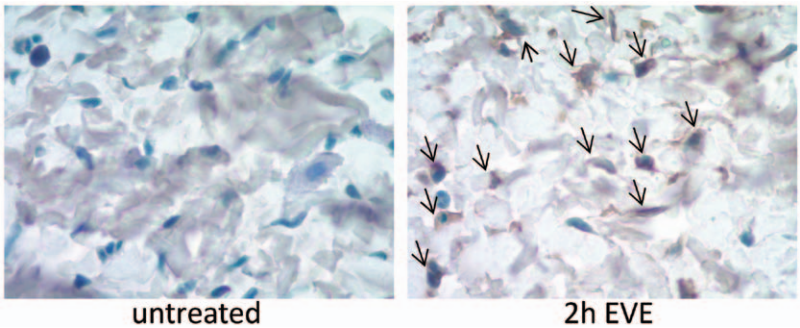

Fig. 2. (*Above*) Immunohistochemistry staining for pimonidazole hydrochloride demonstrates numerous hypoxic cells (*arrows*) in external volume expansion–treated areas that are not found in untreated controls.

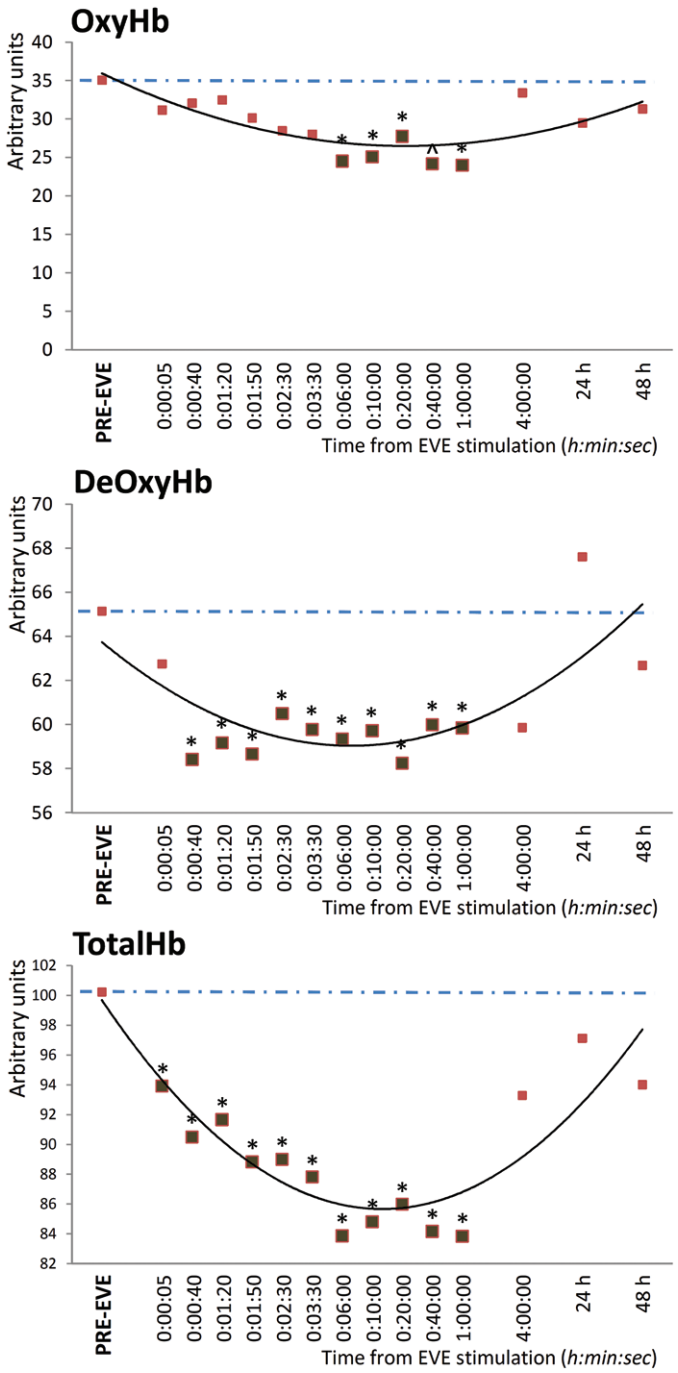

Fig. 3. Hyperspectral imaging of treated areas demonstrates decreased tissue content of oxyhemoglobin (*OxyHb*) after external volume expansion treatment starting from 6 minutes after release of suction. Deoxyhemoglobin (*DeOxyHb*) is significantly decreased from 40 seconds after removal of external volume expansion at all time points up to 1 hour. Total hemoglobin (*TotalHb*), which is the sum of the previous two, is decreased at all time points from external volume expansion release to 1 hour after external volume expansion. All parameters are normalized at the 4-hour time point and in the following 2 days. $^p < 0.05$, $*p < 0.01$ versus pre–external volume expansion baseline. Notice that the time points indicated correspond to the beginning of a 20-second imaging caption cycle. *EVE*, external volume expansion.

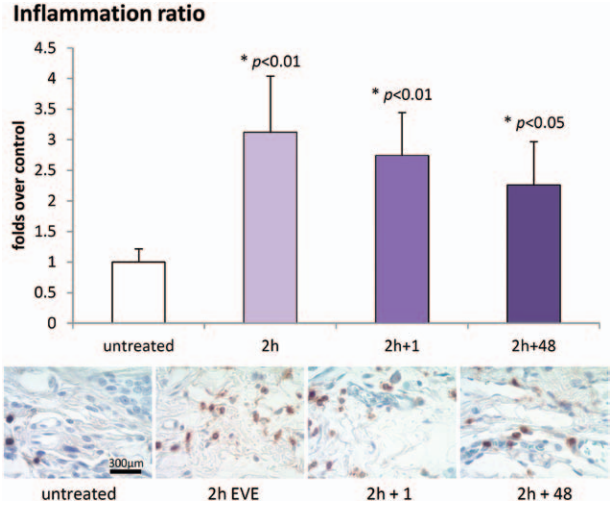

Fig. 4. Semiquantitative analysis of slides stained for pan-leukocyte antigen CD45 demonstrates significant 3-fold increase of density of inflammatory cells in external volume expansion–treated skin at the removal of the device. Inflammation decreases over time but is still significantly higher than baseline 2 days after treatment. *EVE*, external volume expansion.

remained so for 1 hour. Normal oxyhemoglobin levels were restored by 4 hours (Fig. 3, *above*). In parallel, deoxyhemoglobin was significantly decreased at all time points between 40 seconds and 1 hour. A nonsignificant trend toward lower deoxyhemoglobin levels was observed at 4 hours after external volume expansion, with normal levels restored on day 1 (Fig. 3, *center*). Total hemoglobin content of the stimulated tissue was lower than baseline at all time points from the end of treatment up to 1 hour (Fig. 3, *below*), whereas tissue oxygenation was reduced between 6 minutes and 1 hour after treatment.

Inflammation

External volume expansion–treated tissues displayed a significantly increased inflammatory infiltrate by the end of the 2-hour stimulation. Inflammation tended to resolve over time but was still elevated above baseline levels by 2 days (Fig. 4).

Cell Proliferation

Two days after stimulation, the proliferation rate was 1.4- and 1.7-fold higher in the epidermis ($p < 0.05$) and dermis ($p < 0.01$) of treated skin compared with untreated skin, respectively (Fig. 5, *above* and *center*).

Vessel Density

The skin of external volume expansion–treated samples from the 2-hour plus 48-hour group had a significantly higher vascular density than samples from the untreated, 2-hour, and 2-hour plus 1-hour groups. No difference in vascular density was observed when comparisons were made between samples from the untreated, 2-hour, and 2-hour plus 1-hour groups (Fig. 5, *below*).

DISCUSSION

Our results demonstrate that the three-dimensional mechanical forces that external volume expansion exerts on tissues activate multiple pathways ultimately responsible for proliferative, vascular, and even adipogenic responses, as seen in our previous study with this model.[19]

It is likely that the induced extracellular matrix deformation, which in our model is evident from the macroscopic appearance of treated tissues, translates to an increase of the micromechanical strain on the single cells anchored to extracellular matrix fibers at focal adhesion sites. This has largely been shown in vitro to induce a proproliferative state by acting as a gate-control signal on global cellular activity mediated by the cytoskeleton.[10,26]

On a different scale, blood vessel deformation by external volume expansion likely obstructs blood flow either through kinking or stretching, which reduces cross-sectional area and increases vascular resistance. Reduction of the interstitial pressure favors accumulation of edema and increases cell-to-vessel distance, decreasing mass transport because of diffusion. Pimonidazole hydrochloride staining shows that oxygen tension is diffusely reduced during external volume expansion stimulation. As we have demonstrated, edema is not immediately reabsorbed at the removal of external volume expansion. With the removal of the external subatmospheric pressure, edema transforms into a relatively compressive force on individual cells and blood vessels. This proposed relationship of edema and tissue perfusion is further supported by our hyperspectral imaging observations. The oxyhemoglobin content of tissues depends on systemic and local factors. In unmodified systemic conditions, oxyhemoglobin concentration is affected principally by the quantity of blood that passes through the capillaries in a unit of time (blood inflow). Deoxyhemoglobin, in contrast, reflects both the local tissue metabolism at the cellular level and the time red blood cells need to pass through capillaries, during which oxygen is exchanged with local tissues. In our case, the prolonged decrease

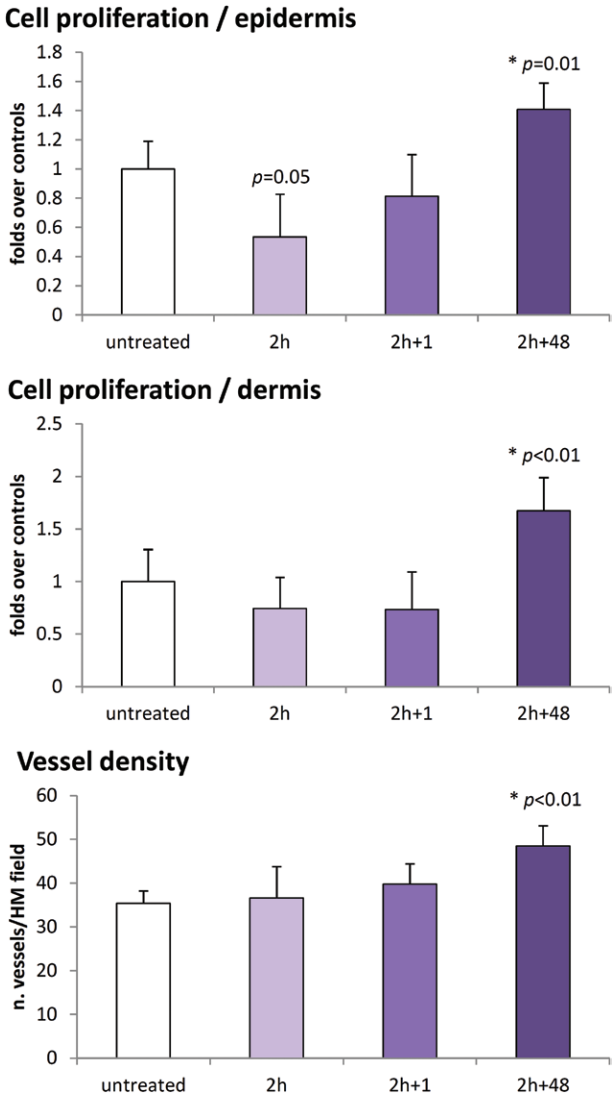

Fig. 5. External volume expansion induces a significant increase in cell proliferation rate by 2 days after treatment in the epidermis (*above*) and deep dermis/hypodermis (*center*), as evaluated by cell counting in proliferating cell nuclear antigen–stained slides; results expressed as fold increase over untreated skin. (*Below*) External volume expansion–treated skin exhibits higher density of platelet endothelial cell adhesion molecule 1–positive vascular structures 2 days after stimulation; results are expressed as number of vessels per field.

in oxyhemoglobin we measured between 6 and 60 minutes after external volume expansion removal suggests a relatively reduced blood inflow, possibly attributable to reactive vasoconstriction secondary to stretch. Decreased levels of deoxyhemoglobin and total hemoglobin are suggestive of the absence of significant venous stasis and the absence of vessel damage with extravasation of red blood cells in the interstitium. A limit of the hyperspectral imaging as used in this study is the time needed for signal acquisition (20 seconds) and processing (20 seconds), which does not allow for true real-time imaging, and impedes detailed exploration of the initial physiology after external volume expansion release. The combination of pimonidazole observations that show hypoxia during stimulation, with normal oxyhemoglobin levels along with decreased deoxyhemoglobin and total hemoglobin after ceasing stimulation, are suggestive of a behavior mildly reproducing hypoxia-reperfusion models. The likely peak in blood flow within the first seconds that can be expected on the basis of those models is probably lost in our analysis because of the limits of the technique. A prolonged state of relative ischemia is established after external volume expansion release that is likely maintained by the combination of vasoreactivity (not explored in our study) and compression by edema.

Although the mechanical stimulus of external volume expansion ends abruptly with cessation of suction, relative ischemia and hypoxia seem to last at least 1 hour beyond the stimulus and normalize by 4 hours. We also observed that an equally powerful stimulus, inflammation, appears to last well beyond the immediate hours following external volume expansion cessation. The onset of inflammation was already seen with 2 hours of external volume expansion stimulation, and persisted through 48 hours after external volume expansion, albeit in a progressively decreasing state. Ischemia and hypoxia are well-known triggers of cell proliferation and the best known stimulus of vascular remodeling and neoangiogenesis by means of activation of the hypoxia-inducible factor 1α/vascular endothelial growth factor pathway.

Based on our observed results through 48 hours after external volume expansion, it appears that all four factors—micromechanical stimulation, edema, hypoxia and ischemia, and inflammation—likely contribute to cell proliferation seen in the epidermis and deep dermis and hypodermis and to vessel proliferation (Fig. 6). Importantly, previous studies suggest that both edema and inflammation, acting in a coordinated fashion, have proadipogenic effects. Brorson et al. observed that lymphedema is accompanied by a net accumulation of fat tissue concentrated in the affected body part,[27,28] which is suggestive of a causal relationship. Zampell et al.[29] and Aschen et al.[30] developed a model of tail lymphedema and reported results that appear to confirm this hypothesis. Harvey et al. demonstrated that defective lymphatic drainage and generalized edema caused by mutation of the Prox 1 allele lead to

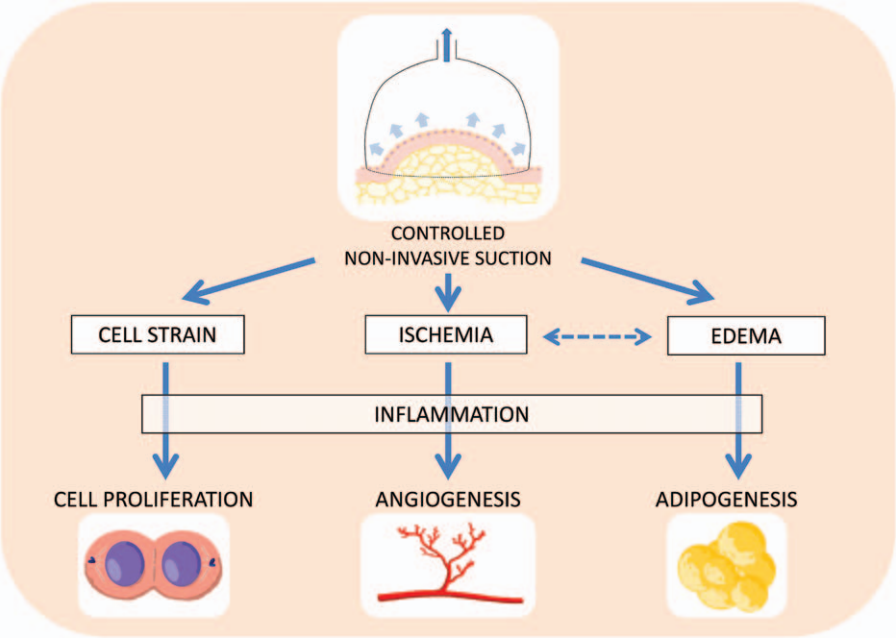

Fig. 6. The controlled noninvasive suction applied by external volume expansion devices determines macroscopic tissue stretch and induces local ischemia and accumulation of edema; all three factors can trigger inflammation, and either independent of or through inflammation are known to activate pathways leading to cell proliferation, vessel remodeling, and adipogenesis.

the development of obesity with increased subcutaneous tissue,[31] and lymph stasis may alter local metabolic balances with the accumulation of molecules, particularly lipids, possessing lipogenic or adipogenic effects.[32] In an elegant series of studies, Cronin et al. and Dolderer et al. demonstrated that the presence of an empty proteic matrix[33–36] in contact with a fat mass[37,38] provides signals for cellular differentiation and attracts cells from blood vessels that are induced to differentiate into adipocytes, promoted in part by inflammation.[39] Their model, which may closely resemble the "empty space" filled with edema in external volume expansion–treated tissues, is now being translated from animals to clinical applications.[40] Our results in this study may then help to explain the observations gathered in our previous study on external volume expansion, in which we demonstrated that 28 days of continuous external volume expansion induces thickening of the subcutaneous adipose tissue.[19] Thickening was reflected by a net increase in the number of adipocytes in vertical columns within the subcutaneous tissue, confirming the claims of neoadipogenic potential by Khouri et al.[8] when they initially developed an external volume expansion system for nonsurgical breast augmentation. Edema is a well-known effect of external volume expansion applied to breasts and is evident even after the first day of application. However, caution should be exercised when attempting to translate the results of this animal model to clinical significance, as important intrinsic differences are present that could affect the relative contribution of the different factors we have identified. In particular, human tissues are stiffer and less deformable than those in the mouse, which affects both the stretch imposed at a given pressure and the amount of edema that can accumulate in the third space. Furthermore, the adipogenesis shown in our previous study resulted from continuous external volume expansion that maintains a stable level of edema remindful of lymphedema and Morrison's chamber model.[33] In contrast to this static state of edema, current clinical external volume expansion uses cyclical wear patterns (6 to 10 hours daily) that generate transient periods of edema formation and subsequent absorption. This can be expected to lead to the influx/outflux of metabolites, and more closely reflects the design of this study. Although the proposed nonsurgical external volume expansion methods have yielded a limited degree of breast enlargement,[41–44] the possibility that continuous wearing of external volume expansion may overcome this barrier shown with cyclical external volume expansion appears to be an appealing hypothesis to evaluate.

Another question that our study generates is whether post–fat grafting external volume

expansion is clinically appropriate, as some authors have reported.[18] Although external volume expansion may release positive pressure from the engrafted fat, it also induces intense levels of edema that may decrease diffusion of metabolites critical to fat survival during the first days. For the same reason, an interesting approach may be to reinitiate external volume expansion stimulation *after* the first week after engraftment—when graft take can be expected to have already occurred—to maximize the adipogenic potential of the grafted tissue that is undergoing remodeling during that period. Further studies will be needed to correlate our data with the clinical scenario and to determine whether an additive effect exists with the daily cyclical wearing of external volume expansion relative to cell proliferation and angiogenesis. This may allow us to help optimize clinical protocols for use of external volume expansion devices to prepare tissues for fat grafting.

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Evidence-Based Medicine: Questions and Answers

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