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TITLE: Microfragmented Adipose Tissue and Blood Plasma-Based Hydrogels for Treatment of Combat-Associated Burn Injuries

PRINCIPAL INVESTIGATOR: Shanmugasundaram Natesan, PhD

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Shanmugasundaram Natesan, PhD; Randolph Stone II, PhD; Robert J. Christy, Ph.D

Email ID: Shanmugasundaram.natesan.ctr@mail.mil

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US Army Institute of Surgical Research,
3698 Chambers Pass,
Bldg 3611, JBSA, Ft. Sam Houston, TX 78234

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Following full thickness burn injuries, the subcutaneous adipose tissue often suffers severe damage. Even when the hypodermal fat layer is not fully involved, surgical intervention usually results in removal of the hypodermis down to muscle fascia. Although this avoids the complications of inadvertently leaving necrotic foci in the wound bed, it negates the benefit of retaining viable hypodermal adipose tissue and microvasculature. Grafting onto fat has been shown to reduce wound contraction, especially in extremity burns located near joints, resulting in better range of motion and improved sensation. This indicates that grafts onto fat may heal better and have improved innervation. In addition, the removal of the hypodermal tissue results in loss of vasculature and poor graft take, leading to wound contraction and scarring. The purpose of this study was to evaluate application of micro-fragmented adipose tissue (Lipogems) as a hypodermal skin substitute using hydrogels in a porcine full-thickness wound model. We hypothesize that early reconstruction of hypodermis using purified Lipogems and hydrogels will improve angiogenesis, healing, and scar appearance.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Subject: According to the National Burn Repository of the American Burn Association (ABA), the annual burn incidence in the United States is approximately 500,000. Approximately 3,400 people lose their lives and 40,000 require hospitalization due to burn related injuries (1). Following full-thickness burn injuries, the subcutaneous adipose tissue often suffers severe damage. The current standard of care to treat deep partial thickness and full thickness burns involves excision to viable wound bed followed by early coverage with a split thickness skin graft. Even when the hypodermal fat layer is not fully involved, surgical intervention usually results in removal of the hypodermis down to muscle fascia to avoid the complications of inadvertently leaving necrotic foci in the wound bed (2). However, grafting onto fat has been shown to reduce wound contraction especially in extremity burns located near joints (3, 4). Patients who received skin grafts onto fat reported better range of motion and reported more normal sensation than patients who received skin grafts directly onto fascia (4). Moreover, currently available skin substitutes do not include the adipose tissue layer in their constructs. A recent clinical study shows that skin grafting on subcutaneous fat to have a substantial influence on reduction of repeated reconstructive surgeries and long term scarring which may reduce serious deformity complications (2). Therefore, our effort in this project focuses on reconstructing/replacing subcutaneous adipose tissue after a full-thickness skin injury. Recently, a point-of-care FDA approved technology has been developed by Lipogems LLC., to mechanically process adipose tissue without any enzymatic digestion. For clinical use, adipose tissue can be isolated at the 'bed-side' in the surgical suite using aspirator and processed with this mechanical device. The processed fat essentially contains mesenchymal stem cells, pericytes, and endothelial cells in a cluster within the collagenous extracellular matrix (5-7).

Purpose: The management of acute burn wounds requires maintenance of a moist environment to both initiate active healing processes and to protect surrounding tissue from further trauma. The major goal of this project is to reconstruct full-thickness wound with microfragmented adipose tissue (Lipogems). Our objective is to replace the hypodermis of a full thickness burn wound after debridement before grafting. We hypothesize that subcutaneous hypodermal replacement will augment revascularization of a full-thickness burn wound and improve healing and scar appearance. In order to accomplish this goal we have non-enzymatically processed allogeneic adipose tissue using a FDA-approved device to isolate the microfragmented adipocytes and stem cell fraction (termed Lipogems). We have then used a blood plasma-based hydrogel as an adjunct to reconstruct the hypodermis. In order to assess the feasibility, and efficacy of using Lipogems to treat full thickness skin loss, we have used a porcine full-thickness burn wound model. Wherein, following a deep partial thickness burn wound, allogeneic adipose layer is spared to prepare hydrogel-Lipogems formulations and used along with autologous meshed split thickness skin graft (mSTSG) to cover the full-thickness wounds.

Scope: We aim at providing a cost-effective, bedside treatment option to regenerate full-thickness burn wounds with better long term healing. To accomplish this goal, we propose to deliver Lipogems using platelet free plasma (PFP) hydrogels. Lipogems will retain the essential cell populations necessary for regeneration, and when delivered via hydrogel matrix will improve healing of full-thickness burn wounds with better scar outcomes. The Lipogems-hydrogel

treatment will allow cells within the Lipogems to recapitulate the 3-D microenvironment and act as a conducive medium to foster better graft take and wound healing outcomes.

1. **KEYWORDS:** (limit to 20 words).

Lipogems, PFP hydrogels, Porcine burn Wound, meshed split thickness skin graft, adipose tissue

ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Goals

Evaluate the optimal Lipogems-hydrogel formulation using a 30 cm^2 full-thickness porcine burn wound model to assess healing and longtime healing

Specific Aim 1: Optimization and characterization of Lipogems and hydrogel based formulations	**Timeline (Months)**	**Status**
Major Task 1: Human Lipogems-hydrogel based formulation	**Y1**	Start: Y1Q1 Completed:Y2Q2
Subtask 1: Lipogems-PEGylated PFP hydrogel formulation	9-12	Completed
Subtask 2: Lipogems-PEGylated fibrin hydrogel formulation	9-12	
Subtask 3: Lipogems-collagen hydrogel formulation	9-12	
Subtask 3: *In vitro* characterization Lipogems-hydrogel formulations	12-15	
Milestone(s): 1. *Human and porcine adipose tissue Lipogems preparation protocol established.* 2. *In vitro Lipogems and Lipogems-hydrogels characterization completed.* 3. *1st manuscript (Pending; consolidating manuscript 1 with the results from excision wound model treated with Lipogems formulations).*		
Specific Aim 2: Screening and evaluation of Lipogems in full-thickness porcine wound model	**Y2**	Start: Y2Q1 Completed:Y2Q4
Major Task 1: Full-thickness excision wound model (30cm^2 wounds)	7-24	Y2Q1-Y3Q1
Subtask 1: Autologous adipose tissue isolation	13-24	
Subtask 2: Surgical procedure	7-24	
Major Task 2: Treatment of full-thickness wounds with Lipogems and Lipogems-hydrogels	10-24	
Major Task 3: End point measurements	13-24	
Subtask 1: Photo-Documentation of Healing	13-24	
Subtask 2: Assessment of graft take and healing	13-24	
Major Task 4: Data Analysis	22-27	
Milestone(s) Achieved: 1. *Treatment protocol established to evaluate Lipogems and Lipogems-hydrogel formulation.* 2. *Effective dose of Lipogems and Lipogems-hydrogels identified.*		

3. *Efficiency of Lipogems and Lipogems-hydrogel formulation evaluated.*
4. *1st manuscript under preparation (Combined effort year 1 and 2).*

Specific Aim 3: Lipogems using full-thickness porcine burn wound model	**Y3**	Start: Y2Q4 Experiments Completed:Y3Q4
Major Task 1: Full-thickness burn excision wound model (5×5 cm wounds)	25-36	Completed Y3Q4
Subtask 1: IACUC approval	Y2Q4	
Subtask 2: ACURO approval	Y32Q1 (*Delayed*)	
Subtask 2: Allogeneic adipose tissue isolation	22-25	
Major Task 2: Treatment of full-thickness burn wounds with Lipogems and Lipogems-hydrogels	27-36	
Major Task 3: End point measurements	30-36	
Subtask 1: Photo-Documentation of Healing	30-36	
Subtask 2: Assessment of graft take and healing	30-36	
Major Task 4: Data Analysis	30-36	*Data analysis pending*
NOTE: No Cost Extension approved to complete data analysis	*NCE: January 2020*	*NCE Approved*

Milestone(s) Achieved:

5. *Burn treatment protocol established to evaluate Lipogems and Lipogems-hydrogel formulation.*
6. *Safety and efficacy of Lipogems and Lipogems-hydrogel formulation evaluated.*
7. *All the experiments completed as proposed.*

What was accomplished under these goals?

1. MAJOR ACTIVITIES (Year 3)

- **Evaluation of Lipogems in full-thickness burn porcine wound model**

2. SPECIFIC OBJECTIVES

- Allogeneic Lipogems isolated and stored.
- Treatment protocol established to evaluate Lipogems and Lipogems-hydrogel formulation in a porcine full thickness bur wound healing model.
- Safety and efficiency of Lipogems and Lipogems-hydrogel formulation evaluated *in vivo*.

3. KEY OUTCOME (*for the current reporting period-Year 3*)

METHODS:

a. **Porcine Lipogems**: Isolation of Lipogems and PEG-PFP hydrogel + Lipogems formulations were carried out as described in our previous report (Year 2 Report). Briefly, emulsion of fragmented adipose tissue forms within the barrel and the saline flow washes the tissue, under gravitational force until saline barrel clears and allowing the processed adipose tissue (Lipogems) to float which are then extruded through the grey filter and collected for further experiments. The

cluster of Lipogems were collected in a separate sterile conical tube and aliquots (5ml) were cryopreserved using 5ml of cryoprotectant media (Mesenpro basal media containing 10% dimethyl sulfoxide.

b. **PEGylated platelet free plasma (PEG-PFP) hydrogel**: PEG-PFP hydrogels were prepared from PFP isolated from both human and porcine whole blood. Briefly, PEG (8mg/ml) was mixed with a PFP at a 1:20 v/v ratio. This mixture was then incubated for 10 minutes in a 5% CO_2 humidified incubator at 37°C. Gelation of the PEG-PFP liquid mixture was then initiated using human thrombin (10U/ml of PEG-PFP) and incubated for 20 minutes in a 5% CO_2 humidified incubator at 37°C to obtain PEGylated PFP hydrogels.

c. **Hydrogel-Lipogems formulations**: Lipogems+PEG-PFP hydrogels for porcine wound healing studies were prepared on the days of treatment by mixing 1ml of porcine Lipogems clusters (1ml) with 6.4ml of PEG-PFP solution and gelled using thrombin (10U/ml of PEG-PFP). The gels were kept 5% CO_2 humidified incubator at 37°C until treatment.

d. **Treatment of 25 cm_2 full-thickness porcine burn wound with Lipogems + hydrogels:**

The animal studies were carried out with ACURO (MB150163) and IACUC approval, and has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Prior to excision and treatment, 100 ml of blood was obtained and used for preparing PEG-PFP hydrogels.

In this study, 5x5 cm_2 full thickness burns (25 sec) were created by placing a thermocoupled brass block heated to 100°C on the dorsum of each anesthetized pig at a constant pressure (~0.4 kg/cm2). Five wounds were placed on each side, separated by 3-5 cm, at same distance (2-4 cm) from the spine in the same anatomical location. Two unburned locations with tattoo were used as a controls (**Figure 1A**). Prior to burning on day -4, up to 200 ml of blood was obtained and used for preparing PEGylated plasma hydrogels. Full thickness burns were created on the dorsum as described above. On day 0, the wounds were sharp debrided to remove all necrotic tissue. Full-thickness skin loss was performed using a surgical blade down to fat for all wounds. The wounds were treated with the PEG-PFP hydrogels ± Lipogems. Widely meshed split thickness skin grafts will be applied to all wounds in addition to the designated treatments.

After application PEG-PFP hydrogels ± Lipogems (**Figure 1B**), all the wounds were covered with a split-thickness skin graft (STSG) that was harvested from the hind legs using a Zimmer pneumatic dermatome (Zimmer Inc, Warsaw, IN) set at the thickness of 12 thousandths of an inch (0.012") and meshed using a skin mesher at 4:1 ratio to get a mSTSG. One of the wounds up to fat served as positive control and the other excised till fascia served as a negative control. Each wound was then covered with sterile antimicrobial Telfa wrapped gauze and overlaid with plain gauze. Additional dressing using tape, Ace bandages, stockinette and/or cloth jacket were applied. Punch biopsies (8 mm) were taken on p. and overlaid with plain gauze. Additional dressing using tape, Ace bandages, stockinette and/or cloth jacket were applied. Punch biopsies (8 mm) were be taken on post treatment days with a strip through the middle also collected on the day of euthanasia (day 90). On day 90 a strip through the middle will be collected on the day of euthanasia (**Figure 1C**).

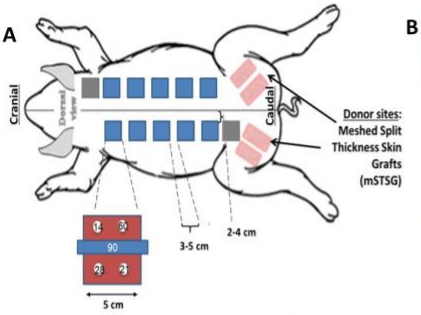
e. **End point measurements:**

Histology: The wound samples were fixed in 10% neutral buffered formalin, blocked in paraffin wax and 5µm section were cut and stained using Masson's Trichrome stain (MTS). Light microscopic images were taken using a Leica microscope (DMI 3000, Buffalo Grove).

Measurement of healing rate: Wound contraction were measured using pictures taken at different day pre and post treatment by Silhouette star (Aranz Medical), a non-contact device. Effect of

Lipogems formulations on wound healing were calculated with measurements of the wound size compared to their original size. The unwounded growth control areas were used to normalize the wound size.

A



B

Group number	Treatment
1	Positive Control (Normal debridement to fat)
2	Negative Control (Debridement to fascia)
3	PEGylated PFP hydrogel
4	Lipogems
5	Lipogems Embedded in PEGylated PFP hydrogels

C

Day -4	Day 0	Days 3, 7, 10, 14	Days 21, 28, 60	Day 90
1) Blood draw 2) Tattoo 3) Burn 4) NIM	1) NIM 2) Setup sterile field 3) Debride wounds 4) Harvest skin grafts 5) Apply Treatments 6) Apply primary and secondary dressings	1) Remove bandaging 2) Clean wounds 3) NIM 4) Biopsy (D14 only) 5) Reapply Lipogems and Hydrogels 6) Rebandage	1) Remove bandaging 2) Clean wounds 3) NIM 4) Biopsy 5) Rebandage	1) Remove bandaging 2) Clean wounds 3) NIM 4) Biopsy 5) Euthanasia

Figure 1: Schematic diagram of,) Experimental wounds on the dorsum, time-line for treatment and assessment protocol (B), and biopsy procedure of pigs are represented above (C).NIM: non-invasive imaging.

Measurement of blood perfusion: The blood flow microcirculation on the surface of the wound bed was measured using a commercially available Laser Speckle Imaging (LSI) system (moorFLPI-1, Moor Instruments). The blood flow perfusion of the entire wound was measured at high spatial and temporal resolution using a standardized setup. Briefly, laser speckle lens was aimed vertically and exactly perpendicular to the wound surface, the device focus and zoom dials were adjusted according to manufacturer's recommendations to achieve optimal image resolution in the field of view. After optimal adjustment, the wound along with tattoo was captured. High-resolution speckle images were acquired using a charge-coupled device camera (CCD). Perfusion data analysis expressed in laser speckle perfusion units (LSPU) was performed offline using the moorFLPI analysis software tool.

Melanin and Erythema Content: Pigmentation and vascularity of wounds treated with Lipogems±PEG-PFP groups and the normal skin site was measured over 60 days using the DermaLab Combo device (Cortex Technology, Denmark). To measure the pigmentation and vascularity, the color probe was place on the wound surface over the clear front and illuminated by the white LED lights. Spectrophotometry readings were recorded at 550±30 nm and 660 nm±60nm for hemoglobin and melanin, respectively. Pigmentation and erythema was determined using the melanin and hemoglobin values, respectively.

RESULTS:

a. **Photo documentation:** Digital images captured during days of revisit were documented to visualize the wound closure and the contraction over entire period of study (**Figure 2**).

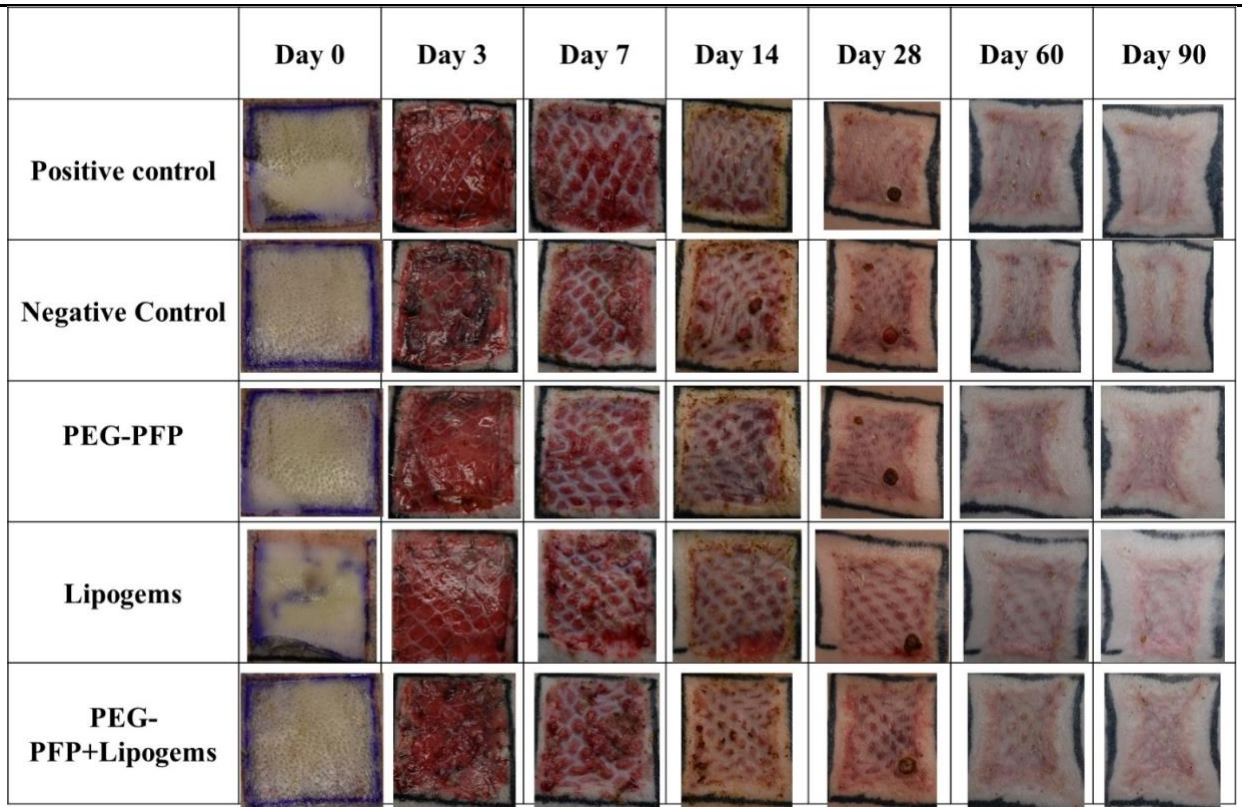


Figure 2: Representative photo images of burn wounds debrided and treated with Lipogems± PEG-PFP hydrogels. Positive control – Burn wounds debrided up to fat and negative control: wounds debrided up to fascia. All the wounds were grafted with 1:4 ratio meshed split thickness skin grafts.

Histology:

α-smooth muscle actin staining: In order to understand the remodeling of the wound after application of Lipogems, and Lipogems+hydrogels, biopsies taken on days (0,7, 14, 21, 28, 60 and 90) were sectioned and immune stained with α-smooth muscle actin (α-SMA). The α-SMA antibody targets actin filaments surround the mature blood vessels and the contractile myofibroblasts. When mSTSG were placed onto fat (positive control), myofibroblasts were observed in all wounds by day 7 and started to decrease by Day 28 (**Figure 3**). Then by day 60, α-SMA decreased, staining mainly the actin around blood vessels and sparsely the myofibroblasts, indicting resolving dermal wound bed slowly returning approaching normal dermal architecture. The mSTSG placed onto fascia (negative control), exhibited strong expression of α-SMA, with significant actin-stained myofibroblasts on day 21. The dermal myofibroblasts exhibited expression of actin fiber along with mature blood vessels on day 28. Day 60 biopsy sections still showed large number of myofibroblasts with less mature collagen (counterstain). These results shows aberrant dermal remodeling in the wounds grafted on fascia with no hypodermal layer. PEG-PFP hydrogel treated wounds less infiltrated with actin myofilaments by day 28 and were seem to have less positively stained for actin by day 60 in comparison to the negative control. The Lipogems treated wounds showed evidence of lesser amount of myofibroblasts by day 28 in comparison to negative control. However, their expression subsided drastically in comparison to the negative controls (Day 60 magnified α-SMA images). In addition, the Lipogems± PFP gels showed better remodel by day

60 (MTS Stained sections). Within the Lipogems groups, there did not show vast difference in actin staining. Collective, our observations shows that grafts on fascia tend to tether to the remodeling dermis with observable stress fibers (actin stained myofibroblasts) which may contribute to more contraction in comparison to positive control and the wounds reconstructed with Lipogems.

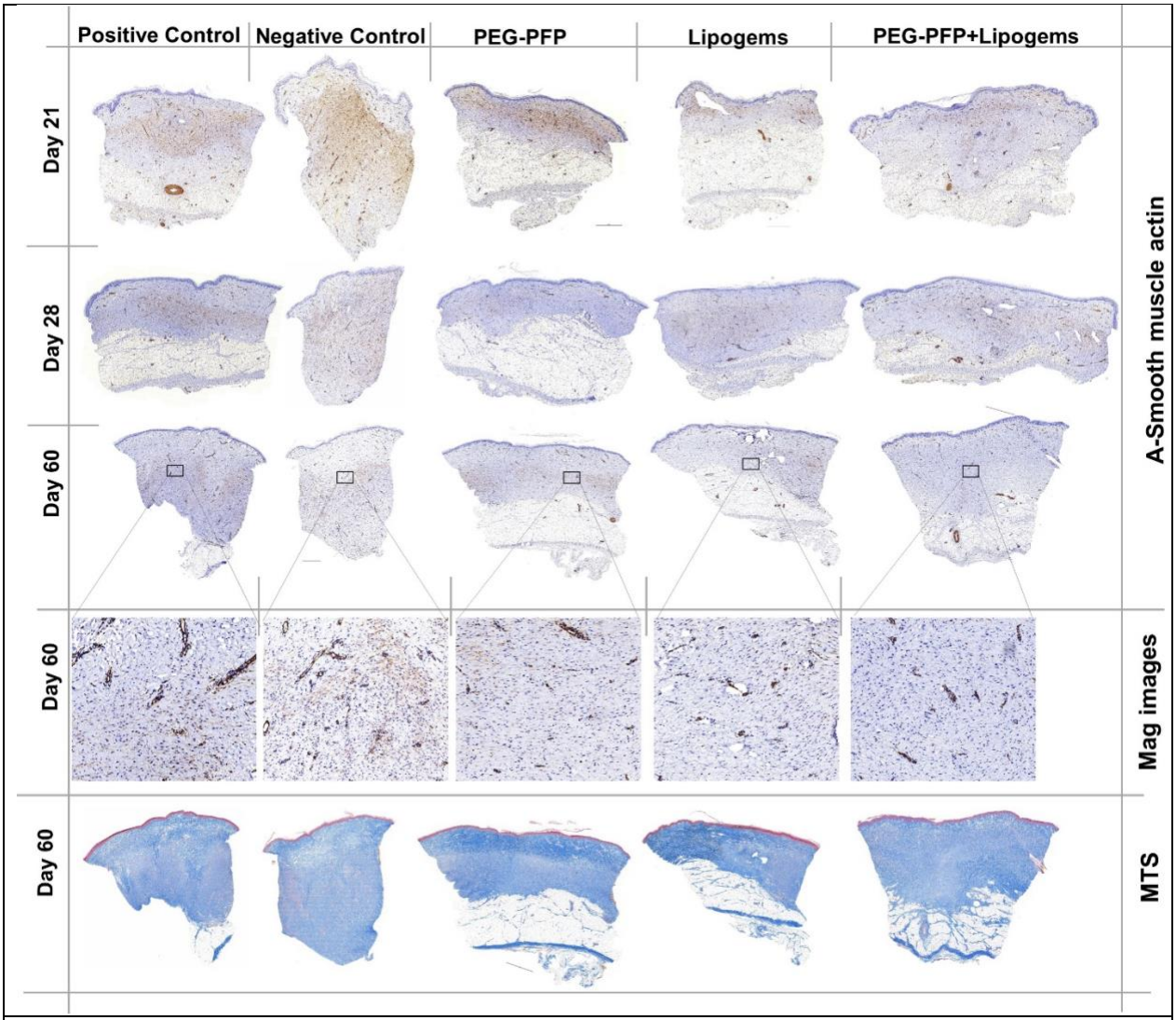


Figure 4: Masson's Trichrome Stained (MTS) sections of tissue biopsies collected from burn wounds treated with 2 ml Lipogems±PEG-PFP hydrogels. Mag: Magnified

Masson's trichrome staining (MTS): The MTS histological images on day 60 corroborates with results observed with α-SMA stained section. Day 60 MTS stained negative controls exhibiting the wound debrided till fascia and then treated with mSTSG were undergoing active remodeling with aberrant collagen bundles directly tethered to the fascial layer of the wound bed. This was further confirmed on day 90, where the negative controls exhibited drastically contracted wound bed in comparison to all other groups (**Figure 4**). The positive controls with mSTSGs grafted on an intact/viable hypodermis were less contracted inundated with resolving dermal layer with observable amount of remodeling nascent collagen. Through PFP hydrogel treated wounds contracted more in comparison to positive controls, they were observed to be less contracted than

the negative controls. Wounds treated with Lipogems, a thin layers of hypodermis was observable indicating reconstruction of wounds with hypodermal layer to have induced hypodermal regeneration. In groups treated with Lipogems and PFP hydrogels a pronounced increase in hypodermal line thickness can be observed (**Figure 4**). We speculate PFP, a vasculogenic adjunct, may have helped maintain viability of wound bed, which in turn may have resulted in better hypodermal regeneration. Collective these results indicates, early fat reconstruction to have positive effect on long term hypodermal regeneration with less contraction. More definitive data to show statistical values of contraction, and perfusion are currently under progress.

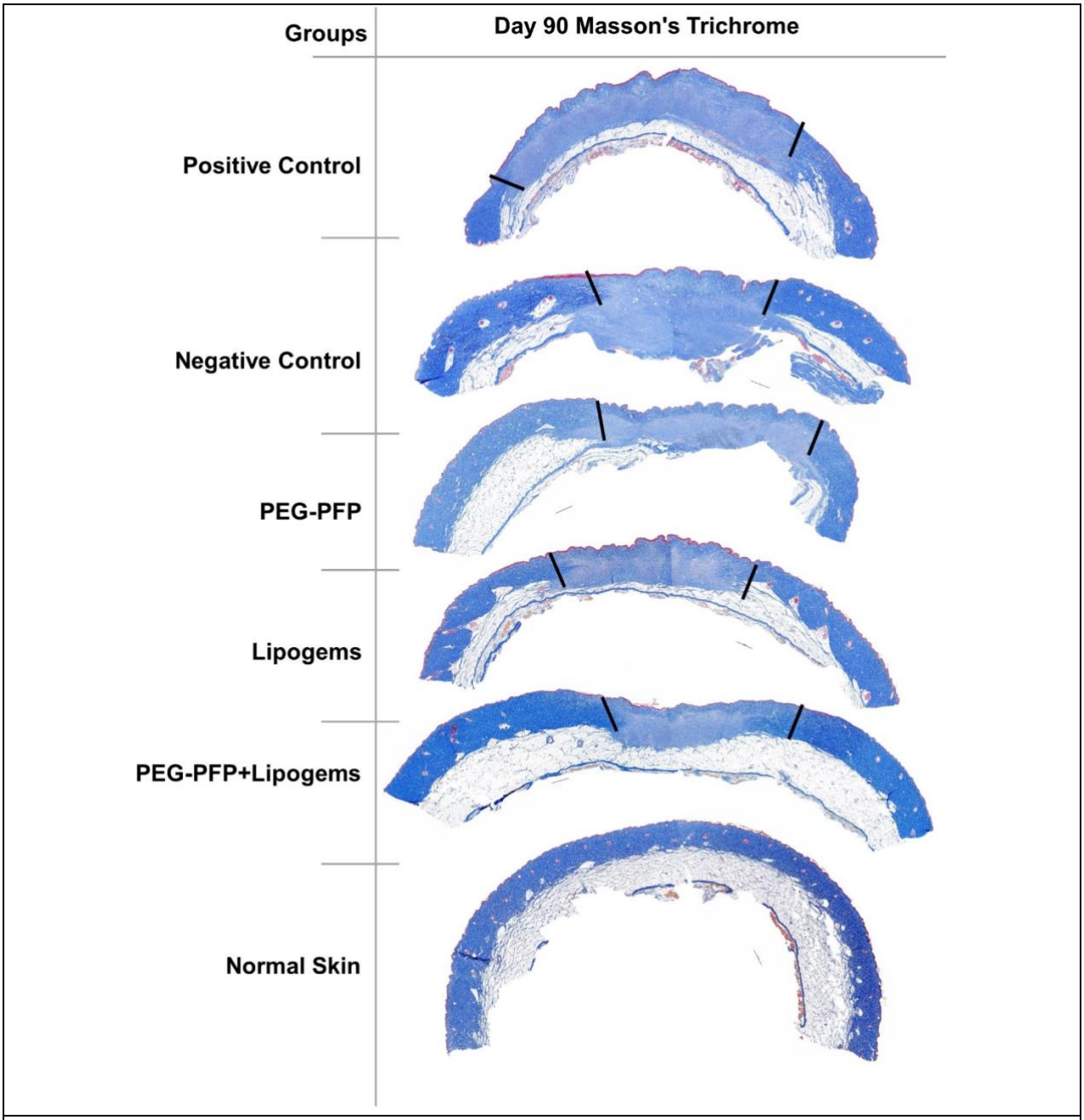


Figure 4: Masson's Trichrome Stained Day 90 tissue strips. Dark lines indicates area of wound bed with remodeled dermal and hypodermal layers.

Key outcomes (Year 2):

- Prepared allogenic porcine Lipogems and used for the current burn wound healing studies.
- The Lipogems±PEG-PFG hydrogel can be successfully delivered to the burn wound without graft loss. The Lipogems formulation exhibited better remodeling with active regeneration of subcutaneous hypodermis..
- The Lipogems+PEG-PFP treatment showed positive effect on long-term healing outcome with resolving dermal layer with less myofibroblasts.

4. DEVELOPMENTS

One of our research goals is to reduce the number of revision surgeries that a patient would undergo after a severe burn. The current standard of care is to utilize the patient's own skin, which imparts a donor site injury. Therefore, it is equally important to achieve desirable outcome with stringent use of autograft. Aligning to clinical practice, in cases where there is limited availability of donor skin and reconstruction materials, we have successfully reconstructed skin with mSTSG meshed at higher mesh ratio (4:1). In addition allogeneic Lipogems did not exhibit any adverse events. In addition Lipogems along with plasma hydrogel as a tissue sparing adjunct were proven to be effective in successful grafting protocol with better remodeling and long term scar outcome.

5. GOALS NOT MET

Year 3 data analysis is still under progress due to delayed experimental start. We were approved of a no cost extension to accomplish the pending work.

We further anticipated to submit manuscripts (total 2) by end of the final reporting period: End of next quarter (Y4Q1).

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

1. We were able to train two technical support staffs, Ms. Michelle Holik and Ms. Lucy Shaffer in performing porcine burn wound protocol, skin harvest and grafting. Various noninvasive imaging techniques and data analysis.
2. PI of this project (Dr. Natesan) and staff scientist (Dr. Stone) were able to self-train and develop formulations combining Lipogems and biomaterials in-house, perform pre-clinical studies.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Presentations at international and nation conferences (listed below)

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

A no cost extension is approved. We anticipate to complete the pending year 3 data analysis and submit manuscripts for peer-review.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

The observation made from this study that stem cells can be non-enzymatically sourced from adipose tissue and used in 3-D hydrogel scaffold to derive similar function of an isolated ASC population, has provided a new, and less-manipulative method to use stem cells for further wound healing application. The results may be more favorable, because of fewer hurdles for future FDA approval.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

- Isolation and storage of adipose derived stem cells involved several different steps, time and labor. Lipogems can impact the process of stem cell storage and use, since the clusters can be easily isolated by a service provider (e.g., research nurse), stored and retrieved and still possess similar potentials of enzymatically isolated stem cells.
- The entire process of Lipogems isolation can be carried out in a clinical suite, therefore requiring sophisticated laboratory space and equipment to isolate stem cells.
- Use of Lipogems may improve functional recovery and cosmesis of burn wound patients.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

LIPOGEMS, USA, has an established CRADA with USAISR, wherein they provide Lipogems isolation kits. Upon mutual agreement and interest, Lipogems-hydrogel technology can be transferred in future to the company for further product development and approval.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report in the year

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to Report in the year

Actual or anticipated problems or delays and actions or plans to resolve them

a. There was a delay in start of the specific aim 3 studies due to delayed ACURO approval b. There was delay in procurement of pigs (number 3 and 4) due to unforeseen delivery delays, which eventually pushed back completion of all 6 porcine experiments proposed.

Changes that had a significant impact on expenditures

There are no significant changes in expenditure, however a no cost extension was requested; which is now approved. We anticipate to complete the pending year 3 data analysis and submit manuscripts for peer-review in this quarter.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

[Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a*

periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

1. Natesan S, Stone R, Coronado RE, Wrice NL, Kowalczewski AC, Zamora DO, Christy RJ. PEGylated Platelet-Free Blood Plasma-Based Hydrogels for Full-Thickness Wound Regeneration. Adv Wound Care (New Rochelle). 2019 Jul 1;8(7):323-340.
2. Shanmugasundaram Natesan, Randolph Stone II, Rodney K Chan, Robert J Christy; Editor: Xiao-Dong Chen Chapter 8 - Mesenchymal Stem Cell–Based Therapies for Repair and Regeneration of Skin Wounds A Roadmap to Non-Hematopoietic Stem Cell-based Therapeutics, From the Bench to the Clinic; 2019, Pages 173-222, *Academic Press*.
3. Stone R 2nd, Wall JT, Natesan S, Christy RJ. PEG-Plasma hydrogels increase epithelialization using a human *ex vivo* skin model. Int J Mol Sci. 2018 Oct 13;19(10). pii: E3156

Other publications, conference papers, and presentations.

1. Randolph Stone II, David Larson, John Wall, Nicole L Wrice, Kyle Florell, Robert Christy, Shanmugasundaram Natesan. Treatment of Full Thickness Wounds with Microfragmented Adipose Tissue and Plasma-Based Hydrogels Improves Healing Outcome; International Society for Burn Injuries, New Delhi, 03 DEC 2018, Poster; acknowledgement of federal support under this award: Yes
2. David Larson, Randolph Stone II, John Wall, Robert Christy, Shanmugasundaram Natesan. Treatment of Full Thickness Wounds with Microfragmented Adipose Tissue and Plasma-Based Hydrogels, Military Health Symposium, August 2019; Poser; acknowledgement of federal support under this award: Yes

- **Website(s) or other Internet site(s)**

None during the reporting period

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

1. Lipogems-hydrogel technology has potentials to be transferred in future. Pre-clinical data in following year will enable us to generate results to evaluate the efficacy of the formulation.
2. The current indications for Lipogems use is in reconstructive surgeries. Dialogues with clinicians may be initiated to use this technology to isolated adipose tissue fragments for burn wound healing application.

- **Inventions, patent applications, and/or licenses**

None during the reporting period

- **Other Products**

Biospecimen: Human Lipogems were isolated and cryopreserved. This can be used for future in vitro experiment purposes.

Model: A porcine burn wound healing model to reconstruct subcutaneous hypodermis is established.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: ***Shanmugasundaram Natesan****, PhD*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-4213-3111
Nearest person month worked: *5*
Contribution to Project: *Dr. Natesan was responsible for overall experimental design, involved in optimizing the process of isolating Lipogems from porcine adipose tissue and oversee progress of this proposal. He conducted animal studies and data analysis. Also, Dr. Natesan was responsible for the submission of animal and IRB protocols for ACRUO and HRPO approval*

Name: ***Randolph Stone II****, PhD*
Project Role: *Research Physiologist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr. Stone was key in optimizing the size range of Lipogems clusters from porcine adipose tissue. He conducted in vitro studies using Lipogems-PEGylated PFP hydrogel formulations. Dr. Stone conducted animal surgeries and data analysis*

Name: ***Mr. David Larson****, MS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *8*
Contribution to Project: *Mr. Larson isolated helped in Lipogems isolation, in vitro culture, and performed animal surgieries.*

Name: ***Mr. Sergio Garcia****, BS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *8*

Contribution to Project: *Mr. Garcia performed animal studies, maintenance of medical records and every day animal care.*

Name: ***Ms. Michelle Holik****, BS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Ms. Holik was a support staff in the animal studies, she was capturing noninvasive images during the experimental revisits.*

Name: ***Ms. Lucy Shaffer****, BS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Ms. Shaffer was responsible for biopsy specimen section and staining. She also performed histological slide scanning and data analysis.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

LIPOGEMS, USA, in-kind provided Lipogems isolation kits

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

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7. Tremolada C, Colombo V, Ventura C. Adipose Tissue and Mesenchymal Stem Cells: State of the Art and Lipogems® Technology Development. Curr Stem Cell Rep.2016;2:304-312.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

APPENDIX 1: Publications abstract

Book Chapter

1.

A Roadmap to Nonhematopoietic Stem Cell-Based Therapeutics. https://doi.org/10.1016/B978-0-12-811920-4.00008-2

Edited by
Xiao-Dong Chen

Chapter 8

Mesenchymal Stem Cell—Based Therapies for Repair and Regeneration of Skin Wounds

Shanmugasundaram Natesan[1], Randolph Stone II[1], Rodney K. Chan[2,3], Robert J. Christy[1]

[1]*Combat Trauma and Burn Injury Research, US Army Institute of Surgical Research, Joint Base San Antonio, Ft. Sam Houston, TX, United States;* [2]*Q-SCARR™ (Quality Skin Collaborative for Advanced Reconstruction and Regeneration) Research Program, US Army Institute of Surgical Research, Joint Base San Antonio, TX, United States;* [3]*Dental and Craniofacial Trauma Research and Tissue Regeneration, US Army Institute of Surgical Research, Joint Base San Antonio, Ft. Sam Houston, TX, United States*

ADVANCES IN WOUND CARE, VOLUME 8, NUMBER 7

2.

TECHNOLOGY ADVANCES

PEGylated Platelet-Free Blood Plasma-Based Hydrogels for Full-Thickness Wound Regeneration

Shanmugasundaram Natesan,[1] Randolph Stone II,[1] Ramon E. Coronado,[2] Nicole L. Wrice,[3] Andrew C. Kowalczewski,[1] David O. Zamora,[3] and Robert J. Christy[1,*]

[1]*Combat Trauma and Burn Injury Research and* [3]*Ocular Trauma & Vision Restoration, U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas.*
[2]*Lester Smith Medical Research Institute, San Antonio, Texas.*

Objective: To develop a cost-effective and clinically usable therapy to treat full-thickness skin injuries. We accomplished this by preparing a viscoelastic hydrogel using polyethylene glycol (PEG)-modified platelet-free plasma (PEGylated PFP) combined with human adipose-derived stem cells (ASCs).
Approach: PEGylated PFP hydrogels were prepared by polymerizing the liquid mixture of PEG and PFP±ASCs and gelled either by adding calcium chloride ($CaCl_2$) or thrombin. Rheological and *in vitro* studies were performed to assess viscoelasticity and the ability of hydrogels to direct ASCs toward a vasculogenic phenotype, respectively. Finally, a pilot study evaluated the efficacy of hydrogels±ASCs using an athymic rat full-thickness skin wound model.
Results: Hydrogels prepared within the range of 11 to 27 mM for $CaCl_2$ or 5 to 12.5 U/mL for thrombin exhibited a storage modulus of ~62 to 87 Pa and ~47 to 92 Pa, respectively. The PEGylated PFP hydrogels directed ASCs to form network-like structures resembling vasculature, with a fourfold increase in perivascular specific genes that were confirmed by immunofluorescent staining. Hydrogels combined with ASCs exhibited an increase in blood vessel density when applied to excisional rat wounds compared with those treated with hydrogels (110.3 vs. 95.6 BV/mm^2; $p < 0.05$). Furthermore, ASCs were identified in the perivascular region associated with newly forming blood vessels.
Innovation: This study demonstrates that PFP modified with PEG along with ASCs can be used to prepare cost-effective stable hydrogels, at the bed-side, to treat extensive skin wounds.
Conclusion: These results indicate that PEGylated plasma-based hydrogels combined with ASCs may be a potential regenerative therapy for full-thickness skin wounds.

Keywords: platelet-free plasma, PEGylated hydrogels, adipose-derived stem cells, vascularization, skin regeneration

3.

Int. J. Mol. Sci. **2018**, *19*, 3156; doi:10.3390/ijms19103156

Article

PEG-Plasma Hydrogels Increase Epithelialization Using a Human Ex Vivo Skin Model

Randolph Stone II, John T. Wall, Shanmugasundaram Natesan and Robert J. Christy *

Combat Trauma and Burn Injury Research, US Army Institute of Surgical Research, JBSA Fort Sam Houston, San Antonio, TX 78234-6315, USA; randolph.stone4.ctr@mail.mil (R.S.II); john.t.wall40.ctr@mail.mil (J.T.W.); shanmugasundaram.natesan.ctr@mail.mil (S.N.)

* Correspondence: Robert.j.christy12.civ@mail.mil; Tel.: +1-210-539-9528

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Abstract: In vitro cell culture methods are used extensively to study cellular migration, proliferation, and differentiation, which play major roles in wound healing but the results often do not translate to the in vivo environment. One alternative would be to establish an ex vivo model utilizing human discarded skin to evaluate therapies in a more natural setting. The purpose of this study was to institute such a model by creating 'wounds' in the center of a piece of discarded skin and treating them with three different biomaterials: collagen, polyethylene glycol (PEG)-fibrin, or PEG-platelet free plasma (PFP). Explants were cultured for 14 days with supernatant and microscopy images collected every 3 days to assess cytotoxicity and epithelialization. After 14 days, the explants were fixed, sectioned, and stained for cytokeratin-10 (CK-10), alpha-smooth muscle actin (α-SMA), and wheat germ (WG). Compared to controls, similar levels of cytotoxicity were detected for 12 days which decreased slightly at day 14. The PEG-PFP hydrogel-treated wounds epithelialized faster than other treatments at days 6 to 14. A 6-8 cell layer thick CK-10+ stratified epidermis had developed over the PEG-PFP hydrogel and cells co-stained by WG and α-SMA were observed within the hydrogel. An ex vivo model was established that can be used practically to screen different therapies exploring wound healing.

Keywords: ex vivo; epithelialization; keratinocyte; discarded skin; wound closure; biomaterials