Creation of a Novel Human Wound Model to Test Novel Wound Healing Approaches

Nathan Northern
Wright State University - Main Campus, nothern.14@wright.edu

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Abstract
In the US there is a growing prevalence of chronic wounds such as leg ulcers, diabetic foot ulcers, and pressure ulcers. These wounds persist for long periods of time and are expensive to manage. Improved human-based model systems that emulate the wound healing process in humans would accelerate the identification of novel healing strategies that are directly translatable to humans. The goal of this study was to develop a novel wound model able to imitate the human wound healing process. To do this, 8mm punch biopsies were taken from human abdominoplasty samples and 2mm wounds were created in the center of the punches. Punch biopsies were maintained in transwells at the air-liquid interface. The viability of the model was confirmed by MTT staining and the structure evaluated with H&E staining. Two approaches to induce wound healing, Adipose Derived Stem Cells (ASC) and Red Light Therapy (RLT), were investigated. It was found that the human tissue wound models were able to persist for at least 4 months without "wound" closure. H&E staining demonstrated that they maintained normal skin structure and MTT indicated that the tissues were alive. ASCs were isolated from fat and their purity and ability to differentiate were confirmed by flow cytometry and differentiation assays. Whereas ASC or RLT were able to partially close the wounds, a combination of ASC and RLT resulted in wound expansion. However, this latter result may have been due to bacterial contamination. These wound models could revolutionize wound healing studies because they are derived from human skin and maintain their integrity. In the future, we will confirm the degree to which they replicate chronic wounds and determine the efficacy of novel stem cell, RLT, human skin equivalent, and other growth-factor based treatments to mediate wound closure.

Introduction: Chronic wounds are an epidemic in the United States with approximately 6.5 million Americans developing them every year. They are differentiated from a normal wound by longevity. Chronic wounds are characterized by the persistence of an open sore for more than 3 months. Often these wounds fall into one of three clinical categories: leg ulcers, diabetic foot ulcers, or pressure ulcers (12). Circulatory problems, such as arterial and venous damage or constant pressure blocking normal blood flow, can cause ischemia and lead to tissue death. The normal wound healing process generally consists of cell proliferation and migration, angiogenesis, re-epithelialization, ECM deposition, and remodeling (7). Angiogenesis is the formation of blood vessels to a wound site and is a key part to the healing process. If blood flow is prevented from reaching the wound site, the normal wound healing process cannot occur and the wound will persist. Chronic wounds can be debilitating for the affected individuals and result in the need for frequent medical care. The market for wound care products is rapidly expanding, with $25 billion spent annually (15). This makes finding innovative ways to study and treat these wounds of tantamount importance and worth researching.

Models to study chronic wounds are developing, but they are still far from emulating the wound healing process of a human. Common models include scratch assays, 3-dimensional skin models, and animal models. A scratch assay is a wound, or "scratch", that is made on a monolayer of cells. The premise of the model is that over time this wound will close and that rate of cell migration can be observed. Practically this could lead to a better understanding of cell migration in vivo. For example, when part of the endothelium in blood vessels is removed, endothelial cells in the blood vessels will begin to migrate to the wounded area in order to close the wound. These models can also provide insight into the effects of the Extracellular Matrix (ECM) on cell migration and cell-cell interactions (11). The assay is a low-cost and well developed method to do basic research on wound closure. However, there are some major
limitations to the model. A relatively large number of cells and chemicals are needed to properly perform the assay. Also, the test takes 8-18 hours for complete scratch closure, which is relatively longer than other methods. However, the largest issues are that real physiological conditions are not able to be replicated on 2-D surfaces and that the complex microenvironment of cells is not able to be replicated in these experiments (5). The epidermis and dermis in human skin are multilayered and there is a physiological process that is enacting to promote wound healing including the onset of inflammation and release of immune factors. The scratch assays are not able to replicate the structure nor the process of healing human skin.

Even though there are severe physiological limitations when using 2D models, they have been useful in cell biology research. However, in order to remedy some of the deficiencies of 2-dimensional models, some researchers began developing 3 dimensional models. 3D models give a more physiologically accurate representation of the architecture of human skin and wounds. Several methods can be utilized to create 3D models including forced-floating, hanging drop, agitation-based approaches, matrices, and scaffolds. The first three methods mentioned function by preventing attachment of cells to the vessel surface. This causes the cells to congregate in the center of the vessel and many times they begin to adhere to each other and form a ball that is hanging in suspension. These methods do not have the potential to be used for wound closure research, but they are essential for developing models that have 3D architecture and finding the manner in which cells are able to communicate to form these structures. Matrices and scaffolds are models that are more applicable to wound closure research. They both have a structure that is based on a basement membrane with integrated cells that are allowed to grow. This allows the interaction of ECM between cells to develop, the addition of growth factors that are found in human skin, and the growth of an endothelial syncytium. However, according to an article by Breslin and O'Driscoll, mouse cancer cells were used to make the matrix model and scaffolds were prefabricated. Both ways of growth take away the human aspect of the model and therefore are not ideal for wound healing studies (2).

Another 3D model is even closer to replicating skin. In this model a thin acellular layer of collagen is constructed and subsequently a collagen gel embedded with dermal fibroblasts is layered over top. Over a 7 day period in media, the dermal fibroblasts remodel the collagen matrix forming a plateau. Keratinocytes are then added to the center of the plateau and allowed to attach in order to form a basal layer of tissue. Tissues are raised to an air-liquid interface to initiate a stratification. Keratinocytes stratify and differentiate to form suprabasal layers that mimic human skin. Over time greater development in the spinous and cornified layers of the tissue occurs. These models can be used for research on skin cancer or wound healing. Both incisions with a scalpel and punch biopsies were performed on these wounds. The main issues with the model include the prolonged period of time it takes to grow the model and the fact that it is not a direct replication of human epithelial tissue (3).

The last types of models that are prevalent for wound care studies are animal models. Common wound models include: rabbit ear ischemia, pig flap ischemia, rat magnet ischemia-reperfusion, diabetic mouse, and pig wound infection. Pig flap ischemia and diabetic mouse wounds are the most commonly used for research purposes. In pig flap ischemia regions on the pig’s back are made ischemic via surgical incision. Wounds created in these regions don’t have adequate blood flow and can become chronic. Diabetic mice models include the creation of wounds on the back of the mice. These wounds have the same issue of little blood flow due to the diabetic condition of the mouse and the wounds tend to become chronic (12). There are however some inherent problems with the animal wound models. These models are formed on animals, which do not directly model the structure and healing process of human skin. For example, wound contraction accounts for 90% of wound healing in rats, 50% in pigs, and 25-50% in humans. Pigs and humans heal more readily through the re-epithelialization process. Also, compared to
humans, pigs have less vasculature in their skin and do not have eccrine sweat glands. In contrast, humans have far more vasculature and also have apocrine and eccrine sweat glands. However, there are some major benefits of the models. All animals have live physiological responses to any treatments for chronic wounds and show a wound healing progression in a dynamic manner. This cannot be seen in any of the other models. They are able to get infections, inflammation, scabs, and have active blood flow. All of which modulate some human responses to wounds (13).

To remedy this lack of a good human model system, Dr. Michael Johnson, Department of Surgery, WSU, and Dr. Katherine Excoffon’s lab has developed a novel wound model to simulate an *in vivo* chronic wound. The organotypic wound model is created from 6-8 mm punch biopsies of abdominoplasty skin-fat specimens with a central 2-3 mm punch biopsy that behaves as a “wound”. These organotypic models contain both the epidermal and dermal layers of the skin and retain morphology when cultured in a Millicell that allows tissue polarization (Figure 1). This model will allow the careful examination of the factors that affect wound healing and the efficacy of potential therapeutics.

![Diagram](image)

**Fig. 1:** A) Punch biopsy instruments are available in a wide range of sizes. B) Schematic of biopsy process. C) 6 mm skin biopsy with a 2 mm central internal wound was created from an abdominoplasty specimen in the Johnson lab. D) Schematic of organotypic chronic wound culture system maintained in a hanging millicell (black outline). E, epidermal keratinocytes; B, basement membrane; D, dermis.

The major benefits of this model are evident. The biopsies come directly from human tissue, therefore the architecture of the skin and the wound healing process are precisely human. Along with these benefits we are also able to take into account the normal environment of the skin and the impact it can have on wounds. Skin has a normal flora of bacteria. However, it is unclear if different types of bacteria are present on the wound models versus on humans. A study performed by Cosseau and colleagues found that there are generally more types of gram-positive bacteria on the skin than gram-negative bacteria (4).

With the development of this new model it is essential to find ways to test the model. Three general techniques are currently being investigated for wound healing including: Biological Skin Equivalents (BSE), growth-factor based therapies, and stem cell techniques. Our lab in particular has investigated growth-factor based therapies in conjunction with stem cell techniques. In recent years, stem cells have emerged as a therapeutic alternative for repair and healing of chronic wounds. Adult stem cells are a viable option for wound treatment because there is an abundant supply available in most Americans, auto-transplant eliminates immune rejection, and, in contrast to embryonic stem cells, there are no ethical issues. Bone marrow-derived stem cells (BMSC) and adipose-derived stem cells (ASC) are adult stem cells that are investigated in translational research (8). In contrast to BMSC which are generally committed to blood cell types, ASC can differentiate into several lineages useful for wound healing. The addition of ASC to wounds has been shown to increase wound closure and enhance wound healing (9).
However, stem cells alone are unlikely to be sufficient to solve the chronic wound dilemma since chronic wounds frequently have factors that inhibit ASC differentiation. The Excoffon lab is taking the approach of bioengineering ASC with an adeno-associated virus (AAV) that is designed to transiently express the vascular endothelial growth hormone (VEGF) which should induce angiogenesis in chronic wounds. Also, in order to hopefully induce even more closure of wounds, we have utilized Red Light Therapy. This method uses red LED lights to induce bio-stimulatory effects, which can increase survivability and viability of ASCs and increase level of growth factor secretion by ASCs with exposure to red light. Our wound models are ideal to test the efficacy of these methods.

The purpose of this study in particular was threefold. First, it functioned as a literature review of current wound models and techniques being utilized with these models. Also, the study has led to the development of novel wound model system which tested the efficacy of several treatments on wound healing.

Materials and Methods:

Literature Search
A literature search was conducted to identify current wound models, applications of wound models, and potential therapies. This literature search was conducted through both the Wright State University and University of Cincinnati Library collections.

Creation of Wound Models
The wounds were created following the IRB protocol established by collaborators at Miami Valley Hospital. Pieces of skin and fat from abdominoplasty procedures were cut using a scalpel to remove the thick subcutaneous layer under the dermis. This created a piece of skin containing just the epidermis and a portion of the dermis. A full thickness 8mm punch biopsy was then taken with a punch biopsy tool and the skin piece was removed. In the center of the 8mm punch a full thickness 2mm punch was taken, creating the wound. The punch was added to a Millicell® in a 24 well-plate, which was hanging from the sides and did not touch the bottom. This allowed the epidermis to be exposed to the air and the bottom part of the model to be exposed to media. ASC complete media (DMEM-F12, 10% FCS and 1% ABAM) was used as nutrition. Media in the wells was changed every 48 hours.

Characterization of Bacteria
Directly after the creation of the punch biopsies, the epidermis of the punch was streaked on a sheep blood agar plate. The process was repeated for the subcutaneous dermal end of the wound. The samples were placed in an incubator at 37°C and left for 24 hours. Following this incubation period, isolated colonies were collected and streaked on separate plates and then placed into the 37°C incubator for 24 hours. The bacteria formed isolated colonies on these plates and samples of bacteria were able to be collected and analyzed by a specialized mass spectrometry instrument at Compunet Clinical Laboratories. The instrument has the ability to identify a specific bacteria from a clinical database with many types of bacteria. This process was also utilized to identify contamination of ASCs in culture.

Isolation and Culture of ASC
ASCs were isolated utilizing the IRB protocol of our collaborators at Miami Valley Hospital. Initially, the subcutaneous tissue was separated from the dermal layer using scalpels and surgical scissors. 50 CCs of the adipose tissue was then minced and added to a collecting cup. The fat was then washed 4 times with PBS/- until the supernatant was clear. Following the washes, collagenase type 1 was added and the
sample was placed in a 37°C water bath for 15 minutes. This broke the tissue down into individual adipose cells, which were then centrifuged and resuspended in PBS for a washing step. Following another centrifugation, the cells were finally resuspended in Stromal Medium and plated in a T75 flask. One day after plating the cells they were washed with PBS and fresh ASC media was added. Every alternate day fresh media was added and every two weeks they were passaged.

**Characterization of ASC**
The ASCs were characterized using the standards set by the Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). An immunofluorescence multicolor flow cytometry strategy was utilized which tested for the positive stem cell markers APC anti-human CD31, CD34, CD36, CD73, CD90, and CD105 and the negative stem cell markers PE anti-human CD45, and APC anti-human CD106. The stem cells were then seeded into 6 well plates at a concentration of $10^4$ cells per well. Several days later the cells were detached using TrypLE, centrifuged, and re-suspended in PBS and 10% FCS. Stem cells were aliquoted in equivalent amounts to each of several tubes following a cell count. Appropriate stem cell marker antibodies were then added to the tubes and allowed to incubate for 25-30 minutes. The cells were then washed with PBS and centrifuged. This process was repeated three times. Following the washes, the cells were characterized via flow cytometry using the FL1, FL2, and FL4 channels.

**Imaging of wound models**
Every alternate day ImageJ and Picture Frame software were used to assess wound healing in the 21 wound models treated with either non-transduced ASC or ASC media (n=10). Wound closure rate was determined by comparing the current size of the wound to the initial size of the wound. The results were analyzed and graphed using Microsoft Excel in order to assess the rate of wound closure in each experimental group and to make comparisons between the groups.

**Red Light Exposure on Wounds**
During a 30 day period, on every alternate day the wound models were exposed to either red light or ambient light for 15 minutes. Two conditions were tested including wounds treated with non-transduced ASC in both concentrated red light and in ambient light. Normal ambient light and a 670nm red light were used as the two treatments for the cells at room temperature. Measurements were taken on the wounds throughout the exposure experiment to determine the effects of red light therapy on wound closure.

**ASC Differentiation**
ASCs were seeded at a concentration of $1 \times 10^4$ cells and were allowed to expand in growth media for several days. The monolayer of cells was rinsed with DPBS and subsequently TrypLE was added to detach the cells. A single cell solution was created and was then centrifuged to pellet the cells. The pellet was re-suspended in an MSC Growth Medium (60% Differentiation Medium, 7% MSC Supplement, 33% Gentamicin). A cell count was performed at this stage. Cells were seeded in a T75 flask at a concentration of $10^4$. For two days the cells were incubated in a 37°C incubator with 4% CO$_2$. After this time period, the media was changed as the cells continued to differentiate. Once the cells were fully differentiated, they able to be stained according to cell types. This process was performed to differentiate ASCs into adipocytes, chondrocytes, and osteocytes. Differentiated adipocytes were stained with Oil Red O. To do this, the cells were rinsed with 1x PBS, which was subsequently removed. From there, 10mL of 10% formalin was added and allowed to incubate at room temperature for 30 minutes. The formalin was discarded, another rinse with 1x PBS was performed, and 10mL of Oil Red O was added and incubated for 1 hour. The stain was aspirated out, the cells were washed 3 times with 1x
PBS, and enough dH2O was added to cover the dish. Cells were viewed under the microscope directly after staining. Chondrocytes were stained with Safranin and osteocytes were stained with Alizarin Red utilizing the same procedure as Oil Red O staining. Safranin was prepared by adding 0.1 grams of safranin to 100mL of dH2O. Alizarin Red was created by adding 2 grams of Alizarin Red S powder into 100mL of dH2O and the pH was adjusted to 4.2.

Cryo-OCT Sectioning
Punch biopsies created via the procedure in “Creation of Wound Models” were placed in a plastic mold that contained tissue freezing medium. These samples were then frozen at -80°C. The frozen samples were then able to be sectioned with the cryostat at -27°C. Each section was sliced at 15 µm. The sections were transferred to microscope slides by touching the frozen sample to the slide. Slides were labeled according to the sample used and stored at 4°C.

H&E Staining
Sections created by Cryo-OCT sectioning were fixed in 4% alcoholic formalin for one minute. The sections were then sequentially washed in tap water and distilled water. Slides were then placed in a hematoxylin stain for 15 minutes and followed by a rinse with tap water. Next, the sections were placed in a bluing reagent for 30 seconds and again washed in tap water. Sections were then placed in an eosin stain for 5 minutes, followed by two washes in 95% alcohol. Finally, the sections were washed 3 times in 100% alcohol and washed twice in xylene. The stained sections were mounted and a coverslip was secured on the slide with Permount and nail polish. Slides were viewed under the microscope or stored at 4°C.

MTT Staining
Wound models, both experimental (supplemented with ASC media) and control (fixed in 4% paraformaldehyde for 2 hours), were tested for viability of the tissue via an MTT assay. The wound models were first washed with PBS, followed by the addition of 100µl of MTT in 1:10 dilution to the base of the transwell containing the wound model. The model in the MTT solution was incubated at 37°C for four hours in the dark. After four hours, the wound models were checked for the formation of a purple color and imaged under the microscope.

Results

Wound Models
The initial purpose of this study was to create a wound model that could effectively mimic the environment of a wound in live human epithelial tissue. A novel wound model in this study was created from full thickness skin acquired via an abdominoplasty procedure. The samples from this surgery left the skin intact allowing the architecture of the epithelial tissue to be preserved in a live state. Being placed in the Millicell well allowed the environment of the human tissue to be preserved, with the epidermis being exposed to air and the subcutaneous layers being exposed to a growth medium. Figure 2 depicts a wound model taken from human tissue and a diagram of the punch biopsy in a Millicell and well.
In the figure the epidermis is displayed in both images, while the dermal layer can be seen as the lighter purple in the diagram in image B. To study the complete structure of the wound, Hematoxylin and Eosin staining was employed on the cryo-OCT sections. As illustrated in Figure 3, the H&E staining was able to highlight the epidermal, dermal, and subcutaneous layers of the wound model. Even more specifically, the layers of the epidermis (Stratum corneum, Stratum spinosum, Stratum granulosum, and Stratum basale) were able to be identified. Dermal papillae, a unique structure of human skin, can also be observed in the figure.
The model system was particularly desirable because the wounds were able to be sustained for months at a time. In one case the wound models were able to be sustained for over four months in a 5% CO₂ incubator at 37°C with ASC media. This time period exceeds the typical three month standard used to determine if a wound is chronic. It was confirmed that the wound models were still viable after a four month period by utilizing an MTT assay. There was formation of a purple color, indicating the breakdown of MTT into formazan. Further, measurements of the wounds showed that the wound area size persisted for periods as long as three months. This indicated that the wounds were chronic and could not heal naturally. Also, it showed that additional therapies would be required for wound closure.

**Characterization of ASCs**

In order to study the potential of ASC for wound healing, stem cells first had to be isolated from adipose tissue. Utilizing a protocol established by Dr. Johnson and his team, ASC were able to be isolated from subcutaneous tissue of abdominoplasty samples. The stem cells were able to be isolated from samples that were both autologous to the wound models and also heterologous. Figure 4 illustrates the morphology of the ASCs several days after an isolation.

![Fig 4: ASC isolated from abdominoplasty adipose tissue.](image)

To confirm the stem-like qualities of the ASC flow cytometry was utilized in accordance with Gold Standard IFATS criteria. More than 95% of the stem cells demonstrated the presence of positive stem cell markers CD13, CD44, CD73, CD90, and CD105 and nearly 98% of the ASC did not express the negative stem cell markers CD31, CD45, CD106 (Figure 5). This confirmed the presence of stem-like qualities of the ASC.
Fig 5: Flow cytometry analysis of stem cell markers show that ASC retain their stemness. (a) Representative gating of the stem cell population, (b) unstained ASC (control), (c) CD106: a negative stem cell marker which was not expressed. More than 98% of the ASC express positive markers (d) CD44, (e) CD73 and (f) CD90. Data collected in collaboration with Upasana Niyogi, Greg Gould, Sunishka Wimalawansa, and Priyanka Sharma.

ASCs are supposed to have the potential to transform into multiple cell types. The efficacy of the ASC was confirmed via a differentiation assay. With the addition of specific differentiation media, the ASC were able to be differentiated into adipocytes, chondrocytes, and osteocytes. Figure 6 illustrates the histological structures of the derived cells. This assay confirmed the ability of the ASC to be multipotent cells.

Fig 6: Both the undifferentiated control group (top images) and the differentiated state (bottom images) of ASC into multiple cell types are shown. The contrast can be seen between the control ASC and the differentiated A) Adipocytes, B) Chondrocytes, and C) Osteocytes.
Effect of ASC and RLT on Wounds

It was hypothesized that wound healing could be promoted by the addition of ASC and/or RLT to a wound site. During the course of this study it was found that alone both the addition of ASC and RLT could potentiate wound healing. The addition of just ASC to a wound caused 17.78 ± 2.3% closure and exposure to RLT alone caused 59.15 ± 0.3% closure. It was expected that the combination of the two therapies would induce even greater wound healing and cause more complete wound closure.

However, during the first week of the experiment it was observed that the wound models became contaminated. Analysis of the wound models showed that the bacteria was *Ochrobactrum anthropi*. The experiment was continued and the bacterial infection was considered as a confounding variable. The combination of ASC and RLT in conjunction with a bacterial infection caused an expansion of the wound area. In ambient light the wound size increased by 3.29 ± 0.09% and with RLT the wound size increased by 6.23 ± 0.051%. As figure 7 demonstrates, both conditions had positive slopes, showing an increase in wound size. A t-test confirmed that there was a significant difference in the rate of wound expansion with a p-value = 0.008 (p<0.05). RLT had a faster rate of wound expansion during the course of the experiment.

![Graph A](image1.png)

No Red Light: Wound Area as a Function of Time

![Graph B](image2.png)

Red Light Therapy: Wound Area as a Function of Time

Fig 7: ASC and ASC combined with RLT failed to induce wound closure. A) Wound models exposed to solely ambient light were allowed to heal for a 33 day period. A positive trend was seen with a positive slope; indicating that the wound area increased during the course of the experiment. B) Wound Models were exposed to red light. A positive trend was observed with an increase in wound area size over the 33 day period. There was a less positive slope when wounds were exposed to red light, indicating less expansion of wound area than when exposed to ambient light.
Bacteria

One benefit of using a full thickness human skin wound model is that it can maintain the bacteria that are common on the skin. However, after the contamination of the wound models in the ASC and RLT experiment we decided to determine the bacteria that were present on the wound model. When the contaminated wound was streaked on a bacterial plate, a bacterial fauna quickly grew. A specialized mass spectroscopy instrument in a clinical microbiology lab identified only one bacteria: *Ochrobactrum anthropi*. Months later when more samples were processed and new wound models were made, the wounds were tested for bacteria again. The second sample tested was a healthy wound model that did not have the appearance of contamination. It yielded the same *Ochrobactrum anthropi* bacteria as well as *Staphylococcus epidermidis* and *Corynebacterium* species. A third sample, also from a live and healthy wound model, again had *Ochrobactrum anthropi* present.

During the process of learning to isolate ASC, there were also several ASC samples that became contaminated. When the contaminated media was streaked on a plate and the bacteria was grown, it was identified as *Ochrobactrum anthropi*. Table 1 provides a concise summary of the results of the bacteriological experiments. In every sample tested during this experiment *Ochrobactrum anthropi* was present.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Gram Negative Bacteria</th>
<th>Gram Positive Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis of contaminated</td>
<td><em>Ochrobactrum anthropi</em></td>
<td>-</td>
</tr>
<tr>
<td>wound model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermis of healthy</td>
<td><em>Ochrobactrum anthropi</em></td>
<td><em>Staphylococcus epidermidis, Corynebacterium</em></td>
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<tr>
<td>wound model</td>
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<tr>
<td>Epidermis of healthy</td>
<td><em>Ochrobactrum anthropi</em></td>
<td>-</td>
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<tr>
<td>wound model</td>
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<tr>
<td>Media of contaminated ASC</td>
<td><em>Ochrobactrum anthropi</em></td>
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Discussion

Wound Models

The novel wound models developed during this study were unique in that they are made directly from human epithelial tissue. Other models available have their merits, but they are not directly translatable to the human wound healing process. Having the punch biopsies that come from tissue that was surgically removed soon before the punches were made allowed them to maintain their identity as a viable human tissue. There were functioning enzymes in the tissue (as shown by a positive MTT test) and a live microbiota on the surface of the skin.

In order to stabilize the structure of the epithelial tissue, the punch biopsies were placed in millicells. These allowed them to maintain the integrity of both the skin and the wound. The millicells also were important in creating an air-liquid interface. An air-liquid interface allowed the wound model
to be in an environment where the epidermis was exposed to the air, while the dermal layer was exposed to a media that provided nutrients. Exposure to the air allowed gas exchange to occur at the epidermis and the liquid media allowed nutrient exchange at the basal surface. The process of caring for the wounds revealed that ASC media alone is sufficient to sustain live tissue.

It was confirmed by an MTT assay that the tissues in the wound models were still living. The addition of MTT to the models yielded a purple color on the live wounds. This can be deduced because when proper enzymes are present MTT can be broken down into formazan, which produces a distinctive purple color to develop. The only way that the enzymes could have been functional is if the tissue was in fact surviving. In the experiment where the wounds were maintained for four months, MTT assays showed that the tissue was alive for the entire time period.

The process of H&E staining was able to confirm the sustained histoarchitecture of the human skin. All of the epidermal layers were present including the Stratum corneum, Stratum spinosum, Stratum granulosum, and Stratum basale. The dermal layer was able to be identified as well. Also, the dermal papillae were visible in images of the tissue, which is not seen on tissues that were grown from cell monolayers or mice.

One major problem with the model is the lack of blood flow. Chronic wounds are often in ischemic regions and receive few nutrients from the blood, however there is generally some interaction. In the case of this model system, a novel flow system could be developed but it is unlikely that there is the potential for the wound model to interact with blood as observed in vivo.

**Characterization of ASC**

After the development of the novel wound model, we wanted an effective way to test its efficacy. ASCs have been known to be positively associated with wound healing. In order to pursue this method of wound healing, ASC first had to be isolated and characterized. ASC can be isolated from adipose tissue and they begin to take on stem cell properties.

Once the ASC have been in culture for a period of time, they can be characterized by the presence of certain surface markers. These markers can be utilized to distinguish between different cell types. The ASC we isolated from brachioplasty samples were confirmed to have true stem like qualities with data developed by flow cytometry. Positive surface markers were present on the ASC and negative stem cell markers were not found to be present.

The ASC were further characterized via a differentiation assay. ASC in particular tend to have properties that define their specific cell type. For example, they are multipotent cells that have the ability to differentiate into adipocytes, chondrocytes, and osteocytes (16). When differentiation media was utilized with the ASC, they were able to transform into those three cell types. This further confirmed that not only did the isolated ASC have true stem-like qualities according to flow cytometry, but they also demonstrated normal characteristics attributed to ASC.

Other qualities that are associated with ASC are the abilities to be easily transfected and secrete growth hormones. With the isolated ASC being confirmed to be functioning stem cells, we anticipated that they maintained these abilities as well. This led to experiments where ASC were added to the wounds present on the punch biopsies.

**Effect of ASC and RLT on Wounds**

ASC in particular show promise for being utilized as a method for wound healing because they can be involved in angiogenesis, capillary formation, and re-epithelization (6). Some of these functions are modulated by the ASC's ability to secrete growth factors. EGF, FGF, VEGF, PDGF, HGF, TGF-β are all growth factors released that are of importance to the wound healing process (14). VEGF in particular is
an essential growth factor because it has been shown to induce angiogenesis, which in a chronic wound could alleviate ischemia (10).

The addition of ASC to the wounds on the novel model system promoted wound closure only to some degree. On average, only about 20% wound closure was observed with the sole addition of ASC. There are several possible factors that could contribute to this. ASC cannot provide all of the growth factors, immunological cells, and cytokines that are important for the wound healing process. Therefore without the help of the blood, the proper nutrients could not be delivered to the wound in order for repair to occur. In the future there is potential for ASC to have the ability to secrete a mixture of growth factors that could potentially cause complete wound closure.

Red Light Therapy is another method of inducing wound closure. The specific mechanism of RLT is unknown, however it is postulated that it can induce angiogenesis, reduce inflammation, and reduce pain (14). It is possible that RLT has a stimulatory effect on cells in damaged areas. This could potentially cause wound healing to be enhanced by increasing cell migration and proliferation in the wounds. In this study the addition of solely red light did result in more wound closure than the use of ambient light. Nearly 60% wound closure was observed by this method, however it is possible that the dosage of the RLT or the time the wounds exposed to the light could affect the efficacy of the treatment. If the correct dosage of light is used in accordance with the right amount of time, there is potential that RLT could induce even more wound healing.

It was expected that if the two therapies were used in conjunction they could potentiate even greater wound closure. There is evidence that ASC supplemented with red light have increased secretion of angiogenic growth factors, increased cell migration, and increased cell proliferation (14). However, when the actual experiment was conducted, the opposite result was observed. The wound area did not decrease significantly, but the wound area actually increased. This can most likely be explained by a concurrent bacterial infection. It was observed during the first week of the experiment that the media was cloudy. The experiment was continued because the bacterial infection could lead to interesting implications for responses to treatment and treatment failure. The addition of the bacteria to the model system could have actually caused the wounds to take on a more chronic-like nature. The wounds expanded instead of closed over the period of a month, which could be indicative of a loss of integrity in the wound and skin structure or simply that they were taking on additional phenotypes consistent with chronic wounds. Future studies should characterize how the bacteria affected the wound models by utilizing H&E staining to track how the structure changed and gene expression studies to determine how the cell environment changed.

**Bacteria**

The bacteria that was found on the skin of the wound models during contamination was *Ochrobactrum anthopii*. This came as a surprise upon identification because this bacteria in particular is not generally found in normal skin microbiota and although it has been associated with human disease, it is infrequently. There is always a diverse fauna of bacteria that can grow on skin and each layer of skin can contain different types of bacteria. These can include both gram-positive and gram-negative bacteria. However, a recent study showed that gram-positive bacteria are far more prevalent (4).

*Ochrobactrum anthopii* is a rod-shaped, gram-negative bacteria, but is not typically found on skin. It is characterized as an environmental pathogen that is prevalent in hospital and laboratory settings. It can occasionally affect patients who have in-dwelling catheters or immunocompromised patients (1). The presence of this bacterium in the samples may have come from the hospital but most likely indicates that our lab has some type of *Ochrobactrum anthopii* that is contaminating the wound cultures. This highlights another weakness of the wound models. Since they are grown in static cultures in 2 antibiotics, they may be more susceptible to infection. While we did not test if the isolated
*Ochrobactrum anthopi* were resistant to streptomycin and penicillin, it is likely they are. The positive aspect is that the addition of a bacterial infection to the wound models may also allow the wounds to develop a more chronic state and allow us to dissect the critical features of wound healing that are inhibited by bacterial infection.

**Summary**

In summary, the novel wound system is derived from human tissue and therefore has the same histoarchitecture as human skin. Different therapies have shown efficacy in causing closure of these wounds, such as the addition of ASC and RLT. When contaminated with a bacterial infection, these therapies fail to facilitate wound closure. It is possible that therapeutic approaches can cause the wound area to enlarge, potentially creating a wound that is more similar to a chronic wound, but the correct control (no therapeutic intervention) was not included in this study. Future studies could potentially use this model to continue investigating the effects of ASC and RLT on wound closure or other therapies could be investigated such as human skin equivalents and growth-based factors.
References


