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The Multiple Use Of Autologous Conditioned Plasma Supports For Wound Healing In A Natural Environment

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Abstract- ***In this chapter the use of a full-thickness murine model was implemented to test the efficacy of ACP combined and activated on demand with electrospun type I bovine collagen scaffolds. A total of four, Severe Combined Immunodeficiency (SCID) 8-week female mice were purchased from Jackson Labs (albino, without hair genotype: A/A Tyr^{c}/Tyr^{c} Foxn1^{nu}/Foxn1^{nju}) and used in this experiment. The mice had 5 full-thickness dorsal dermal punches created in a sterile operating room using sterile techniques. The wounds were assessed at pre-determined time intervals until euthanization occurred, at which time tissue collection was implemented for histological analysis. Histological assessment showed increased epithelial thickness and the presence of intradermal adipocytes indicative of a specific phase in the normal wound healing cycle. Based on both gross wound percent closure and histological assessment the use of ACP and collagen scaffolds reduces wound healing time, can positively impact the reforming stratum corneum, thus can be stated offers a favorable treatment modality for full-thickness wound closure.***

Keywords: Autologous conditioned plasma, wound healing cycle

Introduction

Biological systems rely on complex cellular pathways to maintain homeostasis. This is especially true and present during the normal process of wound healing. The use of the scratch assay in chapter three, focuses on the role of one specific cell type, the human neonatal dermal fibroblasts. The bench-top scratch assay allows for a fast through-put to determine potential biological activity of various treatments and therefore serves as a valuable screening tool. However, it does not completely replicate the living system and therefore does not account for many of the complex processes in play during normal wound healing in an organism. Here a murine model was used as a complexbiological system to evaluate wound healing event(s) in a full-thickness wound defect. In this study, two treatments and a control were evaluated.

The treatments included ACP combined with electrospun collagen scaffolds, stand-alone collagen scaffolds and a control that used current clinical standards (e.g. Vaseline gauze and a Tegaderm dressing). Both ACP and collagen are needed during the wound healing event (Falanga, 2005) (Madden, & Peacock, 1971). Determining how to assess the activity of the ACP and collagen in these wounds is an important tool. As a result, gross wound closure was assessed using photographic techniques with subsequent image analysis to model a clinical mode of assessment (Houghton, 2000) and to determine if a specific tested treatment aided in wound healing. Furthermore, histology was performed to allow for microscopic analysis of the healing event (Yeung, 1999).

Research Aim

Implement ACP with a collagen scaffold into a full thickness murine wound.

Research Hypothesis

If ACP activated with a collagen scaffold is used to treat a full-thickness integumentary wound, then a decrease in wound healing time will occur compared to the stand-along collagen and control (current standard of wound care).

Materials and Methods: Histology Staining and Assessment

On day 6, tissue specimens of each wound site were removed immediately following euthanasization with an 8 mm biopsy sterile punches (n=5 ACP and Collagen) (n= 5 ACP) and (n=5 control). Each punch was sagittally sectioned and one sagittal half was placed into 2% paraformaldehyde (PFA) for 24-hour fixation, the other half in RNA later for future research. Following fixation, the specimens were subsequently dried and paraffin embedded. Sectioning of specimens was performed at a thickness of 5 µ m, followed by hematoxylin & eosin staining (H&E) protocol. In this staining protocol, thesequence of events was as follows:

1. 3 changes of xylene, 2 minuteseach.
2. Re-hydrate in 100% ethanol, 10dips.
3. 95% alcohol, two changes, 10 dipseach.
4. Wash briefly in distilled water, until water runs offevenly.
5. Stain in Harris hematoxylin solution for 15minutes.
6. Wash in tap water, two changes, 10 dipseach.
7. Bluing in 0.25% ammonia water or saturated lithium carbonate0.50% solution, untilblue.
8. Wash in running tap water for 5minutes.
9. Counterstain in eosin, 10 -20 dips each.
10. Clear in 3 changes of xylene, 10-15 dipseach.
11. Mount with xylene based mountingmedium.

The stained slides were assessed for percent re-epithelialization and average epithelial thickness between wounded skin and intact/non-wounded skin. Percent re-epithelialization was calculated by determining the entire width (length of tissue) minus wound width divided by entire width for each individual slide. The average epithelial thickness was determined by measuring the native skin thickness and dividing by wound skin thickness. Figure 1 below demonstrates the visible nature of the wounded site versus the native healthy skin.

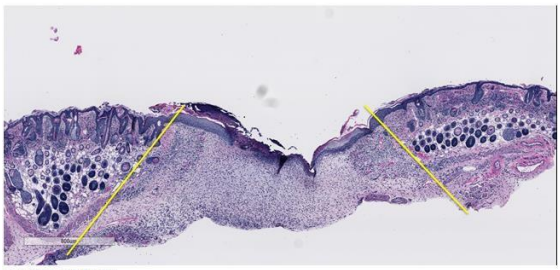
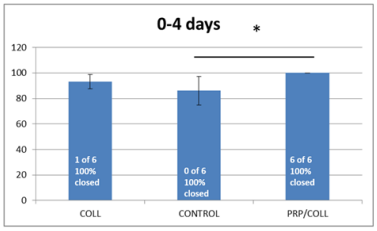


Figure 1: A control wound site from day 6 of the *in-vivo* study. The wound margins are denoted by the yellow lines. The left and right sides of the tissue outside of the yellow lines, represent the non-wounded native tissue (H & E stain, 40x).

Materials and Methods: Adipocyte Manual Counts

Intradermal adipocytes were manually counted in each FOV using APERIO software, ImageScope version 12.1.0.5029.

The same results were seen at 144 hours post wound creation with the combination treatment having a percent closure of 100% while the control had a percent closure of 96.48±2.72% (p<0.02). The standalone collagen treatment had a percent closure of 98.74±2.34% and again lacked statistical significance compared to the control. The graphs below (figures 2, 3 and 4) illustrate the above findings, and the photographic images (figure 6) detail the wound progression at the various time points (48, 96 and 144 hours).



*p = 0.01
n= 6
Alpha= 0.05
ANOVA Tukey-Post Hoc

Figure 2: Percent wound closure at day 4 (96 hours), statistical difference was seen between ACP with collagen activation and control(p<0.01).

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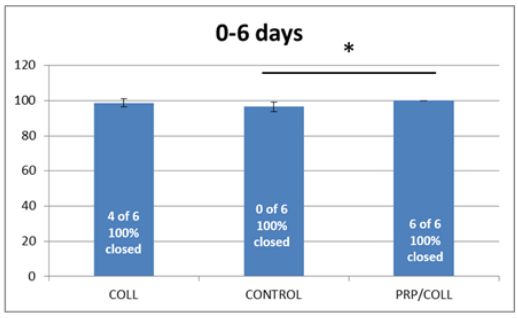

*p = 0.025
n= 6
Alpha= 0.05
ANOVA Tukey-Post Hoc

Figure 3: Percent wound closure at day 6 (144 hours), statistical difference was seen between ACP with collagen activation and control($p<0.02$).

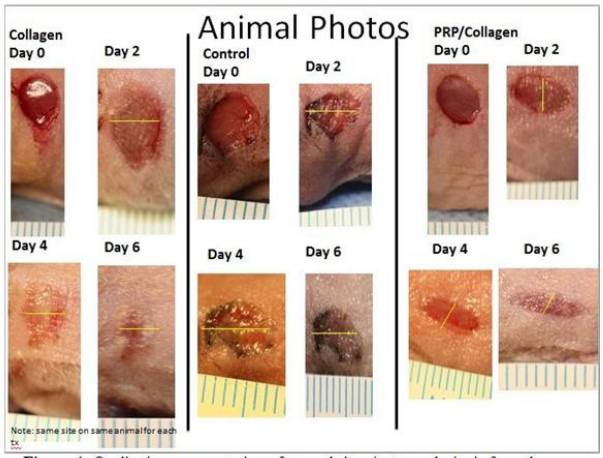

Figure 4: Qualitative representation of wound sites (note: each site is from the same animal). The yellow line represents the wound margin at the intervals post-creation.

Results: Histological Assessment

Re-epithelialization was quantitatively assessed using histological analysis with subsequent digital morphometry to evaluate the thickness of the resulting new epithelium following the wound healing event. No statistical differences were seen between any of the treatment groups ($p<0.19$). However, the ACP activated with collagen did show the greatest amount of re-epithelization.

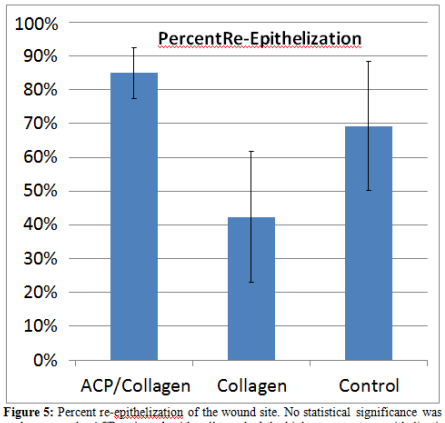

Figure 5: Percent re-epithelization of the wound site. No statistical significance was seen; however, the ACP activated with collagen had the highest percent re-epithelization.

Average epithelial ratio thickness between the wound site and native skin was not statistically different ($p<0.19$). However, ACP activated with the collagen scaffold trended with the highest amount of epithelial recovery i.e. healing compared to the collagen stand-alone and control. The graph below (figure 6) illustrates the data.

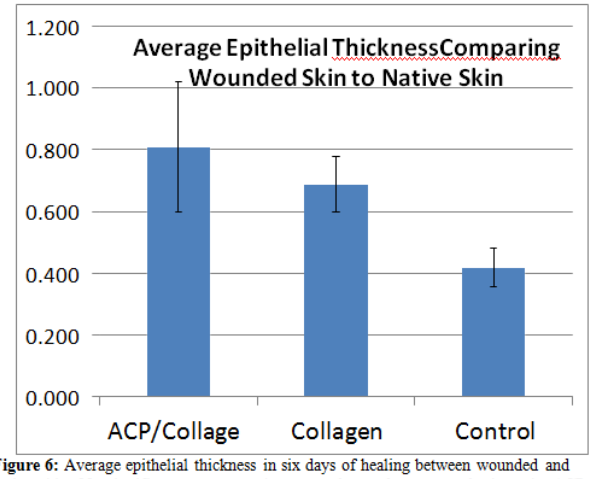

Figure 6: Average epithelial thickness in six days of healing between wounded and native skin. No significance was seen but a trend was demonstrated where the ACP and collagen had a greater epithelial thickness in the 6 day healing time. The closer to 1.00 represents more similar to native thickness.

Further micro-anatomical features were identified to be present in the histological samples, including intradermal adipocyte deposits. These adipocytes have been reported to aid in the wound healing response by the release of adipocyte cytokines signaling recruitment of fibroblasts to the wound site (Schmidt & Horsley, 2013). Fibroblasts are needed to re-epithelialize wounded integument. The presence of epidermal layers, specifically stratum corneum is indicative of a healing event (Ghalbzouri et. al., 2004).

In the ACP and collagen combination samples, there was presence of an intact, reformed stratum corneum layer in 4 of the 5 of the samples (1 histological ACP/ collagen sample was not assessed due to the inability to find the wound during histological sectioning and assessment). The H & E stained histological image (figure 7) below demonstrates theseadipocyte deposits and the presence the stratum corneum found in the ACP and collagen combination treatment. Although the intradermal adipocytes were present in the collagen stand-alone treatment and the control, both groups did not present with the same re- epithelization (stratum corneum) as the ACP and collagen combination treatment group. The collagen stand-alone had no presence of a completely re-formed stratum corneum in

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any of the histological samples (n=6) and the control had 1 of the 5 samples with a re- formed stratum corneum (one sample was not assessed due to the inability to find the wound during histological sectioning and assessment). Figures 08 and 09 below demonstrates the collagen stand-alone and the control histological samples. Figure 11 below shows the average adipocyte cell count for each wound margin field of view (FOV).

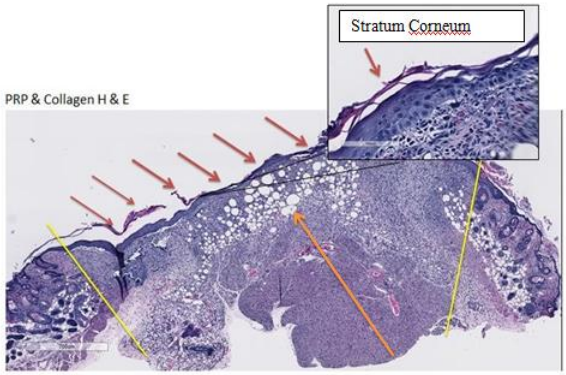

Figure 7: Integument H & E slide with the wound margins identified by the yellow line, this sample was part of the ACP& collagen treatment group. The insert box (higher magnification) demonstrates the detail of the reformed stratum corneum layer of the epidermis. The orange arrow is identifying the intradermal fatty infiltrate.

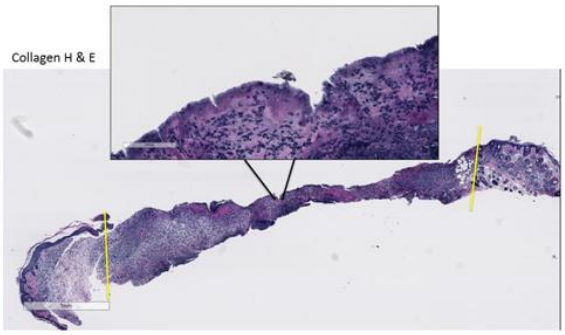

Figure 8: Integument H & E slide with the wound margins identified by the yellow line, this sample was part of the collagen only treatment group. The insert box (higher magnification) demonstrates the lack of stratum corneum presence.

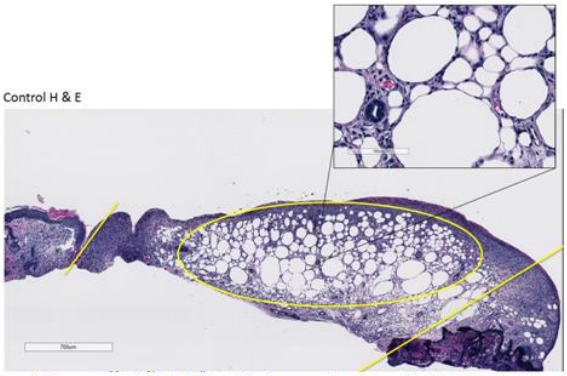

Figure 9: Integument H & E slide with the wound margins identified by the yellow line, this sample was part of the control group. The insert box (higher magnification) demonstrates the intradermal adipocytes presence. Also, note that the top epidermal layer (stratum corneum) is missing.

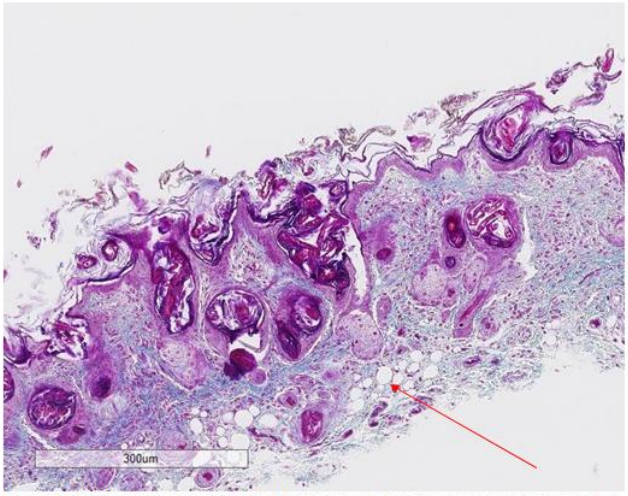

Figure 10: Native integument H & E slide. No wound margins are present. This was due to a lack of the wound not being created in this sample. Here the intradermal adipocytes are not present. The hypodermal layer identified by the red arrow. The lack of the intradermal adipocytes further indicate that the intradermal adipocyte presence may be needed as part of the wound healing event.

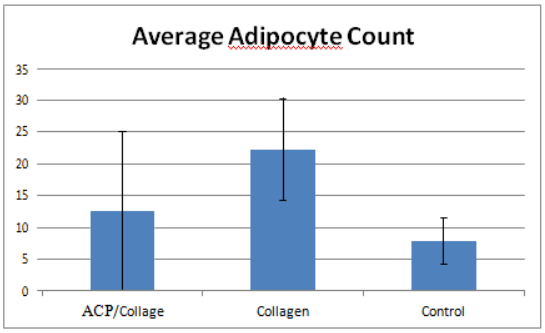

Figure 11: Average adipocyte count for the two treatment groups (collagen and the combination ACP with collagen and the control). No statistical significance ($p<0.20$).

Discussion:

The standalone treatment (electrospun collagen scaffold) was not statistically different than control wounds at any of the time points evaluated. Although quantitative histological assessment did not demonstrate significance, possibly due to small sample sizes, trends were visible with the ACP combined with collagen in re-epithelization and epithelial thickness ratio as well as morphologic evidence of wound re-epithelization and the formation of a new stratum corneum layer, identified in H&E histology samples. Four (4) out of 5 ACP and collagen samples demonstrated desquamation of a stratum corneum layer whereas the collagen only had no samples with stratum corneum presence and the control had one histological sample with desquamation presence. A new stratum corneum layer is indicative of complete wound healing with a full re-epithelization of the wound site (Ghalbzouri et. al., 2004). In the murine model it has been reported that reforming a desquamating corneal layer can take approximately 7-14 days

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(Potten, 1975) (Potten, 1981). The combination therapy, ACP this corneum in 6 days, demonstrating that a decrease in wound healing time is occurring using the morphological corneal layer as an identifying feature. Additionally, it is interesting to note that the average epithelial thickness ratio was most representative to native skin thickness (1.00) in the combination ACP therapy at 0.808 followed by the stand-alone collagen at 0.688 and lastly the control at a thickness ratio of 0.418, further demonstrating the effectiveness of the combination therapy. Additional anatomical evidence of wound healing was also demonstrated by quantifying the amount of intradermal adipocyte presence. Based on manual adipocyte counts, the control group presented with the fewest averageintradermal

adipocytes [7.8 cells/field of view (FOV)], collagen presented with the highest average amount of intradermal adipocytes (22.25 cells/FOV) and lastly the combination therapy of ACP presented with a moderate amount of 12.5 adipocyte cells/FOV. The counts may be indicative of the specific phase or sub-phase of wound healing that has been captured at the day 6 timepoint. The data demonstrated that the control and stand-alone collagen did not have the most amount of re-epithelization nor the same average epithelial thickness ratio compared to the combination therapy of ACP and collagen. The literature has described that the intradermal adipocytes serve a critical role in the recruitment of fibroblasts, which then are able to facilitate the beginning of the matrix deposition phase of wound healing (Schmidt & Horsley, 2013).

Based on thecurrent experimental findings, the control group may have been delayed in its progression through the typical phases of wound healing. In this case, the control group may be in an early stage of wound healing at the day 6 time point compared to the treatment groups, potentially still in the inflammatory stage. The stand-alone collagen treatment group had the greatest amount intradermal adipocytes; therefore, possibly indicating that this group had progressed into a later stage of wound healing by the day 6 time point when compared to the control group. This may suggest the stand-alone collagen is in the later inflammatory stage or early proliferative stage. This finding is also supported by the average epithelial thickness data that was measured using histological assessments of the wound site re-epithelization. The combination therapy of ACP and collagen demonstrated the greatest amount of re-epithelization and average epithelial thickness ratio along witha moderate amount of intradermal adipocyte presence compared to the control group. This could be explained by suggesting that the adipocytes are receding from the woundbedand hence the wound healing event has progressed further, beyond the early proliferative stage compared to the stand-alone collagen and control. In summary, the study outcomes support that the combination ACP and collagen treatment had progressed to a later stage of wound healing and subsequently reduced the wound healing time based on the following:

1)The combination treatment had the greatest percent wound closure.

2)The combination treatment contained the greatest epithelial recovery andepithelial thickness ratio along with all sample containing a desquamating corneal layer at day 6 compared to the standard reforming murine skin between 7-14 days.

3)The combination treatment contained a moderate amount of adipocytes

compared to the standalone collagen and control, suggesting a later stage of the wound healing event, whichfacilitate the remainder of the wound healing process. In conclusion, these research findings provide support for the aim three hypothesis, If ACP activated with a collagen scaffold is used to treat a full-thickness integumentary wound, then a decrease in wound healing time will occur compared to the control and collagen treatment.

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