Development of Supercritical CO_2-Treated Human Amniotic Membrane Combined with Adipose Derived Stem Cells for Wound Treatment

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Introduction: Combat related burn injuries often involve large total body surface area, frequently requiring skin allografts due to lack of availability of autologous skin. [1] Amniotic membrane (AM), the innermost layer of the placenta, is well known to improve wound healing. Unfortunately, the current methods used to sterilize AM, including gamma irradiation, are detrimental to the microstructures of the AM. Supercritical CO_2 ($SCCO_2$) treatment combines the diffusivity of a gas with the solvent properties of a liquid to remove unwanted compounds, such as blood and lipids, while the low processing temperatures and non-toxic nature of CO_2 allow for the preservation of both the physiological and mechanical integrity of the tissue. Thus, $SCCO_2$ provides a novel method for amniotic membrane tissue graft preparation and sterilization. Studies have shown that stem cell based therapies, specifically adipose-derived stem cells, can improve wound healing by differentiating to skin-specific epithelial cells as well as increase vascularization of the wound bed.[2] In this study we hypothesize that human ASCs (hASCs) when combined with $SCCO_2$ treated AM can enhance the repair and regeneration of skin after wounding. We have evaluated the effect of $SCCO_2$ sterilization on the structural and biological integrity of the AM and determined its efficacy when combined with hASCs to promote wound healing *in vivo*.

Methods: Human placentas from caesarean sections were obtained from the University Hospital (San Antonio, TX, Material Transfer Agreement #W81XWH-11-0441). The amnion was separated from the chorion, sterilized with $SCCO_2$, and stored at -80 °C. AM was stained with picrosirius red stain (Polysciences, Inc.) to visualize the organization of collagen. The amount of Hydroxyproline (BioVision), elastin (Fastin™, Biocolor), collagen IV (ELISA) and glycoaminoglycans (GAGs) present in native and $SCCO_2$-treated AM were analyzed. Human ASCs (isolated from discarded skin samples collected in accordance with a protocol reviewed and approved by the U.S. Army Medical Research and Materiel Command Institutional Review Board # HSC20080290N) fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CSFE), were seeded on the epithelial side of AM (50,000 cells/membrane) and cultured under standard cell culture conditions for up to 4 days in MesenPRO RS™ growth media. Fluorescent images were taken to assess ASCs attachment after 24 hours. Cell proliferation was monitored over a period of 4 days using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-dipenyltetrazolium bromide) assay. AM membrane seeded with hASCs was then evaluated *in vivo* using a full thickness excision wound model in athymic rats. The efficacy of treatment on wound healing was evaluated histologically using skin biopsies stained with Masson's trichrome stain (MTS) and/or hematoxylin and eosin (H&E).

Results: $SCCO_2$ technique rendered ready-to-use, sterile AM retaining the overall ultrastructural collagen organization observed through picrosirius red staining (Figure 1). Biochemical analysis of the $SCCO_2$-treated AM did not show a significant decrease in the total amount of hydroxyproline, elastin, glycoaminoglycans or collagen IV compared to native tissue. *In vitro*, $SCCO_2$ treated AM showed the ability to act as a tissue engineering platform scaffold supporting attachment and proliferation of hASCs. Finally, AM seeded hASCs improved the epithelialization and increased the blood vessel formation within the wound bed, indicating its positive effect to promote wound healing.

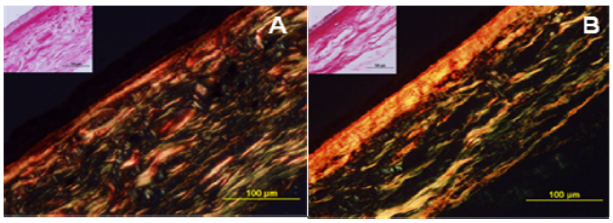

Figure 1. Amniotic membrane before (A) and after (B) $SCCO_2$-treatment. Images show polarized light microscopic sections of AM stained with picosirius red stain. The inset shows the non-polarized image of the $SCCO_2$ treated AM.

Conclusions: In this study we have shown through physical, biochemical and histological analysis that $SCCO_2$ treatment provides a viable method to produce sterile AM while still retaining characteristics of native AM. Additionally, we have shown that hASCs readily attached and proliferated on the $SCCO_2$-treated membrane. *In vivo* studies revealed that an AM-hASCs construct promotes wound healing as evidenced by improved re-epithelalization and increased vascular networks within the wound bed. These results demonstrate that $SCCO_2$-treated AM in combination with hASCs are efficacious in wound healing and skin regeneration.

References:

1. Chung, KK., et al. J Burn Care Res 2006; 27: 606-611.
2. Heng, BC., et al. Exp Dermatol 2005; 14: 1-16.
3. Yang, L. et al. J Derm Sci 2009; 56: 188-195.