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● *Original Contribution*

HIGH-FREQUENCY ULTRASONIC IMAGING OF GROWTH AND DEVELOPMENT IN MANUFACTURED ENGINEERED ORAL MUCOSAL TISSUE SURFACES

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Abstract—This study uses high-resolution ultrasound to examine the growth and development of engineered oral mucosal tissues manufactured under aseptic conditions. The specimens are a commercially available natural tissue scaffold, AlloDerm, and oral keratinocytes seeded onto AlloDerm to form an *ex vivo*-produced oral mucosal equivalent (EVPOME) suitable for intra-oral grafting. The seeded cells produce a keratinized protective upper layer that smooths out any remaining surface irregularities on the underlying AlloDerm. Two-dimensional acoustic imaging of unseeded AlloDerm and developing EVPOMEs was performed on each day of their growth and development, each tissue specimen being imaged under aseptic conditions (total time from seeding to maturation: 11 d). Ultrasonic monitoring offers us the ability to determine the constituents of the EVPOME that are responsible for changes in its mechanical behavior during the manufacturing process. Ultrasonic monitoring affords us an opportunity to non-invasively assess, in real time, tissue-engineered constructs before release for use in patient care. (E-mail: Fwinterr@umich.edu) © **2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Acoustic elasticity microscopy, Oral mucosa, Non-invasive assessment, Tissue engineering, Ultrasound, Image analysis, Keratinocyte, Cell spreading.**

INTRODUCTION

Scanning acoustic microscopy (SAM) has been reported to be an effective tool in the study of both the mechanical properties and non-linear elastic characteristics of cells and tissues (Cohn et al. 1997a, 1997b; Hollman et al. 2002; Kolios et al. 2002). The advantages of SAM over conventional light and electron microscopy include the ability to image cells and tissues without damaging or altering them; this provides a more accurate representation of the natural properties of the tissues (Cohn et. al. 1997a). We have compared SAM imaging results with those of standard optical microscopy (Winterroth et al. 2011a) and, in single-blind studies, have compared engineered tissue specimens with those subjected to an elevated thermal stress (damaging the cells) (Winterroth et al. 2011b). We further tested the physical elastic properties of *ex vivo*-produced oral mucosal equivalent (EVPOME) and commercially available unseeded acellular cadaveric dermis, AlloDerm (LifeCell, Branchburg, NJ, USA), which serves as the scaffold for EVPOME construction and development (Wagner et al. 2009).

Previously, we used SAM to compare changes in radiofrequency (RF) data with differentiation, apoptosis and keratinization in EVPOME and natural human oral mucosal cells (Winterroth et al. 2009; Zuber et al. 1999). The spectral analysis results from SAM can be compared with histologic images of the EVPOME tissues at different stages of growth and development. By correlating changes in the RF data with differentiation, apoptosis and keratinization in EVPOME (and mucosal cells in general), we can better understand the physiologic

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processes of these cells as they evolve and proliferate along the AlloDerm surface.

AlloDerm has been found to be a viable scaffold for producing the engineered oral mucosal tissue. It is obtained from allograft donor skin and produced by a carefully controlled process that removes the epidermis and dermis cells without altering the extracellular matrix structure and while maintaining an intact basement membrane, thus reducing immune responses and transmission of diseases (Harrison et al. 2006; Vendramini et al. 2006; Wagner et al. 2009).

SAM is an effective tool for studying both the morphology and non-linear elastic characteristics of natural and engineered oral mucosal tissues (Cohn et al. 1997a). Non-invasive and non-destructive imaging of cells and tissues not only provides accurate assessments of these organisms (as they are alive when being imaged), but also provides evidence of the degree of differentiation the cells are undergoing *in situ* (Amelink et al. 2008; Holland et al. 1997; Kolios et al. 2003; Saijo et al. 2004). Examining the acoustic properties of tissues also allows us to study their density and elasticity.

The reflectivity of the superior portion of the EVPOME allowed us to quantify the degree of surface roughness, which exhibited a strong linear correlation in quantification of the surface characteristics between optical and SAM imaging (Winterroth et al 2011a).

Although SAM was used to study the morphology and density of skin tissue under both normal and pathologic conditions (Moll et al. 2008; Barr et al. 1991; Broekhaert and Van Oostveldt 1988), it has not been used to gain an understanding of the growth and development of the cellular component and finalized engineered tissues during the manufacturing process, as was done in a recent study using Raman spectroscopy (Khmaladze et al. 2013). In this study, we successfully used SAM to assess the cellular component and final tissue properties of the EVPOME during its manufacture.

METHODS

Tissue preparation: EVPOME

The methods used to prepare both AlloDerm and EVPOME devices are similar to those described elsewhere (Hotta et al. 2007; Izumi et al. 2004; Izumi et al. 2003). Briefly, oral mucosa keratinocytes were enzymatically dissociated from the tissue sample, and a primary cell culture was established and propagated in a chemically defined, serum- and xenogenic product-free culture medium, with a calcium concentration of 0.06 mM. The AlloDerm specimens were soaked in 5 μg/mL human type IV collagen overnight at 4°C before seeding cells to assist the adherence of cells; then approximately 2.0×10^5 oral keratinocytes/cm^2 were seeded onto the type IV collagen pre-soaked AlloDerm and cultured in medium containing 1.2 mM calcium. The composites of keratinocytes and AlloDerm were then cultured, while submerged, for 4 d to form a continuous epithelial monolayer. On day 4 post-seeding, samples of the submerged EVPOME were collected for SAM imaging. After 4 d, the equivalents were raised to an air-liquid interface and cultured for another 10 d, resulting in a fully differentiated, well-stratified epithelial layer on the AlloDerm. AlloDerm specimens (used as controls) were treated in the same manner as EVPOMEs, except that they were never seeded with the oral keratinocytes. The setup for scanning of all tissue specimens is illustrated in Figure 1. Each specimen was approximately 1.0 cm^2 in area.

A total of three scanning sessions were conducted for this study; three specimens, one AlloDerm and two EVPOMEs, were prepared for each session. We started the SAM scans of the specimens on day 4 post-seeding.

SAM logistics: setup and imaging

The setup for SAM built in-house has been detailed previously (Cohn et al. 1997a, 1997b; Hollman et al. 2002). Briefly, AlloDerm and EVPOME specimens were immersed in sterile de-ionized water and imaged with a single-element fixed-focus transducer (NIH Resource Center for Medical Ultrasonic Transducer Technology, University of Southern California, Los Angeles, CA, USA) producing ultrasonic B-scans. The transducer has an approximate frequency of 50 MHz; the element is 3 mm in diameter and focused to a depth of 4.1 mm, giving an *f*/number of approximately 1.4. The transducer was fastened to an optical mount, and the angular position was adjusted until the ultrasonic beam was normal to the deflecting plate. Stepper motors control the positioning motion of the transducer; the stepper motors are controlled by a stepper motor driver (MID7604, National Instruments, Austin, TX, USA) and motion control card (PCI-7354, National

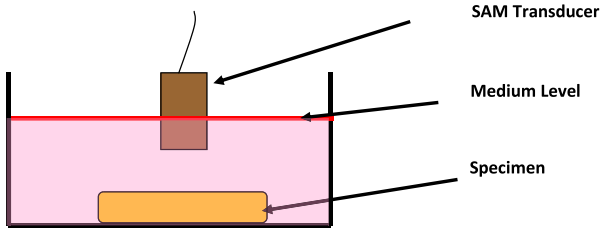

Fig. 1. Basic setup for scanning acoustic microscopy (SAM) imaging of AlloDerm and the EVPOME (*ex vivo*-produced oral mucosal equivalent), on different days post-seeding. The transducer was immersed in the culture medium and imaged under sterile conditions daily for the duration of the growth and development of the EVPOME.