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Non-Linear Stress-Strain Measurements of *Ex Vivo* Produced Oral Mucosal Equivalent (EVPOME) Compared to Normal Oral Mucosal and Skin Tissue

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Abstract—**Stress-strain curves of oral mucosal tissues were measured using direct mechanical testing. Measurements were conducted on both natural oral mucosal tissues and engineered devices, specifically a clinically developed *ex vivo* produced oral mucosal equivalent (EVPOME). As seeded cells proliferate on EVPOME devices, they produce a keratinized protective upper layer which fills in surface irregularities. These transformations can further alter stress-strain parameters as cells in EVPOME differentiate, more similar to natural oral mucosal tissues in contrast to an unseeded scaffold. In addition to tissue devices grown under normal conditions (37^0C), EVPOMEs were also produced at 43^0C. These thermally stressed specimens model possible failure mechanisms. Results from a mechanical deformation system capable of accurate measurements on small (approximately 1.0 - 1.5 cm^2) cylindrical tissue samples are presented. Deformations are produced by lowering a circular piston, with a radius smaller than the sample radius, onto the center of the sample. Resulting force is measured with a precision electronic balance. Cultured EVPOME was less stiff than AlloDerm®, but similar to native porcine buccal tissue. Porcine skin and porcine palate tissues were even less stiff. Thermally stressed EVPOME was less stiff than normally cultured EVPOME as expected because stressed keratin cells were damaged reducing the structural integrity of the tissue.**

I. Introduction

While teeth are the primary food contact, soft tissues such as oral mucosa need to be durable and elastic enough to endure interaction with food as it is being chewed and moved around in the mouth. When developing a laboratory grown transplant replacement for oral mucosa, monitoring mechanical integrity is therefore important. One such oral mucosa replacement is *Ex Vivo* Produced Oral Mucosal Equivalents (EVPOME). Recent studies [1-3] have shown success with a scaffold of AlloDerm®, an acellular matrix derived from cadaveric skin. EVPOME is important to oral reconstructive surgery, its usage ranging from repairing catastrophic facial trauma to replacing removed oral cancerous tissue. As with other transplantations, there are shortages of available tissue due to shortages of donors. Even when donated tissue is available there are issues of biocompatibility. EVPOME overcomes these challenges because it can be grown in the laboratory from cells harvested from the patient.

While current studies [4-6] use laser based imaging to examine the biochemical processes which EVPOMEs undergo from seeding to implantation in the mouth, they are not capable of measuring one very important property of these tissue devices: mechanical integrity. In this study mechanical properties of EVPOME are determined by measuring stress-strain curves with a piston compressor and a precision electronic balance. This initial study will indicate whether there is difference in mechanical properties of the scaffold, a mature specimen grown under normal conditions and a specimen in which failure has been induced through thermal stress. Mechanical difference in these specimens will provide insight into pre-transplant integrity and determine if elastic moduli are an important criterion for implanting EVPOME.

II. Materials and Methods

A. Specimens

To create an EVPOME specimen, oral mucosa keratinocytes were dissociated from human oral tissue samples and then seeded onto a scaffold of acellular cadaveric dermis. EVPOMEs were cultured submerged for 4 days to form a continuous epithelial monolayer and then raised to an air-liquid interface for another 7-10 days.

Mature EVPOME consists of an underlying AlloDerm® scaffold, a layer of active keratinocytes on top of the scaffold, and then layers of keratinized cells forming a

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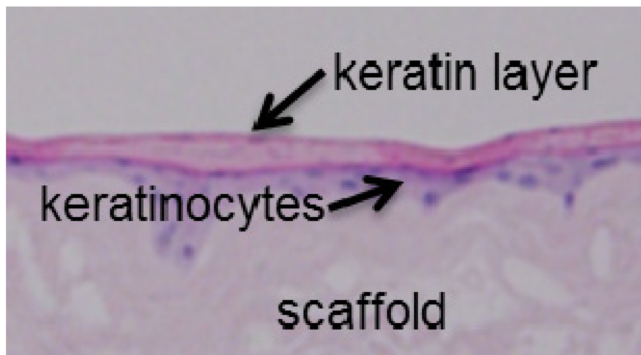

Figure 1. Photomicrograph of mature EVPOME. Scaffold is light pink, keratinocytes are purple, and the keratin layer is dark pink.

protective barrier as shown in Fig. 1. During *ex vivo* culturing, keratinocytes grow only on top of the scaffold and do not invade into it. Only after it is implanted does vascularization take place and the scaffold is gradually integrated into native tissue.

After measuring mechanical properties in specimens grown under ideal conditions, culturing parameters were altered to induce failure in some of the tissue device. For this study temperature was chosen as the failure-inducing parameter.

Optimal *in vitro* growth temperature for EVPOME is 37°C. On Day 9 post-seeding, some EVPOME devices were incubated at 43°C for 24 hours, then reverted to 37°C for another 24 hours. This process is termed "thermal stress". It always creates damaged tissue devices that are unsuitable for implantation. As shown in Fig. 2, histology of a thermally stressed EVPOME shows that the stratified

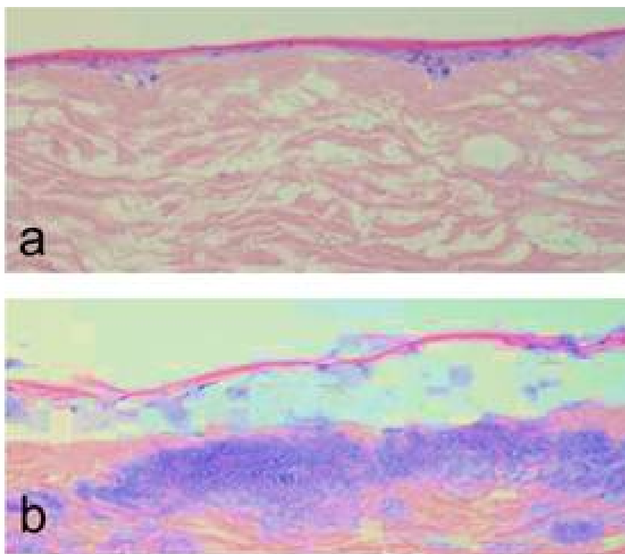

Figure 2. Histology of EVPOME devices. The normal (unstressed) device a) shows a stratified layer (dark pink) attached to Alloderm® (light pink). The thermally stressed device b) shows the stratified layer detached from the Alloderm®.

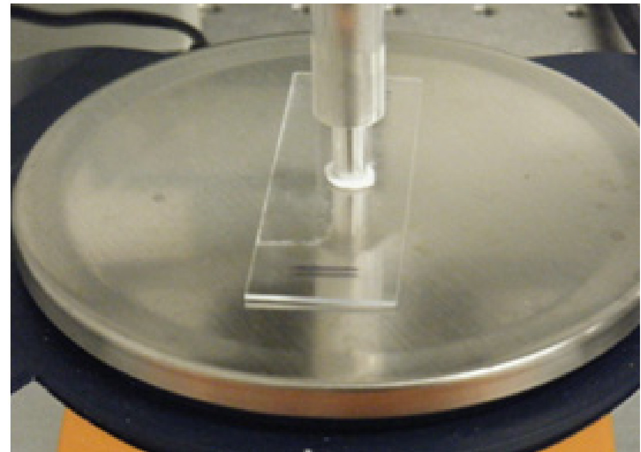

Figure 3. Photograph of compression system testing a tissue specimen placed on top of a glass slide. The slide sits on an electronic scale which has been tared for both the tissue and slide.

layers of the developing cells was so weakened that it lifted off from the AlloDerm®. This result is likely either caused by a disruption of attachment proteins in the basement membrane or a dysfunction of cell metabolic activity in the stratified layer. It is clear from histology that thermally stressed EVPOME have reduced mechanical properties and therefore lack structural integrity. Can this reduction in structural integrity be measured quantitatively?

B. Methods

Compression testing was performed on both unseeded AlloDerm® and mature EVPOME (11-days post-seeding) specimens by removing each specimen from its aqueous environment, measuring their initial thickness with an electronic caliper (Mituyo Corp., Japan), and placing it under an in-house built compression unit with cylindrical pistons which were either 540 mm^2 or 28.26 mm^2 in area. Two measurements in different regions were made on one specimen of each tissue type. There was no preconditioning of the specimens. A digital scale (Ohaus SPE602) was placed directly beneath the specimen, tared to the weight of the specimen, and used to record changes in force for each successive step size (10 μm) that the cylindrical piston applied to the specimen. There was about a 1 second delay between each displacement step. Details of the mechanical set-up are illustrated in Fig. 3. The equipment is a simpler version of the setup used to measure nonlinear elastic properties [7,8]. All stress levels were measured as kilopascals in relation to strain levels of known compression step length. These same methods were performed on all natural porcine skin and oral mucosal tissues obtained from a local abattoir.

Another set of normal and thermally stressed EVPOME were also measured with the same equipment and in the same manner. Both the thermally stressed devices and the normally cultured ones were seeded at the same time and

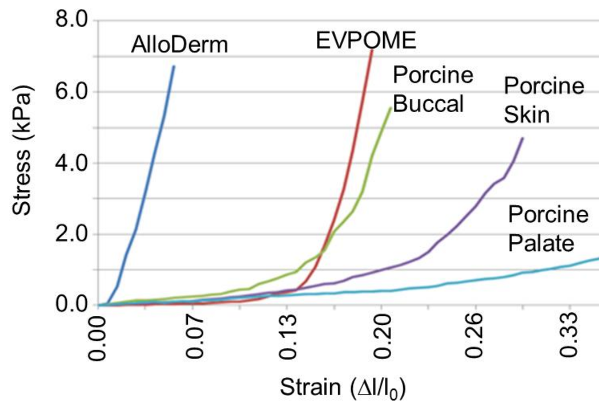

Figure 4. Tissue stess-strain curves measured by the piston deformation device. Shown are results for unseeded AlloDerm®, normally cultured EVPOME, and natural porcine tissues.

from the same batch of seeding cells. In addition to mature devices, unseeded AlloDerm® scaffolds used as controls were also subjected to the same culturing processes. Of course without seeding no keratinocyte layer grew on these scaffolds. One piece of unseeded scaffold was subjected to normal culturing and one piece subjected to the thermal stress protocol.

III. Results

The stress-strain curves of the normally cultured EVPOME, the unseeded AlloDerm®, and natural porcine tissues are shown in Fig. 4. AlloDerm® shows the stiffest and most linear behavior. EVPOME and porcine buccal oral mucosa show the greatest non-linear behavior, with an initial compliant region followed by a stiffening region. Porcine skin and palate also exhibited non-linear behavior, but had a less stiffening characteristic than the EVPOME and buccal mucosa. Especially note the striking difference

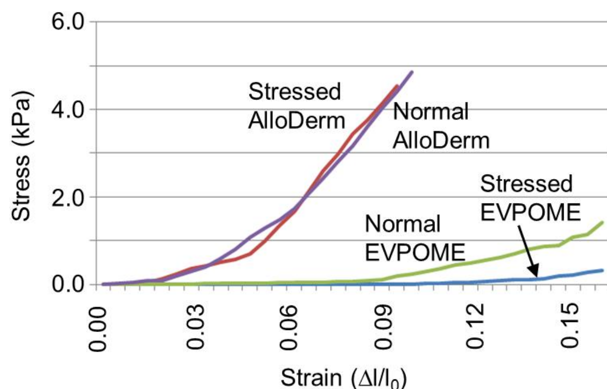

Figure 5. Stress-strain curves for the thermal stress experiments. There is no substantial difference in the results for the unseeded scaffolds subjected to normal culturing and thermal stress. There is a difference between curves for mature EVPOME grown under normal verses thermally stressed conditions.

between the unseeded scaffold and the mature EVPOME even though the scaffold is part of this tissue device.

Fig. 5 shows stess-strain curves for the thermally stressed data set. Curves for both thermally stressed and normal unseeded scaffolds were similar to each other and to the unseeded scaffold curve shown in Fig. 4. (Note that ranges on both axes in Fig. 4 are different from Fig. 5.) The curve in Fig. 5 for EVPOME grown under normal conditions is less stiff than the scaffolds but similar to the normal EVPOME in Fig. 4. The thermally stressed device showed a difference in the non-linear behavior compared to the normal EVPOME, with less stiffening occurring at high strains.

IV. Discussion

As expected, the scaffold is a relatively stiff material as indicated by its curve. It was originally developed and is currently most used for hernia surgery and breast reconstruction. These applications require a strong material to keep tissues and organs in place and prevent a hernia from reoccurring. AlloDerm® is produced from cadaveric skin by removing all the cells that would incite an immune response. The remaining material mostly consists of a dermal collagen matrix which is the main contributor to its strong mechanical properties.

To understand what happens after EVPOME maturely develops, consider that the tissue device consists of layers, one of which is the scaffold. The piston probes perpendicular to these layers. Under these circumstances EVPOME behaves similar to springs in series (at a fairly simplified level). For springs in series the reciprocal of the constituent spring constants average so that softer materials dominate. In this case, the EVPOME curve is dominated by the softer living cell layers on top of the scaffold. Of course the effective tissue elasticity is a more complicated function of the scaffold and cell layer elastic coefficients. There may even be differences as a function of depth in the stratified layers.

The normal EVPOME curve closely resembles the porcine buccal curve because buccal is closest in functionality to EVPOME. Although similar in structure, skin is produced and functions under slightly different conditions then mucosa. Unlike skin, mucosa is constantly moistened with saliva and must protect against food during chewing.

Of course these functional differences do not explain the much less stiff curve of the porcine palate. Further investigation is needed to explain what is happening with the mechanical properties of the palate.

The same simple layered model can help in understanding results of the thermally stressed EVPOME. First, similar curves for the unseeded scaffolds in Fig. 5 indicate that the scaffold is unaffected by thermal stressing. Therefore, mechanical properties of the scaffolds in the normal and stressed curves of Fig. 5 cannot be contributing to the

differences between these two curves. The difference must be primarily due to differences in the keratinocytes growing on similar scaffolds. Not only does thermal stressing affect biochemical function in keratinocytes, the disruption of cellular function shows up as weakened mechanical properties in the tissue devices.

This study shows that the presence of cells (particularly in a stratified pattern) changes the elasticity of these tissue devices. Even minimal presences of cells – including seeded layers that are rather poorly developed – still demonstrate resistance to stress at increasing strain deformations.

Although the sampling size is extremely small, differences in the stress-strain curves shown in Fig. 4 & 5 are fairly substantial and the positions of the curves are reasonable as expected examining their tissue structure. Obviously, more specimens need to be measured to confirm these initial results.

One aspect of further investigation is the relationship between thermal stress temperature and resulting mechanical properties. This study only examined two culturing temperatures. Understanding what happens between those two points will provide a better understanding of the failure modeled by thermal stress. For example, are the mechanical properties a linear relationship with stressing temperature? Are they uniformly affected throughout the thickness or are they depth dependent? Knowledge of these relationships will give a better idea of what to examine when checking for failure in clinical situations.

Also, only mature EVPOME specimens were examined for this study. A study of EVPOME mechanical properties at various stages in growth would be beneficial. As keratinocytes grow on the surface of the developing tissue device, the cells change in form and function. Monitoring their mechanical properties could help separate out poorly developing devices earlier, reducing the waste of time and materials.

While this simple measurement system can determine the composite mechanical properties of an entire EVPOME specimen, it cannot separate out only mechanical properties of the keratinocyte, the portion of the tissue device most of interest. It is important that not only the device as a whole is structurally sound prior to implantation, but also that the keratinocyte layers remain attached to the scaffold. A system such as the ultrasonic elasticity microscope [9-11] has much better resolution both perpendicular and parallel to the layers. The results in this study justify research with this more advanced equipment.

With further development, these results show that difference in mechanical properties of EVPOME can provide additional insight into pre-transplant integrity. Furthermore the results determine that elastic moduli can be one of several important criteria for evaluating EVPOME.

V. Conclusions

The results of this study demonstrate a substantial difference in mechanical properties between an unseeded scaffold and mature normally cultured EVPOME. They also show a difference in mechanical properties between normally cultured EVPOME and thermally stressed devices. However, thermal stressing does not affect the scaffold.

These promising results show that mechanical properties are affected by the growth of keratinocytes and differences in these properties can be measured. Not only is further study of EVPOME mechanical properties important, these results show that these measurements are possible. More detailed studies and more advanced measurements are therefore warranted. In addition, we are currently fitting non-linear elastic models to these results which will allow more quantitative characterization of the difference in non-linear elastic behavior.

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