The dermal equivalent (DE), a dermis substitute consisting of human skin fibroblasts growing into a three-dimensional collagen matrix, is extensively used in many applications: wound-healing response, pharmacological studies, skin grafting, fibroblast proliferation and migration, extracellular matrix remodeling, and efficacy of cosmetic products. The widespread growth of numerical modeling in biomechanical research has placed a heightened emphasis on accurate material property data for soft biological tissues, in particular for equivalent dermis which has not been so thoroughly investigated. Under unconfined compression loading, the effects of the strain rate, time culture, and cytoskeleton-disrupting agents are experimentally investigated. In order to model the observed mechanical behavior of the DE under the above conditions, the internal state variable approach is adopted for finite deformation viscoelasticity and the optimized material parameters are identified with respect to the stated thermodynamic restriction (i.e. positive viscous dissipation).

Keywords: Biomaterial; dermal equivalent; finite strain viscoelasticity; unconfined compression; cytoskeletal disrupting agents.

1. Introduction

The success in performing equivalent tissue and organ replacement is certainly due to the interdisciplinary approaches to tissue engineering. Indeed, today scientists

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with very diverse backgrounds such as biologists collaborate with mechanical engineers to develop tissue analogs that allow physicians to improve, maintain, and restore tissue function. Among other approaches, matrices containing specific cells have been used as tissue replacements.

Fibroblasts populated in three-dimensional (3D) lattices of collagen bind collagen fibrils and organize them into more dense and compact structures.\(^1\)–\(^5\) This cellular process is thought to be an \textit{in vitro} model of the collagen morphogenesis found in a wide variety of biological processes such as embryogenesis, organogenesis, tissue remodeling, and wound healing.\(^6\)–\(^9\)

The cell cytoskeleton is formed predominantly by three classes of filamentous biopolymers: the actin filaments, the microtubules, and the intermediate filaments.\(^10\) These biopolymers are interconnected by linker molecules such as \(\alpha\)-actin and actin-binding protein (ABP) to give form and structure to the cell.\(^11\),\(^12\) Structurally, the cross-linked actin filaments form an outer cortical membrane supported by an underlying microtubule network that emanates from the nuclear periphery.\(^13\)

Cytoskeleton-disrupting agents such as cytochalasin D, which is an inhibitor of actin polymerization, and nocodazol, which is known to disrupt microtubules, have been used in many studies.\(^10\),\(^14\)–\(^16\) Ingber \textit{et al.},\(^10\) for example, confirmed the importance of actin-containing microfilaments in shape-dependent growth control by culturing capillary endothelial cells in the presence of cytochalasin D (25–100 ng mL\(^{-1}\)); dose-dependent inhibition of cell spreading, nuclear extension, and DNA resulted. In contrast, induction of microtubule disassembly using nocodazol had little effect on cell or nuclear spreading and only partially inhibited DNA synthesis. Interestingly, the combination of nocodazol with a suboptimal dose of cytochalasin D (100 ng mL\(^{-1}\)) resulted in potent inhibition of both spreading and growth, suggesting that microtubules are redundant structural elements which can provide critical load-bearing functions when microfilaments are partially compromised.

It is well known that changes in the cytoskeletal structure can lead to alterations in the mechanical force balance.\(^17\) For example, the role of cytoskeletal elements in L929 fibroblast-like cell line mechanics, under indentation loading, was assessed through atomic force microscopy (AFM) measurements carried out on cells treated with cytochalasin D or nocodazol; it was found that cytochalasin D treatment reduced both elasticity (\(\sim 45\%\)) and cytoplasmic viscosity (\(\sim 65\%\)), whereas cells treated with nocodazol exhibited a marked increase of elasticity (\(\sim 100\%\)) and a slight increase in viscosity (\(\sim 15\%\)).\(^16\)

The dermal equivalent (DE), a dermis substitute consisting of human skin fibroblasts growing into a three-dimensional (3D) collagen matrix, is extensively used in many applications: wound-healing response, pharmacological studies, skin grafting, fibroblast proliferation and migration, extracellular matrix remodeling, and efficacy of cosmetic products. The mechanical properties of this tissue construct
are important, especially in load-bearing applications.\textsuperscript{18} The widespread growth of numerical modeling in biomechanical research has placed a heightened emphasis on accurate material property data for soft biological tissues, in particular for equivalent dermis which has not been so thoroughly investigated.

Most soft biological tissues simultaneously exhibit elastic and viscous material behavior, and are often subjected to large deformations.\textsuperscript{18–24} Under unconfined compression loading, the effects of the strain rate, time culture, and cytoskeleton-disrupting agents are experimentally investigated. In order to model the observed mechanical behavior of the DE under the above conditions, the internal state variable approach — previously used for soft biological tissue\textsuperscript{21,22} — is adopted for finite deformation viscoelasticity and the optimized material parameters are identified with respect to the stated thermodynamic restriction (i.e. nonnegative viscous dissipation).

2. Model Formulation

2.1. General considerations

Let $\mathbf{F}$ denote the deformation gradient at a material point $\mathbf{X}$ of a body at time $t$, relative to a reference configuration for which the corresponding right Cauchy–Green tensor is $\mathbf{C} = \mathbf{F}^T \mathbf{F}$. As an assumption, let the stress at a given material point depend on the deformation at that point at actual time $t$ considered, disregarding the contribution of an arbitrary small neighborhood of that material point during the previous deformation history.\textsuperscript{20} Then, the general constitutive law, valid for large deformation and containing the strain rate contribution as an explicit variable, is successfully applied to human knee ligaments and tendons,\textsuperscript{21} as summarized below.

Let the second Piola–Kirchhoff stress tensor $\mathbf{S}$ be expressed as

$$\mathbf{S} = \mathbf{S}(\mathbf{C}(t), \dot{\mathbf{C}}(t)),$$

where $\dot{\mathbf{C}}$ is the first time derivative of the right Green–Cauchy strain tensor.

For our purpose, the thermodynamic principles reduce to the satisfaction of the Clausius–Duhem inequality, which takes the following form for an isothermal process\textsuperscript{25,26}:

$$\left( \mathbf{S} - 2\rho_0 \frac{\partial W_e}{\partial \mathbf{C}} \right): \dot{\mathbf{C}} \geq 0, \quad \forall \mathbf{C}, \dot{\mathbf{C}}.$$  \hspace{1cm} (2)

The viscous contribution is taken into account by assuming the existence of viscous potential $W_v = W_v(\mathbf{C}, \dot{\mathbf{C}})$ accounting for energy dissipation such that

$$\mathbf{S} - 2\rho_0 \frac{\partial W_e}{\partial \mathbf{C}} = 2 \frac{\partial W_v}{\partial \dot{\mathbf{C}}},$$  \hspace{1cm} (3)
where \( W_e = W_e(C) \) is the specific elastic potential and \( \rho_0 \) is the density expressed relative to the reference configuration. The inequality (2) leads to the following:

\[
\frac{\partial W_v}{\partial \dot{C}} : \dot{C} \geq 0, \quad \forall \dot{C},
\]

which holds true when the potential \( W_v \) chosen is continuous, positive (or zero), and convex.

### 2.2. Elastic potential, viscous potential, and viscous dissipation

The specimens are considered as homogeneous, incompressible, and mechanically isotropic. In that case, and for our purpose, the elastic potential \( W_e \) is chosen as a function of the two invariants of the deformation tensor \((I_1, I_2)\), whereas the viscous potential \( W_v \) is expressed as a function of the two invariants of the deformation and the rate of deformation tensors \((J_2, J_7)\). For more details, the reader can consult Refs. 21, 22, and 25. The selected invariants are as follows:

\[
\begin{align*}
I_1 &= \text{tr } C, \quad I_2 = \frac{1}{2}((\text{tr } C)^2 - \text{tr}(C^2)) \\
J_2 &= \text{tr } \dot{C}^2, \quad J_7 = \text{tr}(C^2 \dot{C}^2)
\end{align*}
\]

Classically, the incompressibility constraint introduces an arbitrary hydrostatic pressure \( S = -pC^{-1} \) to the general constitutive law, which in our case is reduced to

\[
S = -pC^{-1} + 2\rho_0 \left[ \left( \frac{\partial W_e}{\partial I_1} + I_1 \frac{\partial W_e}{\partial I_2} \right) \mathbf{I} - \frac{\partial W_e}{\partial I_2} C \right] \\
+ 4 \frac{\partial W_v}{\partial J_2} \dot{C} + 2 \frac{\partial W_v}{\partial J_7} (C^2 \dot{C} + \dot{C}C^2).
\]

In order to obtain an admissible constitutive law, the elastic potential must verify some conditions such as stress-free initial state, convexity of the elastic potential, etc. The elastic potential, previously stated by Veronda and Westmann,\textsuperscript{24} and recently used by Pioletti and colleagues,\textsuperscript{21,22} is taken as

\[
W_e = a \exp(b(I_1 - 3)) - \frac{ab}{2} (I_2 - 3),
\]

where \( a \) and \( b \) are two elastic parameters obtained at the lowest strain rate during the unconfined compression experiments.

The viscous potential parameter, recently proposed by Pioletti \textit{et al.},\textsuperscript{21} is not sufficient to take into account the short-time memory behavior of our DE. Following the same physical arguments as in Pioletti and Rakotomanana\textsuperscript{22} (i.e. free
initial stress state, convexity of the viscous potential, etc.), we have constructed the following parameter for viscous potential:

\[
W_v = \frac{\eta_1}{4} J_2 \sqrt{(I_1 - 3)} \exp(\eta_2(I_1 - 3)) + \frac{\eta_1 \eta_2}{2} J_7(I_2 - 3),
\]

for which the thermodynamic restriction (4) becomes

\[
\Phi = (\eta_1 \sqrt{(I_1 - 3)} \exp(\eta_2(I_1 - 3)) \dot{\mathbf{C}} + \eta_1 \eta_2(I_2 - 3)(C^2 \dot{\mathbf{C}} + \dot{\mathbf{C}} C^2)) : \dot{\mathbf{C}} \geq 0,
\]

and the viscous material parameters \(\eta_1\) and \(\eta_2\) are identified at the highest strain rate with respect to the above inequality (9).

3. Experimental Setup

3.1. DE specimens

The process followed to achieve the reconstruction of a living DE involves a combination of the following states\(^1,^2\): (1) the fibroblasts were taken from a skin biopsy specimen in monolayer until the cells reached confluence or subconfluence. They were detached from the plastic substrate and suspended in culture medium consisting of 10% fetal calf serum, fungizone, penicillin, and streptomycin; (2) type I collagen was extracted from rat tail tendons and immersed in acetic acid solution; (3) the fibroblasts were added, in precise proportions, to a mixture of culture medium and collagen solution. The culture medium used was Earle’s modified Eagle’s medium (EMEM); and (4) the mixture polymerized rapidly when placed in an incubator at 37\(^\circ\)C, while the fibroblasts dispersed uniformly throughout the mixture. The collagen concentration was 5 mg · mL\(^{-1}\), whereas the fibroblast concentration was \(1.5 \times 10^5\) or \(15 \times 10^5\) cells per DE.

3.2. Contraction process of the DE

Reorganization of the extracellular matrix (ECM) plays a crucial role in many biological and pathophysiological processes, including tissue remodeling and wound healing. The ability of cultured fibroblasts to reorganize and contract 3D type I collagen gels is regarded as an \textit{in vitro} model for the wound-healing process and as a suitable way to study mechanical interactions. Harris \textit{et al.}\(^6\) were the first to propose that the reorganization of fibrils around cells dispersed in a reconstituted type I collagen gel is a direct consequence of cell traction forces, cytoplasmic forces transmitted by cell protrusions to the collagen fibrils. The mechanical forces generated by fibroblastic cells that lead to wound contraction are large enough to not only cause cosmetic scarring, but also cause major body deformation and loss of joint motion in cases where contraction persists after wound closure. It has been suggested that fibroblasts reorganize the collagen lattice as a result of either isometric tension applied to the collagen fibrils or traction forces generated by cell movement.\(^9,^{27,28}\)
We performed two experiments by measuring the evolution of the number of fibroblasts within the DE. One can see from Fig. 1 that during the first 24 hours, the relative cell concentration decreased rapidly; moreover, the decreasing rate was greater for the higher initial cell concentration. The second evolution step is the predominant one and corresponds to a long-time increase period of the relative concentration, leading to values greater than the initial cell concentration. In order to interpret, from a macroscopic point of view, the internal cell traction force simply in terms of the rate or extent of contraction, we also measured the evolution of the outer diameter of cell-populated collagen discs during the contraction process. In Fig. 2, the strain (\(\frac{D(t)}{D_0} - 1\)) is plotted against time (days), where \(D_0\) and \(D(t)\) are the initial and current outer diameters, respectively. We can distinguish two phases in the contraction process: the first one has a very high strain rate comparatively to the second phase, which is characterized by a long-time decrease period.

As reported by Moulin et al., the mechanism of tissue contraction during wound healing is not completely understood. Two main theories have been advanced to explain this complex process. The first theory suggests that fibroblast locomotion within the connective tissue induces wound contraction; in other words, it is the fibroblasts, as single units, that generate the forces necessary for contraction by reorganizing the ECM. The second theory, which was suggested by Gabbiani et al. who originally described myofibroblasts, posits that the highly specialized contractile fibroblasts found in granulating wounds are attached to one another by cell–cell connections and to the ECM. The mechanism of the second theory is different from that of the first theory in that forces generated by myofibroblasts are transmitted to other cells and surrounding connective tissue through their gap junctions and basement membrane. Thus, they are capable of contracting synchronously to generate the centripetal force of wound contraction.
3.3. **Unconfined compression experiments**

Unconfined compression tests were performed at fixed displacement rates $v_1$ and $v_{10}$, equal to 0.02 mm s$^{-1}$ and 0.2 mm s$^{-1}$, respectively. For these experiments, the initial cell number per DE was $2.10^5$ cells/DE. The final DE tissue dimensions were approximately 35 mm radial and 2 mm thick. The elastic parameters $a$ and $b$ were identified from unconfined compression experiment performed at a lower strain rate (0.01 s$^{-1}$), whereas the viscous parameters $\eta_1$ and $\eta_2$ were identified at the highest strain rate (0.1 s$^{-1}$).

4. **Results and Discussion**

Untreated DE tissues were first analyzed. The effect of strain rate was evident with respect to the stress level, as presented in Fig. 3. The effect of the time of culture was not notable for strains under 15%, irrespective of the imposed strain rate. Beyond 15%, at low strain rate, stress increased with the time of culture [Fig. 3(a)]; whereas at strain rate 10 times greater, the inverse effect was observed and the stress level was approximately 1.5 times greater on the 10th day [Fig. 3(b)]. What happened between day 10 and day 20 of culture? In fact, the DE has contracted, inducing the reorganization of the ECM and the redistribution of internal tractions traduced by the increasing collagen network density, leading to an increased elastic modulus observed at low strain rate. For higher strain rates, and beside 15% deformation, some contacts between cells and the ECM were broken, leading to a softening of the DE. We have previously shown in Fig. 1 that the number of cells per DE is more important at day 20 of culture and, consequently, the diminution of the elastic modulus is more pronounced at this time of culture.
Before proceeding with the analysis of our results, let us recount some important results stated at the cellular level and reported by Wu et al.\textsuperscript{16}:

- What is important to note is that the cell structure can be thought of as three elastic elements in series, representing (1) the outer cortical membrane formed by the actin filament, (2) the underlying microtubule network, and (3) the cell nucleus. The overall elasticity of the cell depends on all three components, but is governed by the actin filament network since it is much softer than the other components. It is not surprising that the cross-linking of the actin filament network via surface receptors enhanced the rigidity of the cell, while cell rigidity...
decreased in the presence of cytochalasin D. In contrast, the disruption of the microtubule network by nocodazol resulted in an increase in rigidity of the cell. Together, these observations support the conventional view that the elasticity of the cytoskeleton is primarily due to the actin filament network, which is supported by underlying microtubules acting as struts. Disruption of microtubules transfers the contractile load to the extracellular environment and, thus, augments the elasticity of the cell.

- The viscosity of the cytoplasm is commonly attributed to the kinetics of actin filament network rearrangement by a combination of depolymerization-repolymerization processes and by the inactivation and re-establishment of cross-links among the actin filaments. Depolymerization of the actin network by cytochalasin D reduced the viscosity of the cell, while disruption of the microtubules by nocodazol had minimal effects on the viscosity.

The parameters of the model are identified and given in Tables 1 and 2. Figure 4 shows that viscous dissipations $\Phi$ are positive such as imposed by Eq. (9). Broadly, the predictions of the model are faithful to the experimental results.

Under low strain rate, the DE is assumed to respond elastically (Fig. 5). For 10 days’ time of culture, one can observe in Fig. 5(a) the macroscopic response for each treatment. As expected, nocodazol has the best effect, increasing the elastic rigidity of the DE. The effect of the combined drugs is better, but not substantial. For 20 days’ time of culture [Fig. 5(b)], the same effects are observed, except that nocodazol leads to the best increase in elastic rigidity whereas the combined treatment has less effect.

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<tr>
<th>Table 1. Identified viscoelastic parameters for 10 days of time of culture and various treatments.</th>
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<th>Table 2. Identified viscoelastic parameters for 20 days of time of culture and various treatments.</th>
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Fig. 4. Simulated viscous dissipation $\Phi$ versus time (days) at (a) 10 days of time of culture and (b) 20 days of time of culture.

Under higher strain rate (10 times more or 0.1/s), the viscous contribution is mobilized and, as a consequence, the levels of stresses reached are much larger (Fig. 6). Compared to the control DE after 10 days of time of culture, one notes in Fig. 6(a) that both cytochalasin D and nocodazol drugs lead separately to a diminution of the tangent viscoelastic modulus, the reverse from what was obtained at low strain rate; whereas the combined effect of both drugs affects in a substantial way the tangent viscoelastic modulus of the DE. For the DE under 20 days of time of culture [Fig. 6(b)], the viscoelastic response is appreciably the same regardless of whether the type of treatment is combined or not.
It is clear that both the strain rate and the time of culture are important. Analysis of the identified parameters given in Tables 1 and 2 is made difficult by their respective couplings. However, there is probably a bond between the macroscopic response to unconfined compression loading and the well-known mechanisms stated at the cellular level affected by anticytoskeletal treatments. This bond is difficult to comprehend and appreciate because of the many and complex interactions (cell–cell, ECM–cell, etc.) that occur at the cellular level.

The widespread growth of numerical modeling in biomechanical research has placed a heightened emphasis on accurate material property data for soft biological tissues, in particular for equivalent dermis which has not been so thoroughly investigated. Our hope is that the proposed model will lead to the design of
new therapeutic strategies for wound contraction management, and thus to an improved understanding of the ability of cells to macroscopically reorganize their ECM through cell traction in response to anticytoskeletal treatments. The authors believe strongly that this work brings new elements, will open new perspectives, and provides a basis for investigating various aspects with reconstructed tissues submitted to complex loadings and altered by anticytoskeletal drugs.

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