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Rate process analysis of thermal damage in cartilage

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Abstract

Cartilage laser thermoforming (CLT) is a new surgical procedure that allows *in situ* treatment of deformities in the head and neck with less morbidity than traditional approaches. While some animal and human studies have shown promising results, the clinical feasibility of CLT depends on preservation of chondrocyte viability, which has not been extensively studied. The present paper characterizes cellular damage due to heat in rabbit nasal cartilage. Damage was modelled as a first order rate process for which two experimentally derived coefficients, $A = 1.2 \times 10^{70} \text{ s}^{-1}$ and $E_a = 4.5 \times 10^5 \text{ J mole}^{-1}$, were determined by quantifying the decrease in concentration of healthy chondrocytes in tissue samples as a function of exposure time to constant-temperature water baths. After immersion, chondrocytes were enzymatically isolated from the matrix and stained with a two-component fluorescent dye. The dye binds nuclear DNA differentially depending upon chondrocyte viability. A flow cytometer was used to detect differential cell fluorescence to determine the percentage of live and dead cells in each sample. As a result, a damage kinetic model was obtained that can be used to predict the onset, extent and severity of cellular injury to thermal exposure.

1. Introduction

Cartilage laser thermoforming (CLT), also known as laser reshaping, is a new surgical procedure that allows *in situ* treatment of deformities in the head and neck with less morbidity than traditional approaches (Sobol *et al* 2000a). During laser irradiation, cartilage softens and can be stretched and shaped into new stable configurations. Clinically reshaped cartilage can be used to reconstruct the framework of structures within the head and neck, such as ear, nose, larynx and trachea. Since CLT can be performed using minimally invasive techniques, it has the potential to alter radically the practice of aesthetic and reconstructive cranio-maxillo-facial surgery. While some animal and human studies have shown promising results (Sobol *et al* 2000b, Sobol 1995), the clinical feasibility of CLT depends on the preservation of chondrocyte viability after thermal exposure, which has not been extensively studied. Frenz *et al* (1999)

employed confocal microscopy in combination with a live/dead staining viability test to assess laser-induced damage in bovine hyaline cartilage. Their results showed that conventional histology and light microscopy greatly underestimates the extent of thermal damage. Lu *et al* (2001) report similar results in their study of chondrocyte viability following exposure to radiofrequency energy. Zuger *et al* (2001) also used a staining test based on cell viability to evaluate thermal damage of chondrocytes in bovine articular cartilage. Though not rigorously defined, they report a threshold temperature of 54 °C for thermal damage of chondrocytes in 5 min water baths. At a temperature of 52 °C most cells were still viable, but at 54 °C some dead cells became visible and with higher temperatures the majority of chondrocytes was necrotic or had compromised membranes. Even though these studies successfully quantify the extent of thermal damage, they do not provide sufficient information on the time and temperature dependency of injury. Mathematical descriptions of the cellular damage rate process are useful for predicting injury in response to thermal exposures. The rate process essentially describes an alteration of tissue molecules from their native state to a dysfunctional damage state (Diller and Pearce 1999). Unfortunately, there are only a few damage processes that have been characterized (Pearce and Thomsen 1995). Some of the methods used for characterization are quantitative histologic markers (e.g., cell shrinkage and colour change), changes in birefringence (Thomsen *et al* 1989, Pearce *et al* 1993) and temporal changes in the light scattering coefficient (Agah *et al* 1996). In the present study, we employed flow cytometry (also known as fluorescence activated cell sorting (FACS)) to assess chondrocytes viability within rabbit cartilage following immersion in constant-temperature water baths. Exposed specimens were enzymatically digested to isolate individual chondrocytes and then stained by a fluorescent dye system that stains live and dead cells differently, based on plasma membrane integrity. Stained cells (in suspension) were then analysed with a flow cytometer that excites cells with an argon laser beam ($\lambda = 488$ nm). In response to the excitation, viable cells emit light (green fluorescence) at a peak wavelength different from non-viable cells (red fluorescence). The flow cytometer distinguishes live and dead cells based on this differential emission using different detectors optimized for specific spectral bandwidths. Additional detectors are used to measure the forward and side scattering of laser light which provide information on cell size, granularity and post-irradiation debris content of the sample. Ultimately, thermal damage kinetics coefficients were obtained from the analysis of cell viability versus exposure time plots at each temperature.

The purpose of this study was to characterize the thermal damage process in cartilage and to derive a representative first-order kinetic model which can be used to predict cell injury in response to complex temperature profiles generated by different types of heating sources such as focused ultrasound, radiofrequency, microwave and laser radiation.

2. Theory of rate process analysis

Thermal damage is typically quantified using a single parameter, Ω , which is calculated from the Arrhenius integral formulation as

$$\Omega(\tau) = A \int_0^\tau e^{-\frac{E_a}{RT(t)}} dt \quad (1)$$

where A (1/s) is a pre-exponential constant, τ (s) the total heating time, E_a (J mole⁻¹) the activation energy of the transformation, R (J mole⁻¹ K⁻¹) the universal gas constant and T (K) the absolute temperature (Pearce and Thomsen 1995). E_a corresponds to the amount of energy needed to start the transformation process and is an important descriptive factor of the exponential temperature dependence of the rate process. A more useful form of equation (1)

may be obtained by giving a physical significance to Ω . For thermal injury, Ω is described as the logarithm of the ratio of the initial concentration of healthy cells, C_0 , to the remaining fraction of healthy cells, C_τ , at time τ :

$$\Omega(\tau) = \ln \left\{ \frac{C_0}{C_\tau} \right\} = A \int_0^\tau e^{-\frac{E_a}{RT(t)}} dt. \quad (2)$$

This form of the damage integral has the advantage that it can be coupled to computer models of the temperature distribution $T(x, y, z, t)$ in cartilage during laser irradiation (Díaz *et al* 2001) to predict the onset, extent and severity of thermal injury.

3. Materials and methods

3.1. Tissue preparation

New Zealand white rabbit crania were obtained from a local abattoir. Fresh cartilage specimens were extracted from the nasal fossae following the removal of skin and fracture of the bony nasal dorsum. After the septum was removed, the thin mucoperichondrial layer was stripped from both sides of the specimen. Next, irregular edges of the specimen were trimmed to obtain rectangular slabs measuring approximately 25 mm $\times$ 10 mm and thickness varying from 0.2 to 0.8 mm. Each slab was then cut into eight uniform pieces. Four pieces underwent thermal exposure in a hot saline solution bath while the other four were kept as controls and maintained in solution at ambient temperature. Sections of cartilage from different regions of the septum comprised each group (experiment and control) to account for cell density variability within the specimen (Wong *et al* 2001).

3.2. Thermal exposure

Samples of rabbit nasal cartilage were exposed to constant temperature saline solution (0.9% NaCl, Phoenix Pharmaceuticals, St Joseph, MO) baths for different immersion times (general purpose water bath M280, Precision Scientific, Winchester, VA). A minimum of three experiments was made at each time–temperature data point. Bath temperatures ranged between 48 and 62 °C (± 0.2 °C) and exposure times varied between 4 and 160 s. After immersion in hot solution, samples were immediately transferred to a container with saline solution at ambient temperature to minimize the progress of the damage process. Specimens were then placed in tissue culture plates for enzymatic digestion.

In effect, no real thermal exposure occurs at constant temperature since the cartilage temperature has to rise from about 20 °C to the water bath temperature. However, experiments for which exposure time (τ) is long compared to the time constant (t_c) approximately satisfies this assumption. t_c represents the time required for the temperature difference driving force, $\Delta T = T_{\text{waterbath}} - T_{\text{cartilage}}$, to fall to e^{-1} of its initial value (ΔT_0). That is, t_c represents the time required for the total change in the temperature of the cartilage (ΔT_0) to become 63.2% complete. In a complementary experiment to the cell viability assessment, t_c was measured by embedding a thermocouple (Teflon insulated type E, 0.005 inch wire diameter, 5TC-TT-E-36–36 with cold junction compensator, M60/1290, MCJ Series, Omega Engineering Inc., Stamford, CT) into the thick region ($h \approx 0.8$ mm) of a cartilage specimen (not used in the viability assessment) during immersion in hot saline and was found to range between 1 and 2 s. This time range is short compared to the experimental exposure time range 4–160 s, which validates the assumption of a constant temperature immersion. Furthermore, since t_c is proportional to the square of h , thinner regions of the cartilage rapidly reach thermal equilibrium ($t_c < 1$ s).

Figure 1. Forward (FSC) versus side (SSC) light scatter dot plots (linear scale) of cartilage specimens exposed for 6 s in constant-temperature baths; (a) non-heated specimen and (b) 60 °C.

3.3. Chondrocyte isolation and staining

Cartilage specimens were digested using a three-step enzymatic process previously described (Chao *et al* 2000). After digestion, isolated chondrocytes were transferred to centrifuge tubes and pelleted by spinning at 1300 rpm for 5 min. The two-colour viability dye solution was prepared using SYTO® 10 as the green fluorescent cyanine dye and DEAD Red™ (both from Molecular Probes, Eugene, OR) as the red fluorescent ethidium homodimer-2 dye. 1.0 µl SYTO® 10 and 1.0 µl DEAD Red™ were used per 1.0 ml of Hanks buffered saline solution (HBSS) (Gibco, Grand Island, NY). After removing the supernatant, pelleted chondrocytes from each specimen were re-suspended in 200 µl of the two-component dye solution and incubated in total darkness at room temperature for 15 min. Samples were re-pelleted. The dye was removed and 200 µl of 4% electron microscopy grade glutaraldehyde was added to the solution. Samples were then diluted with HBSS to 5 ml in preparation for flow cytometric analysis.

3.4. Live/dead chondrocyte quantification

Flow cytometry (FACScan, Becton–Dickinson, Franklin Lakes, NJ) was performed as described in (Rasouli *et al* 2002) and is summarized here. The instrument is equipped with a 35 mW argon ion laser ($\lambda = 488$ nm) and scattered laser light is collected using a series of photo multiplier tube photodiodes. For the two-colour viability assay, filter configuration included a DF530/30 barrier filter for green fluorescence (FL1), and an LP 650 for red fluorescence (FL3). Forward and side scattered laser light was also recorded. The cytometer analysed 60 µl of re-suspended chondrocyte solution per minute. A population gate was established using forward and side scatter profiles to include only intact cells and exclude debris, as shown in figure 1. All events (including debris) were recorded, but analysis was terminated when 10 000 population-gated events were detected. Light scattering and fluorescence data were analysed using CellQuest acquisition and analysis software (Becton–Dickinson) operating on a personal computer.

Figure 2. Red (FL3) versus green fluorescence (FL1) density contour plots (log scale, smoothed once, 1% display threshold) of cartilage specimens exposed for 6 s in different constant-temperature water baths; (a) non-heated specimen, (b) 58 °C, (c) 60 °C and (d) 62 °C.

Density contour plots were generated for each sample by plotting all events in terms of FL3 intensity (red fluorescence, x -axis) and FL1 intensity (green fluorescence, y -axis), as illustrated in figure 2. The plot was divided into four quadrants: live cells appeared in the upper left (high green, low red emission), dying cells in the upper right (higher red emission), dead cells in the lower right (high red, low green emission), and unstained tissue and cellular debris in the lower left. The percentage of population-gated events in each quadrant was obtained and used to estimate chondrocyte viability. The percentage of viable cells in each specimen was then normalized relative to the viability of its respective non-heated control.

Flow cytometric data can be displayed using either a linear or a logarithmic scale. When the control is set at linear, the voltage measured is directly proportional to the fluorescence intensity. The use of a logarithmic scale is required when there is a broad range of fluorescence, as this can then be compressed. Linear scaling is used when there is not such a broad range of signals. Most analogue-to-digital converters (ADC) in analytical cytometers are 10 bit, i.e.,

Figure 3. Cell viability (%) in cartilage specimens following immersion in constant-temperature water baths for 8 s.

they divide data into 2^{10} , or 1024, channels. On a logarithmic scale, the x -axis is still divided into 1024 channels but is displayed as a 4-log decade scale.

4. Results

Forward (proportional to cell diameter) versus side (proportional to the quantity of granular structures within the cell) light scatter plots were used to establish a population gate (subset) to include only intact cells and exclude debris from each solution sample. When cells die or their membranes become compromised in some way, they exhibit a change in light scatter characteristics, which is identified as a reduction in the forward scatter signal, and an initial increase in the side scatter signal. Both are indicative of cell morphological changes such as shrinkage, shape change, cytoplasm and/or nuclear condensation, and nuclear fragmentation. These variations in the light scatter signal are clearly observed in figure 1 where the scatter plot of a non-heated specimen, figure 1(a), is compared to that of a specimen heated for 6 s at 60 °C, figure 1(b). In both cases, cellular debris at the bottom-left corner is excluded by the gate demarcated by the black-line polygon.

Typical viability density contour plots expressing red (non-viable) versus green (viable) fluorescence intensity for each event are shown in figure 2. The plots include only the population-gated events. As illustrated in figure 2(a), non-heated control samples have a large proportion of live chondrocytes, localized in the upper left quadrant of the plot (high green, low red emission). In contrast, only thermally treated specimens showed an increase in the population of dead chondrocytes (lower-right quadrant). As the temperature of the water bath increased, fewer chondrocytes are observed in the upper left (live) quadrant and there is an increased count in the lower right (dead), as shown in figures 2(b)–(d).

The characteristic behaviour of a first-order kinetic damage model is that, below a threshold temperature, the rate of damage accumulation is negligible and increases steeply when this value is exceeded. Therefore, initial efforts were focused on identifying the temperature range in which damage accumulation increased precipitously. Cartilage specimens were immersed into hot water baths at different temperatures. The exposure time in all cases was 8 s. Figure 3 shows the percentage of cell viability (concentration of healthy cells, C_T) as a function of temperature. A sharp decrease in cell viability was detected near 56 °C.

Figure 4. Thermal damage (Ω) in cartilage specimens as a function of exposure time (τ) to constant-temperature water baths.

Table 1. Cell viability (%) and standard deviation as a function of exposure time (τ) at different temperatures.

τ (s)	Temperature (°C)						
	62	60	58	56	53	52	51
4	3.0 (3.2)	25.7 (11.5)	62.0 (7.2)	86.0 (7.3)		98.4 (3.1)	
6	0.9 (1.5)	5.3 (0.7)	42.1 (18.7)	69.6 (6.5)			
8	0.1 (0.1)	1.3 (1.1)	33.9 (7.8)	55.9 (8.9)			
10				65.2 (14.7)		98.4 (0.6)	100.2 (2.6)
15				27.5 (6.5)			
18					91.7 (5.0)		
20						97.5 (0.8)	100.2 (0.8)
24				32.2 (8.4)	80.8 (6.1)		
40						86.2 (3.4)	93.6 (4.1)
60							83.2 (5.9)
80						50.2 (16.2)	86.2
100							69.5 (6.5)
120							63.1 (14.8)
140							58.6 (12.2)
160							86.5 (6.4)

Having identified the temperature range of interest, cell viability quantification was performed keeping the temperature parameter constant while varying the exposure time. Table 1 lists the percentage of cell viability and standard deviation for constant temperature exposures as a function of time. These data were used in combination with equation (2) to produce figure 4 which shows the calculated Ω versus τ for temperatures 53–62 °C. An advantage of this type of plot is that the experimental data can be fit with a linear regression. In addition, since by definition no damage is expected at $\tau = 0$, the origin is included as an extra data point in the calculation of the slope of each individual line. Figure 4 was then used to calculate the threshold time at each temperature, defined as the τ for which $\Omega = 1$ or $C_\tau = 1/e$ of C_0 . Similarly, the threshold temperature is defined as the temperature at which Ω is 1 for a given exposure. These threshold values are shown in figure 5 for each experimental temperature.

Under isothermal conditions and when the damage parameter $\Omega = 1$, equation (2) simplifies to the logarithmic form of the Arrhenius equation:

Figure 5. Threshold temperatures (temperature T at which $\Omega = 1$) as a function of exposure time (τ).

Figure 6. Best linear fit of $1/T$ versus the natural logarithm of the threshold time (exposure time τ at which $\Omega = 1$).

$$\ln(\tau) = \frac{E_a}{R} \frac{1}{T} - \ln(A). \quad (3)$$

Through inspection of equation (3) and rearranging figure 5 into a $\ln(t)$ versus $1/T$ plot, the thermal damage kinetic coefficients $A = 1.2 \times 10^{70} \text{ s}^{-1}$ and $E_a = 4.5 \times 10^5 \text{ J mole}^{-1}$ were estimated from the intercept and slope, respectively, of the best-fit line to the calculated threshold values, shown in figure 6. Finally, figure 7 illustrates a comparison between the experimental results listed in table 1 and predictions obtained by substituting the kinetic coefficients into equation (1). The correlation coefficient between experimental data and kinetic model was obtained as a measure of the quality of the fit. It varies between 0.9 and 1.0 in this temperature range, indicating that the model fits the observed data well.

5. Discussion

The present work quantified cellular thermal damage in rabbit cartilage and used these results to develop an analytical model for estimating thermal injury. Classically, thermal injury in living tissues has been studied by focusing on either cell viability or protein denaturation in the matrix, with the analysis of damage primarily determined by the subjective assessment of structural changes using microscopy. We focused on cell viability, although the same thermal events that lead to altered cell structure and function likely produce similar changes in matrix

Figure 7. Cell viability (%) as a function of exposure time (τ) at different temperatures. Comparison between experiments and kinetic model (—).

proteins. The differential fluorescent staining of cells combined with flow cytometry allowed the rapid determination of viability in over 10 000 cells per sample. Fluorescent agents used in flow cytometry can target enzymes responsible for specific cell functions such as replication and apoptosis, or structural changes such as membrane integrity. We measured the reduction in the fraction of viable chondrocytes with increasing exposure time as a function of water bath temperature and used this information to develop a first order rate process model of chondrocyte viability in response to heat.

Despite the technical advantages of flow cytometry over classical microscopy methods to quantify cell viability, our measurements resulted in data points with relatively large variance likely due to variations in specimen thickness, which affects the temperature history experienced by the cells, and the reduced number of measurements made at each experimental condition data pair (exposure time, temperature). In addition, one can argue the validity of using equation (3) to derive the kinetic coefficients in this study, since the fundamental assumption that thermal injury takes place under isothermal conditions is only partially achieved. The cartilage specimens experience a temperature excursion from ambient to the water bath temperature before reaching thermal equilibrium. However, as discussed in section 3.2, this temperature transition period is in most cases short, compared to the steady state condition that follows and is thought to be of little effect on the damage measurement since the specimen reaches a temperature capable of producing significant thermal injury (>50 °C) only for a fraction of this transition period. For this reason, we presume that the overall trend of the experimental data set represents the essential behaviour of the thermal damage process in cartilage. Nevertheless, further experiments are required to complete a thorough description of the damage rate process.

Kinetic models of thermal damage can be used to predict the occurrence of cellular injury in tissue during exposures that involve various types of heat sources, allowing determination of appropriate therapy parameters to avoid irreversible injury. For tissue damage processes studied to date, the pre-exponential coefficient A varies from about 10^{40} to 10^{105} s^{-1} , whereas E_a ranges from 10^5 to 10^6 $J\ mole^{-1}$ (Pearce and Thomsen 1995). The rate coefficients obtained in this study are within such ranges.

Our results have significant relevance to the use of tissue heating sources for clinical applications such as orthopaedic, arthroscopy, ear, nose and throat surgery. However, in most cases the heating source is used for ablation and coagulation of soft tissue rather than its preservation. In particular, cartilage reshaping depends on preservation of chondrocyte

viability, which has not been extensively studied. Helidonis *et al* first proposed laser cartilage reshaping in 1993 (Helidonis *et al* 1993). However, to date, little is known about thermal damage processes in cartilage and only the few studies reported address to a limited extent the issue of determining appropriate laser dosimetry to attain effective shape change (Sviridov *et al* 1998, Jones *et al* 2001). Furthermore, these reports indicate that temperatures of 60–70 °C are necessary to thermoform cartilage. Our preliminary analysis of thermal damage indicates that within this temperature range, chondrocyte viability is very much compromised at exposure times longer than about 5 s.

6. Conclusion

The present work describes the formulation of a mathematical model of thermal damage in cartilage. Damage was represented as a first order rate process for which two experimentally derived coefficients were determined by quantifying the decrease in concentration of healthy cells in tissue samples as a function of exposure time to constant-temperature water baths. After heat exposure, flow cytometry was used to determine the number of live and dead cells in each cartilage sample. Good agreement between model and experimental data was found using the rate coefficients $A = 1.2 \times 10^{70} \text{ s}^{-1}$ and $E_a = 4.5 \times 10^5 \text{ J mole}^{-1}$. This kinetic model can be coupled to computer simulations of the thermal response of cartilage to laser irradiation to make predictions of the onset, extent and severity of thermal injury, which are necessary for the development of dosimetry guidelines for medical applications of lasers or other thermal sources.

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