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Boundary conditions during biaxial testing of planar connective tissues. Part 1: Dynamic Behavior

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Current mechanical testing methods used to determine the biaxial properties of planar connective tissues may lead to artifactual observations of material behavior. The method of sample gripping affects the constraint on the extracellular fibers at the bounds of the sample. This applied constraint not only affects how the load is transferred to the sample, but also how the load is transmitted throughout the rest of the material – thereby influencing the resulting mechanical behavior of the tissue. In this study, we compared the dynamic biaxial mechanical response of pericardial tissue samples under two different gripping methods: (i) the common method of suturing sample edges and (ii) a new biaxial clamping method. Tissue samples were repeatedly testing using both gripping methods under the same conditions. The tissue samples appeared to be stiffer and less extensible when mechanically tested with clamped sample edges, as opposed to when tested with sutured sample edges. Thus, the influence of the sample boundaries affected the response of the material – precisely the situation to be avoided for reliable material testing. This casts doubt on whether any *in vitro* mechanical testing method can used to determine the ''real'' properties of the tissue since the boundary conditions of the tissue *in situ* are presently unknown.

Introduction

Due to the inherent difficulties associated with the measurement of tissue mechanical behavior *in vivo*, mechanical testing of biological materials *in vitro* is typically preferred. The underlying assumption is that the method of gripping the sample *in vitro* does not influence the observed behavior. Nonetheless, for *in vitro* testing of soft planar connective tissues, the continuous fiber reinforcement *in vivo* is inevitably cut for the preparation of test samples. Some method of constraint must then be applied at the sample boundaries in order to transfer load from the testing apparatus to the material. The resulting behavior is then a function of both (i) the material and (ii) the boundary conditions applied to the test sample. Any measured properties could be to some extent artifactual, with properties reflecting the gripping method.

The notion that boundary conditions affect the observed mechanical behavior of engineering material test samples was first postulated by the French mathematician and engineer Adhémar Barré de Saint-Venant (1797–1886). Saint-Venant's principle essentially states that stress distribution may be assumed to be independent of the actual mode of load application – except in the immediate vicinity of where the load is applied [1, 2]. In other words, gripping imposes local stresses at the grip–sample interface and the influence of these local stresses becomes negligible at some distance away from the grip. Application of this principle has led to the widespread use of long slender samples in uniaxial material testing. For biaxial testing of planar connective tissue materials, however, there has been no analogous work; indeed the bulk of biaxial tissue testing is based on the first reported technique [3]. In this method, the edges of the square biaxial test sample are held by numerous suture lines which extend from the actuators. With this technique, it is probable that only the discrete groups of fibers within the vicinity of the suture attachment point are actually loaded. This could result in a discontinuous load transfer to the underlying fibrous network. It also follows that the number and position of suture attachments may also affect the observed mechanical behavior of the tissue sample. This method has not been standardized and reported testing parameters vary widely between studies (Table I).

Loosely based on Saint-Venant's principle, biaxial strain is typically measured at the center of the test

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TABLE I Reported biaxial mechanical testing parameters

	Mean	Range	n	Present study
Sample Length (mm)	31	5–50	26	22
Sutures per side	6	4–10	25	5
Suture spacing (mm)	4	0.8–12.5	25	4.4
Suture size (mode 5–0)		2–0 to 6–0	15	2–0

n is the number of studies that reported the specific parameter.

The spacing between suture attachment points may affect the constraint on the tissue sample directly leading to altered material behavior. This parameter varies the most among reported studies, complicating comparisons of measured material properties between studies. Data presented here were compiled from 30 previous studies published between 1974–1999. The testing parameters of the present study are shown for comparison.

sample. This region is presumed to experience a uniform strain distribution and for that reason to be free of the suturing edge effects. Two previous studies have assessed the uniformity of the central strain distribution. Unfortunately, both of these studies only calculated the distribution. Humphrey *et al*. [4] reported that the normal strains were uniform within the central 4% of the specimen area. These strains were determined from the bilinear interpolation functions enforced on this region, and therefore, do not necessarily represent the true variation of material strain. Nielson *et al*. [5] a performed finite element analysis of a sutured biaxial test sample to find the region of uniform strain. Strains were found to be uniform within the central 25% of the sample area for a homogeneous, isotropic material. Although the authors indicated that the uniform strain region would be smaller for anisotropic and/or heterogeneous materials, their assumptions are generally too restrictive to model the behavior of biological materials. In a strict interpretation of the Saint-Venant principle, the center of sample can be deemed free of edge effects only if the stress distribution within this region is uniform. Since stress is not a measurable quantity, the uniformity of strain distribution has been quantified and the corresponding stress distribution is assumed to vary in a similar manner. For homogeneous, elastic materials this approach would be applicable; however, it might lead to erroneous conclusions if applied to more complex connective tissue materials. Connective tissues are heterogeneous and typically display anisotropic, nonlinear viscoelastic properties, implying that the stress and strain fields would not necessarily vary similarly. Stubbs [6] described another method to deal with Saint-Venant edge-related effects during biaxial mechanical testing. The procedure was to quantify the magnitude of the edge artifacts and then correct the observed behavior. The analysis, however, requires the mechanical properties of the material a priori – thus not an applicable approach if the ''real'' properties of the material have yet to be determined.

The use of clamps, instead of sutures, should ensure a more continuous load transfer across the grip-sample interface since all the fibers at the bounds of the sample would be constrained. We hypothesized that this added constraint would result in an apparently stiffer and less extensible material compared to the common method of suturing biaxial sample edges. The purpose of the first part of this study was to assess the apparent changes in the observed mechanical behavior between these two gripping methodologies under dynamic loading conditions.

Methods

Materials

Eight intact bovine pericardia were obtained from 1–2-year-old cattle immediately after slaughter. The left ventral surface was stripped of any adherent fat and the base-to-apex direction was marked with two reference sutures: one placed at the aortic root and the other at the apex of the heart. A portion of the parietal pericardium immediately over the left ventrical was excized. Biaxial samples were cut into squares, 22 mm on edge, and aligned in the base-to-apex and circumferential directions. Sample thickness was measured using a non-rotating Mitutoyo thickness gauge (Mitutoyo Corporation, Tokyo, Japan) [7] and was defined as the average from four random test locations.

Sample gripping

Two gripping arrangements were used for each sample: clamped and sutured edges. The order of the gripping methods was randomized for each sample as not to bias a particular gripping method. Care was taken to ensure that the same region on the sample edge was used to grip the sample.

The suturing of sample edges was accomplished with a continuous strand of 2–0 silk suture material (Davis & Geck, Wayne, NJ) with five attachment points per side. Suture strands were wrapped around pulleys on a central shaft which was attached to the actuators. Pulleys were also allowed to freely rotate and translate along the shaft (Fig. 1). The clamping arrangement consisted of four brass clamps lined with silicon carbide water-proof sandpaper (220 grit). The edge of each clamp was angled outward 45° to the grip face in order to maximize the area

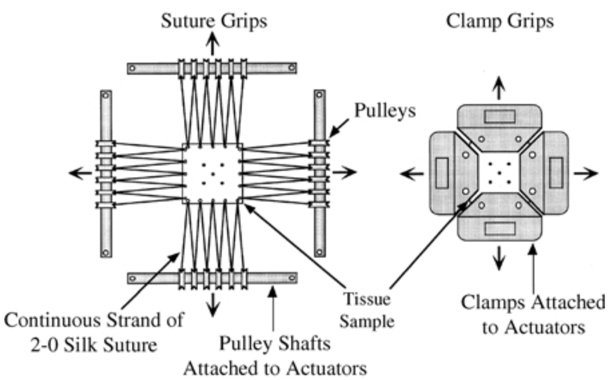


Figure 1 This figure displays the two different gripping methods used in this study. The sutured samples (left) were held in place with a continuous strand of 2–0 silk suture material with five attachment points per side. Each suture strand end was tied to an additional pulley with a slip knot. The clamped samples (right) were constrained by brass clamps lined with silicon carbide waterproof sandpaper. The edge of each clamp was angled outward at 45° to the grip face to maximize the constrained area of tissue. A small, but required, gap between the clamps (~ 0.8 mm) was present on mounting.

of tissue contained within the clamps. A small, but required gap of approximately 0.8 mm existed between the clamps at the time of mounting (zero load condition) (Fig. 1).

Mechanical testing

Biaxial testing was conducted using an MTS (Minneapolis, MN) servo-hydraulic biaxial test device equipped with phased stroke/load waveform synthesis [8]. Samples were preloaded to 0.5 g/axis which was then defined as the zero strain state. Materials were cyclically deformed under stroke control using a haversine waveform at 1 Hz for 30 cycles. Actuator displacements were set to achieve peak equibiaxial loads of 200 g/axis [8]. All tests were conducted in a temperature-controled fluid bath with Hanks' physiologic solution maintained at 37 °C.

Generalized large deformation biaxial strain fields were quantified, at the sample center, through the video tracking of surface deformation markers. Five particles of sand (nominal diameter of 300 μm) were arranged in a square array (approximately 10 mm on edge) with one central marker that corresponded to the center of the sample. Markers were attached to the sample surface with a small amount of cyanoacrylate glue (Elmer's Productions Canada Inc., Brampton, ON). Video images were collected with an overhead CCD camera (Cohu 5000, San Diego, CA), an 8-bit grayscale video acquisition board (Scion Corporation LG-3, Frederick, MD), and image analysis software (NIH Image 1.61, US National Institutes of Health) on a Macintosh computer (PowerMac 7200, Apple Computer Inc., Cupertino, CA). Resulting load behavior was collected using a 12-bit A/D acquisition board (National Instruments NB-MIO-16, Austin, TX) and LabVIEW 2.2.1 data acquisition software (National Instruments, Austin, TX) on another Macintosh computer (Centris 650, Apple Computer Inc., Cupertino, CA). Both analog data acquisition and video capturing were collected at 30 Hz and synchronized to the actuator displacement waveforms with a trigger pulse generated by the MTS console to collect only the last three deformation cycles.

Data analysis

Particle position histories were determined by subsequently analyzing captured video images with image analysis software (NIH Image 1.61, US National Institutes of Health). The position of a particle was defined as the geometric center of a ''best fit'' ellipse placed around the particle. The deformation gradient was determined from the surface marker displacement histories using an isoparametric finite element procedure [9, 10]. Bilinear interpolation functions of a 4-noded isoparametric element were used to calculate the deformation gradient at the fifth node which corresponded to the center of the sample. The associated Green strain and Kirchhoff stress tensors were then determined from the deformation gradient [11, 12]. This procedure has been well described by others [13–15]. Calculation of the deformation gradient and corresponding stress and strain tensors were performed using mathematical analysis software (Mathematica 3.0, Wolfram Research, Champaign, IL). The data from the last three deformation cycles were averaged and the following parameters were determined from this cycle: (i) maximum normal stress and strain, (ii) tangent modulus, (iii) maximum shear stress and strain, and (iv) maximum sample rigid body rotation. The maximum stress and strain components were defined as the difference between the values measured at the valley and peak in the average deformation cycle. The three-cycle-averaged stress–strain curves were also interpolated at incremental levels of stress (5 kPa) to calculate tangent moduli. The tangent modulus was calculated at each incremental stress level and was defined as the slope of the average stress–strain curve calculated from a second-order central-difference numerical derivative. Since the central portion of the sample is not directly constrained, this region free is to rotate during the deformation process – often referred to as the rigid body rotatation. The rigid body rotation was calculated from a polar decomposition of the deformation gradient [16] and was determined in the same manner as described by Waldman and Lee [17]. Briefly, rotational data was curve-fitted to a general sinusoidal waveform and the maximum rotation was defined as twice the calculated amplitude. Note that the measured shear behavior and rigid body rotation are separate phenomena since the shear strains were corrected for rigid body rotation during the analysis.

Statistics

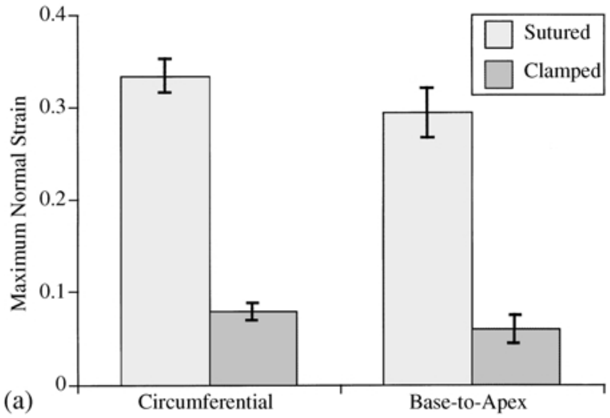
All results were analyzed using repeated measures ANOVA statistics (SuperANOVA and StatView 4.1, SAS Institute, San Francisco, CA) with the ''between subject'' effect assessed through the use of Fisher's Protected Least Significant Differences. The minimum level of significance was associated with p-values less than 0.05.

Results

The measured mechanical properties of bovine pericardial tissue samples were dramatically affected by the gripping technique. All samples appeared to become less extensible (Fig. 2(a)) and stiffer (Fig. 2(b)) in both anatomical directions when clamped (as opposed to being sutured). Comparison of the average stress–strain curves revealed that the observed differences in mechanical behavior were present throughout the entire deformation cycle (Fig. 3). The gripping method also affected the observed shearing behavior of tissue samples. The suturing of sample edges produced greater shearing stresses and strains (as opposed to the clamping method, Table II). The measured rigid rotation of the sample during deformation was similarly affected, with a greater amount of rotation observed under suturing (Table II).

Discussion

This study demonstrated that the observed mechanical behavior of biaxial bovine pericardial tissue was strongly



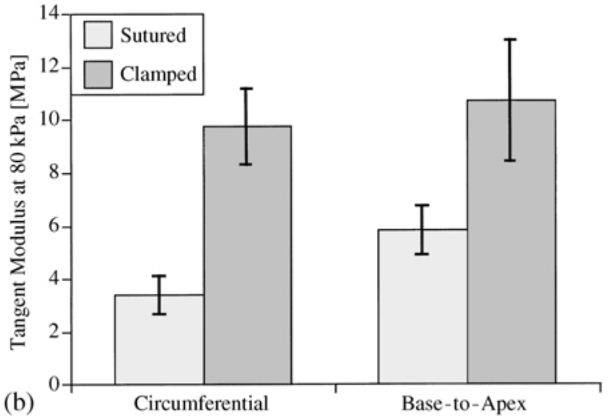
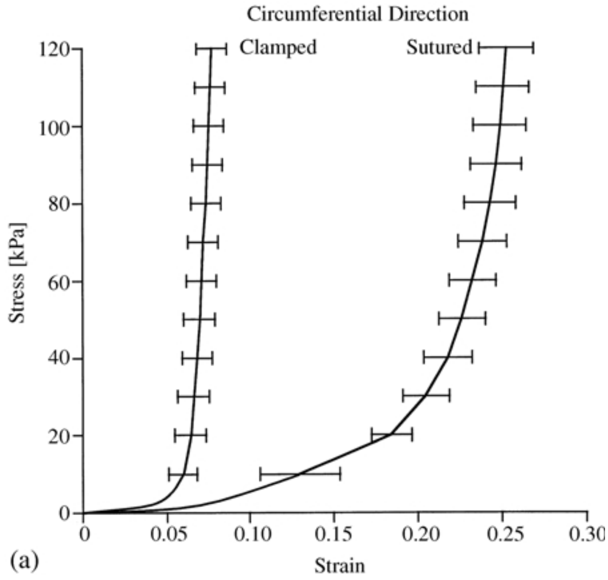


Figure 2 Both the maximum normal strain (a) and tangent modulus at 80 kPa (b) were significantly affected by the gripping method. The clamped samples experienced a lower extension in both anatomical directions ($p < 0.01$) and also appearing to be stiffer ($p < 0.03$ for the circumferential direction and $p < 0.07$ for the base-to-apex direction) ($n = 8$). Presented moduli were determined at a stress level of 80 kPa, for comparison purposes only. Note that the trends observed at this stress level were consistent throughout the entire deformation cycle. Results presented as mean $\pm$ standard error.



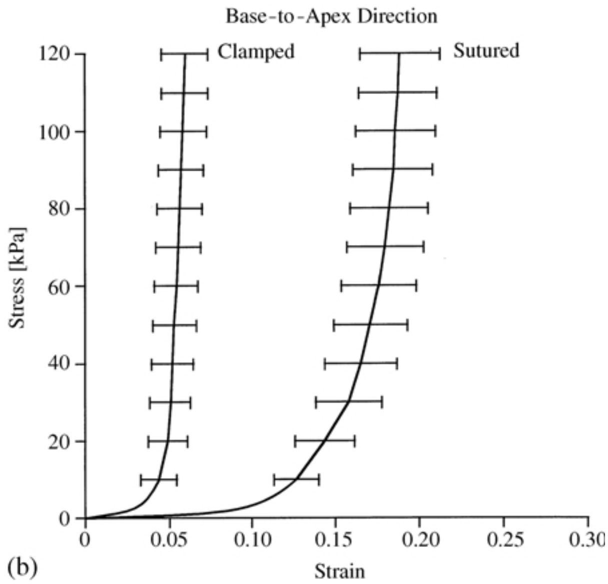


Figure 3 Average stress–strain curves for the circumferential direction (a) and the base-to-apex direction (b) are displayed for each gripping method ($n = 8$). Note that the observed alterations in mechanical behavior are present throughout the entire deformation cycle. Error bars represent standard error of the mean.

dependent on the sample gripping method. These apparent alterations in mechanical behavior can be attributed to the boundary constraints that were applied to the underlying collagen fibers at the edge of the sample. Clamping ensured that all the fiber ends resident at the sample edges were loaded due to the continuous lateral constraint of the clamps. With suturing, however, the load was transferred to the sample at discrete points and only those fibers within the vicinity of these suture attachments were directly loaded. Load transfer to the remainder of the specimen would then have to occur through interactions between adjacent collagen fibrils (e.g. interweaving and/or crosslinking), since the matrix is presumed not to mediate stress transfer between neighboring fibrils [18]. This discontinuous load transfer to the specimen could presumably result in an apparently more compliant and extensible material; indeed, both of these effects were observed. The increased sample shearing and rigid body rotation during deformation were also indications that the sutured samples were less constrained than their clamped counterparts.

The number of suture attachment points should also affect the observed mechanical behavior of the tissue sample with an increasing number providing greater constraint. Any comparisons of measured material behavior could be complicated because of the lack of standardization of this method. It is interesting to note that Mönch and Galster [19] abandoned similar mechanical testing methodology for engineering materials due to unwanted edge effects propagating from the

TABLE II Observed shear and rigid body rotation results

	Sutured	Clamped
Shear strain* (E_{12})	0.04 ± 0.012	0.006 ± 0.002
Shear stress** (S_{12}) (kPa)	9 ± 1.8	2.2 ± 0.67
Shear stress*** (S_{21}) (kPa)	5 ± 2.1	1.0 ± 0.36
Rigid body rotation* (°)	2.7 ± 0.5	0.20 ± 0.06

* A significant difference exists between treatments ($p < 0.01$).
** A significant difference exists between treatments ($p < 0.03$).
*** A trend exists between treatments ($p = 0.06$).

The observed maximum shear stress, shear strain, and rigid body rotation were all significantly affected by the applied gripping method. Clamped sample edges produced dramatically lower values compared to the common method of suturing sample edges. All results are presented as mean $\pm$ standard error ($n = 8$).

discrete grip attachment points on a square sample. This led to the use of the cruciform test specimen widely used in the biaxial testing of engineering materials [20–22]. We have investigated use of this sample geometry for planar connective tissue specimens [8, 17] but found that the apparent material properties were highly dependent on the cruciform arm length [17]. The changes noted with shortened cruciform arms were: (i) increased stiffness, (ii) reduced extensibility, (iii) reduced sample shearing, and (iv) reduced rigid body rotation. Identical trends were observed in the present study with the added constraint supplied by clamping biaxial tissue sample edges.

The underlying assumption in the present study was that the extracellular reinforcing fibers in planar connective tissues are continuous in nature. Although some evidence does exist which suggests that individual collagen fibrils are discontinuous [23, 24], observations of tissue structure at a light microscopic level (100–200 × magnification) reveal continuous bands or sheets of collagen fibrils with interspersed elastic fibers that also appear to be continuous in nature. For example, the pericardium has been ascribed to contain multiple laminated sheets of unidirectional collagen and elastic fibers that span the length of the tissue [25–28]. Also the fibers within these sheets have been claimed to be continuous in nature [29]. In order to distribute the applied load throughout the tissue, there must be some level of continuity within these fiber layers since the amorphous matrix does not exclusively mediate stress transfer between adjacent fibers [18]. Although certain proteoglycans do bind specifically to collagen [30] it is unlikely that these relatively long proteoglycan filaments are completely responsible for holding the entire structure together. The more plausible explanation is that the physical interactions between adjacent fibers (such as crosslinking and/or interweaving) are the predominant mechanism by which load is transmitted between the extracellular fibers and thus throughout the tissue – suggesting that an ''effective'' continuous fiber reinforcement exists within the tissue.

Apart from the observed changes in the apparent biaxial material behavior with different gripping arrangements, the general biaxial behavior of bovine pericardium was consistent with previous studies of pericardial mechanics. The pericardium shows nonlinear viscoelastic mechanical behavior indicative of most collagenous tissues. Although not explicitly measured in this study, all of the common viscoelastic behavior modes have been observed, including: hysteresis, stress relaxation, and creep – although there is not much agreement regarding the creep properties of pericardium [31–34]. The pericardium also displayed anisotropic behavior with the base-to-apex direction typically being stiffer and less extensible compared to the circumferential direction [8, 33–37] as observed here. This anisotropic behavior is probably as a result of the structural orientation of the extracellular reinforcing fibers within the pericardium. Sacks *et al*. [38] observed that over the left ventral surface of bovine pericardium, collagen fibers appeared to be preferentially orientated along the circumferential direction. This does not quite explain the increased extensibility of this direction, but could be the reason why the circumferential direction has been accredited with a greater tensile strength [39]. The degree of anisotropy is also dependent on the magnitude of the imposed strain, with less anisotropy experienced at higher strains [35]. This phenomenon has been observed in other planar connective tissues such as myocardium [15, 40].

The order of the applied gripping method for each sample was randomized to avoid the possibility of inadvertently biasing the results towards a particular grip method. However, by using this approach, the effects of repeated mechanical testing of the tissue sample cannot be eliminated. Although not explicitly determined, the dramatic differences in the observed mechanical behavior suggest that any effects of repeated testing would have been overwhelmed by the effects of the boundary conditions. In a previous study we have shown that similar repeated mechanical testing produced no significant effects on the observed mechanical properties of the connective tissue sample [17].

Under the suturing method, the displacement of the actuators during the deformation process was inevitably shared between the sample and the sutures. With deformation measured at the sample center – and not from the grip-to-grip displacement – this would not be a problem. However, it is prudent to have the material testing system less compliant than the material to be tested and thus the stiffness of the 2–0 silk suture material was quantified. Suture lengths of 44 mm were mechanically deformed at a displacement rate of 25 mm/min and suture stiffness was then defined as the initial slope of the stress–strain curve (in large deformation theory). The suture stiffness was 3.7 ± 0.19 GPa ($n = 8$), approximately three orders of magnitude stiffer than the tissue samples (Fig. 2(b)). This was assumed to be adequate.

The results of this study suggest that any biaxially measured properties of planar connective tissues do not necessarily reflect the properties of the material *in vivo*. The response of a material sample to a set of imposed forces or displacements is not just a function of the mechanical properties of the material, but also of the sample geometry and the boundary conditions applied to the sample. Thus, any test method inherently biases the observed behavior. Typically, mechanical testing methods are designed to create uniform stress fields at the sample center in attempts to minimize the effects of the boundaries (Saint-Venant's principle). In this study, the extent to which the boundaries affected the observed behavior of the sample center is unknown. Analysis of the collagen fiber orientations during deformation may provide additional information and is the focus of the second part of this study [41]. In any event, it is probable that neither the suturing nor clamping methods used to grip the edges of biaxial test samples actually reflects the boundary conditions on the tissue *in situ*. Therefore, reliable estimates of the ''real'' material properties only be obtained through *in vivo* testing where the continuous nature of the reinforcing fibers is still intact; however, this is not always feasible or practical. Until the time when either: (i) feasible *in vivo* mechanical test methods are developed or (ii) information regarding the boundary conditions on the extracellular fibers *in situ* is determined, the choice of applied sample boundary conditions should be carefully addressed prior to mechanical testing. Realistically, since at present there does not exist many suitable mechanical testing methods for planar connective tissues, some attempt must be made to standardize those methods that are commonly used. For example, with the suturing method developed by Lanir and Fung [3], experiments should be conducted to ascertain the optimum sample size, number of suture attachments as well as the size of the measured strain field.

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