Collagen / Hyaluronic Acid Matrices for Connective Tissue Repair

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Abstract

Subcutaneous tissue loss from pressure sores or trauma is commonly treated by reconstructive surgery, using a myocutaneous flap rotated from an adjacent unaffected site. This is a preliminary report on a semi-synthetic graft intended to perform the same function as the surgical flap but without donor site morbidity; it should have the same properties as lost tissue and contain autologous cells. We are studying composites of Type I bovine or rat collagen with hyaluronic acid (HyA).

Three physical forms of 1:1 collagen:HyA have been made:

- (1) homogeneous dispersions of HyA particles,
- (2) continuous-strand esterified HyA mats or felts,
- (3) loosely-packed HyA ester beads.

Physical properties were assessed by a sphere indentation test for creep compliance and a tensile relaxation test using sutured junctions simulating clinical use. Creep (combined compression and shear) and tensile moduli were lower but comparable to published values for rabbit mesentery including effects of pre-loading history and continued creep over long periods (>16 hours). Early (<2 minute) indentation is influenced by a collagen-rich surface layer. Cell interaction: Rat cells were added in the form of: neonatal fibroblasts layered onto the matrix, adult omentum explants inset into the matrix, and adult fibroblasts and adipocytes distributed throughout the matrix. Cell survival and attachment were assessed after 7-14 days by vital staining and post-fixation histology. Survival was poor (<50%) in homogeneous collagen:HyA, due to residual solvent in the HyA. Cells migrating from explants tended to envelop individual HyA beads or strands. Surface cells entered the matrix principally along flaws in the collagen phase. HyA prevented contraction seen in cell-seeded collagen-only preparations. Matrices of stranded HyA with cells distributed in the collagen phase have better strength and surgical handling, and are being pursued further.

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Problem

Pressure sores ("decubitus ulcers") occur in hyposensitive skin of immobilized individuals, where prolonged compression of tissue between the supporting surface and a bony prominence impairs capillary blood perfusion in the region [1] . Sufferers typically include paralyzed spinal cord injured and stroke patients, postsurgical and casted fracture patients, and frail institutionalized elderly [2] . It is estimated that every year 7% of the 200,000 spinal cord injured persons in America will develop an ischial pressure sore. Additionally, 14% of the general population over 70 years of age will develop pressure sores. Between 3 and 8.8% of sores will require surgery [3] [Figure 1].

Recurrence rates after surgery vary from 7% in trochanteric ulcers to 11% of sacral sores [4] ; recurrence is frequently due to continued stress on deep tissues despite adequate skin coverage. All pressure sores are difficult to treat; failed surgeries result in some of the longest hospital stays (>70 days on average) and highest costs.

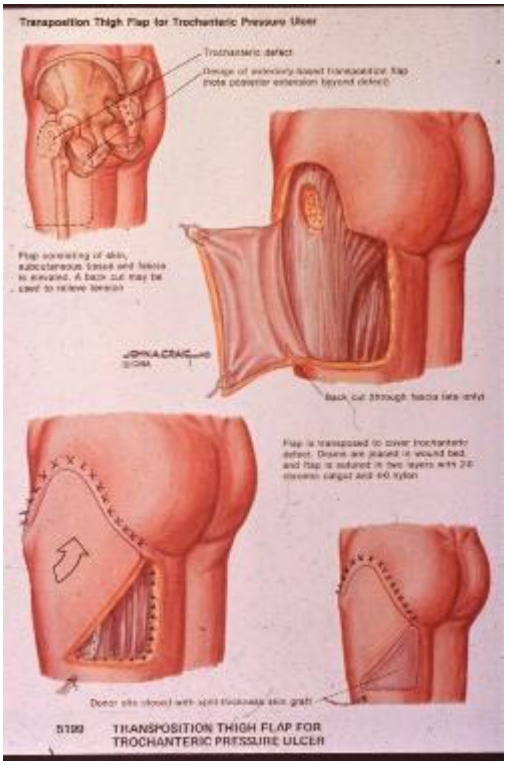
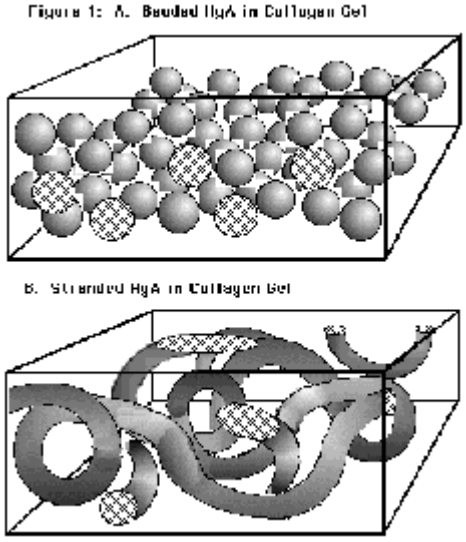


Figure 1 - *Pressure sore surgery*

Conservative dressings: The range of nonsurgical approaches to enhance healing of open pressure sores has recently broadened. Gauze dressings have been superceded by a variety of packings (usually based on sodium alginate [5]) and film barriers for controlling moisture content of the wound environment while excluding contamination. Collagen [6,7,8] and collagen:HyA [9] have been investigated as dressings, but without tailored microgeometry to encourage incorporation into living tissue.



Two-phase collagen:hyaluronic acid composite tissues

Reconstructive surgery using prefabricated flaps: Since restoration and maintenance of vascular continuity within any flap is essential to its survival, prefabrication is used to either place a larger vessel within tissue to be transferred or to prepare a donor site in advance, thus achieving in a single procedure what would otherwise require multiple operations [10] . Experimentally, synthetic biomaterials have been combined with mobilization of a vascular pedicle, which accelerates ingrowth of connective tissue into the synthetic graft [11]. Similar concepts have been explored by Walton and Brown [12] , with vascular pedicles to accelerate vascularization of Teflon discs, and Mikos and co-workers [13] , who implanted polylactic acid discs into folds in the rat mesenteric membrane.

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Objectives

We hypothesize that a cell-seeded synthetic matrix could perform some of the functions of the myocutaneous flap autograft, specifically:

- Restore connective tissue depth and contour better than a skin graft
- Restore mechanical properties for protection against vascular stasis under load
- Restore viable tissue more rapidly than healing by peripheral ingrowth

The success of the tissue-engineered graft would be enhanced by:

- Rapid revascularization
- Reducing the magnitude of the interfacial barrier between graft and wound bed, so as to prevent fibrous encapsulation

The immediate goals reported in this paper are to:

- Fabricate tissue matrices having properties approximating subcutaneous connective tissue
- Examine interaction of these matrices with cells in vitro
- Implant matrices with a vascular pedicle into animals and retrieve after 3, 5, and 8 weeks

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Approach

The graft is constructed of a biopolymer matrix inoculated with fibroblasts and/or epidermal cells and is either microsurgically reconnected to blood vessels near the wound, or perfused with a culture medium in lieu of blood circulation.

The matrix consists of collagen, which provides a substrate for cell attachment, and hyaluronic acid, which provides bulk, viscous damping and compression resistance without impeding cell mobility [14] , similar to fat in the intact tissue. Collagen is an appropriate material for such matrices, since it is a structural protein with minimal interspecies immunoreactivity and capable of dissolution and controlled repolymerization [15] .

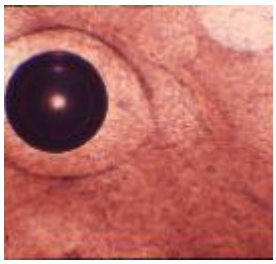

Figure 2 - *HyA:collagen matrix*

Three physical forms have been created [Figure 2]:

- homogeneous 1:1 collagen:HyA dispersions of random-geometry HyA particles
- continuous-strand HyA mats or felts
- loosely-packed HyA beads

Note that because HyA is in the form of a discrete micron-scale phase, it does not interact with collagen at the molecular level as in cartilage [16] , nor is it free to flow under load [17].

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Methods 1

The matrices under investigation were mixtures of Type I collagen (from bovine skin or rat tail tendon) with hyaluronic acid (supplied in 1:9 DMSO solution). Collagen (1-2% in pH 3.5 acetic acid, neutralized by mixing 1:1 Hanks BSS + stoichiometric NaOH) was added to preformed HyA bead or strand preparations and allowed to polymerize in situ in 35-100 mm Petri dishes. Cell-free matrices were cross-linked by UV illumination (254 nm, ¸ 200 mw/cm2) to avoid cross-linking agents such as glutaraldehyde known to have cytotoxic effects [18] .

Homogeneous Collagen/HyA - Foams of Type I collagen are produced by neutralizing the acid collagen solution and homogenizing to disperse entrapped air and CO2 formed during neutralization into micron-scale bubbles. The homogenized collagen is

mixed at various ratios with 5% HyA in DMSO using the same method but lower shear forces, then is layered into a Petri dish and allowed to polymerize or gel *in situ* for two hours at 37· C. The microbubbles dissolve and are replaced by liquid over the course of a few days, leaving a porous fluid-filled microstructure.

Collagen/HyA bead matrix - Droplets or beads of hyaluronic acid are formed in an immiscible liquid using extrusion nozzles driven by a low-frequency oscillator. The beads are uniform in diameter (50 µm - 1 mm) and therefore incite minimal foreign-body reaction compared to dispersions formed by sonication[Figure 2b]. Similar methods are used for microencapsulation of cells for suspension culture and xenogeneic transplantation [19] .

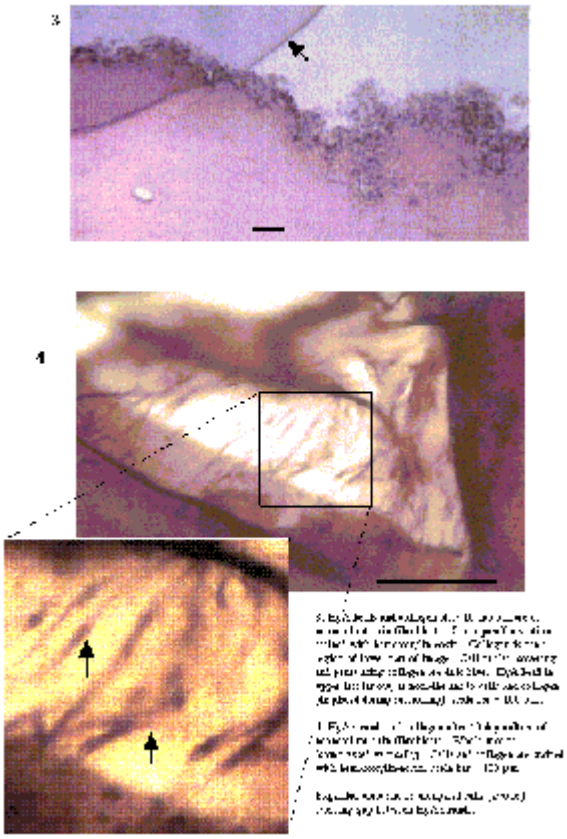

Figure 2b- *Collagen/HyA bead matrix* -

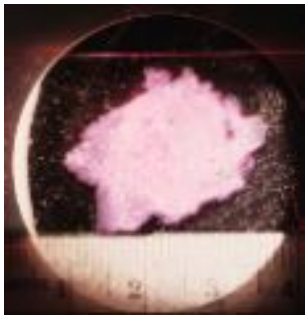

Figure 3a - -*Beaded HyA:collagen*

Packing density of the beads [Figure 3a] is such that spaces between them permit immigration of cells from an inoculum or the periphery of the wound.

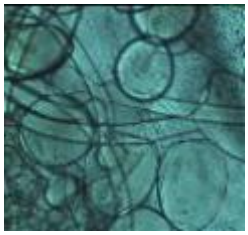

Figure 3b - *Collagen/HyA felt matrix*

Collagen/HyA felt matrix - Fibers or strands of HyA are made by extrusion into a bath (e.g.: absolute ethanol) that is miscible with the DMSO solvent but immiscible with HyA [Figure 3b].

Prolonged soaking (2-7 days) in a similar bath results in condensed HyA strands that have high tensile strength and

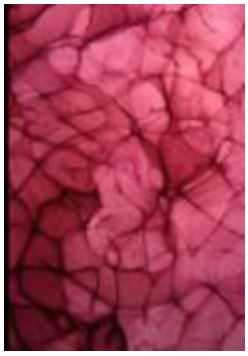

Figure 4a - *Stranded HyA:collagen*

resistance to rehydration compared to bulk HyA [Figure 4a].[17].

Figure 4b - *Stranded HyA:collagen*

The soaking bath is removed by filtration and washing with distilled water; this and mechanical treatment to interlock the strands yields a felt or mat 1-2 mm thick with interstices between strands of 50 µm to several mm [Figure 4b].

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Methods 2

Donor Cells - For *in vitro* testing, tissues were obtained from neonatal or young adult Fisher rats. Specimens were dissected using aseptic microsurgical technique into deep and superficial components, then enzymatic or immunoadhesion techniques were used to separate epidermal cells, fibroblasts, adipocytes and/or myoblasts. For clinical use, autologous cells would be obtained from the wound margin or from biopsy of soft tissues remote from the injury site.

Figure 5 - *Explant culture in wells or dishes*

In Vitro Culture Model - Cells were cultured on experimental matrices in either static Petri dishes [Figure 5] or in perfused chambers [Figure 6]. After expanding cell population in culture, cells were suspended in DMEM + 10% FBS and added at concentrations of 500-2000 cells/mm3 of matrix. In some static cultures cells were layered onto the matrix in the same dish in which it was gelled; for replicate cultures, the matrix was cut into 1 cm squares and distributed among 4-8 wells or dishes.

Perfused cultures were in molded silicone chambers with a 25 mm square cavity; passing through the midline of the 5 mm depth of the cavity were parallel polysulfone hollow ultrafiltration fibers through which culture medium (DMEM + 5% FBS) was pumped at 2 ml/min [Figure 6].

Figure 6 - *Perfused silicone chambers*

One or two layers of matrix were placed on each side of the row of hollow fibers before sealing the chamber between aluminum plates. Cells were inoculated by injection through the silicone chamber wall. In vitro cell survival and attachment were assessed after 7-14 days by vital neutral red staining and post-fixation thin section histology with hematoxylin-eosin staining. Relative cell population was estimated by comparing area occupied by cells in and perpendicular to the plane of the matrix layer, with cell-culture plastic as a control substrate. Matrix geometry was evaluated in compressed whole mounts stained for collagen with Sirius red F3BA and for HyA with alcian blue.

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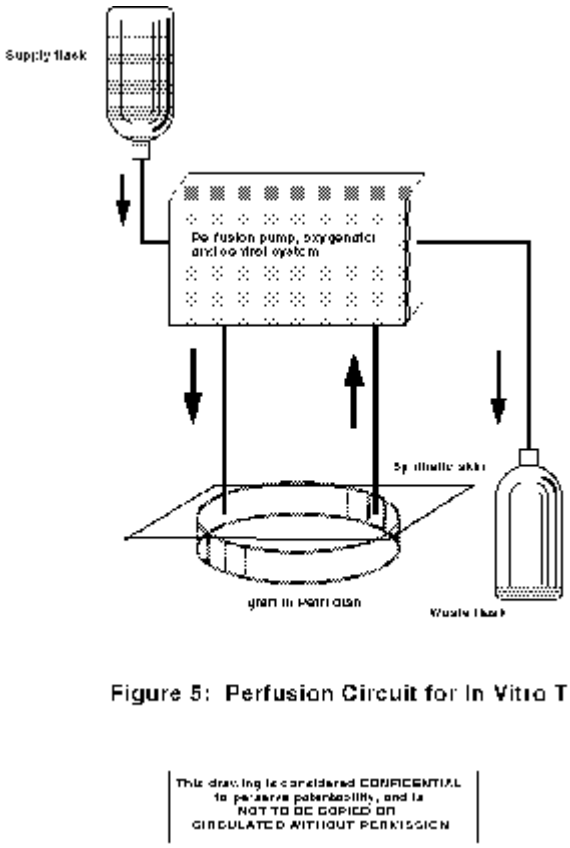

Figure 5: Perfusion Circuit for In Vitro Test

Perfusion circuit for in vitro test

Methods 3

Surgical implantation **-** Male F344 Fischer rats weighing approximately 400 grams were anesthetized with intraperitoneal sodium pentobarbital. All surgical repairs and dissections were performed under a Zeiss operating microscope with 10X to 25X magnification. A 25 - 30 mm square patch of skin over the abdominal region was shaved, incised on 3 sides and elevated HyA strand or bead-containing matrix layers were cut to 20 - 25 mm square, then laid in place and attached to muscle fascia with 2 or 3 4-0 nylon sutures. The arteriovenous pedicle was constructed from the saphenous vein dissected from one leg, looped over the deep matrix layer, and anastomosed to the femoral artery. Sufficient collateral veins exist so that circulation to the leg, although initially decreased, is not static. One or more layers of matrix were laid over the vascular loop before closing the incision with wound clips.

Rats were sacrificed and the implant region was excised and fixed at 3, 5 and 8 weeks post-surgery. Paraffin sections were cut at 6 to 8 µm and stained with hematoxylin-eosin to evaluate inflammatory reaction, and with Masson's or Gomori's trichrome for connective tissue. Because HyA was not infiltrated with paraffin, displacement and folding of HyA in sections could not be avoided. Light microscopy was used to visualize: presence and orientation of blood vessels, location and morphology of cells, orientation and density of the extracellular matrix, location of specific matrix components dimensions of voids within the matrix, adhesion to the surrounding tissue, and extent of biodegradation of the matrix.

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Methods 4

Mechanical Testing - In order to compare synthetic matrices with host site tissue and with published values for similar materials, physical properties were assessed by a sphere indentation test for creep compliance, and a tensile stress relaxation test using sutured junctions simulating the clinical application.

Figure 7a - *Sphere indentation compression test*

Both tests were performed at ambient temperature and humidity.

In the sphere indentation test [Figure 7a], layers of matrix 15 mm in diameter were stacked on a glass slide to achieve 6-7.5 mm depth. A steel sphere (1/8 to 7/16 inch diameter stainless ball bearing) was gently placed on the matrix, and the rate and distance it sank into the matrix were measured on a video image at 15X magnification. A time-dependent compression modulus, E(t), was calculated from:

$$E = \frac{2(1-\mu^2)P}{2Rh-h^2}$$

where μ is Poisson's ratio (=0.5), P is the total force generated by the sphere's weight, R is the sphere's radius, and h is the depth of indentation at time t. This rudimentary calculation takes into account the change in surface stress as contact area increases, but assumes internal stresses in the matrix are roughly equivalent to a flat indentor and ignores effects of finite thickness [20] . Samples of previously-frozen rat abdominal fat and skeletal muscle were tested by the same method.

Figure 7b - *Tensile stress relaxation test*

Tensile tests were conducted on 10 x 25 mm samples typically 2 mm thick [Figure 7b]. Monofilament polypropylene suture (4-0) was used to make 3 or 4 stitches in each end; the upper end was tied to a beam attached to one arm of a Cahn microbalance. After taring the balance, the other end was fixed to a 20 gram weight on an elevating stage. Weights (1-14 gram) were added to the opposite arm and the balance was re-leveled by lowering the stage a measured distance. Stress relaxation was measured by nulling the balance at intervals during a 10-180 minute period. Tensile modulus was calculated assuming a constant cross-section. The same apparatus can be used for suture pull-out tests, which typically require forces greater than 20 grams.

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Results 1

Matrix Geometry - Sections of homogeneous collagen:HyA matrices showed that HyA was uniformly dispersed in the form of sharp-edged laminated particles less than 200 μm,

typically 20-50 µm, in diameter.

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Figure 8 - *HyA strands under tension relative to collagen gel*

HyA strands were 100-200 µm diameter and in compressed whole mounts were interlaced and effectively coated with collagen. The stiff HyA strands were subject to residual bending stress, as seen at cut edges where they spring out of the surrounding collagen gel [Figure 8]. HyA beads were 0.5-1.0 mm diameter and sparsely distributed, separated by 2 or 3 diameters of collagen gel.

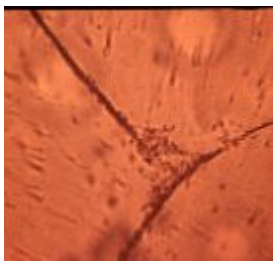


Figure 9 - *Cells penetrating defects in collagen phase*

Cell growth - Cells were initially concentrated at the surface of matrices, and over the first week of culture penetrated into defects in the collagen phase [Figure 9]. Neither cells nor collagen adhered to HyA beads other than by mechanical entrapment.

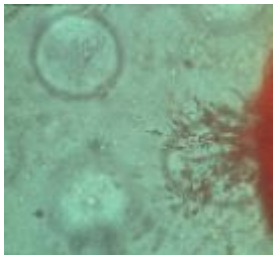


Figure 10 - *Cells migrating through collagen phase in vitro*

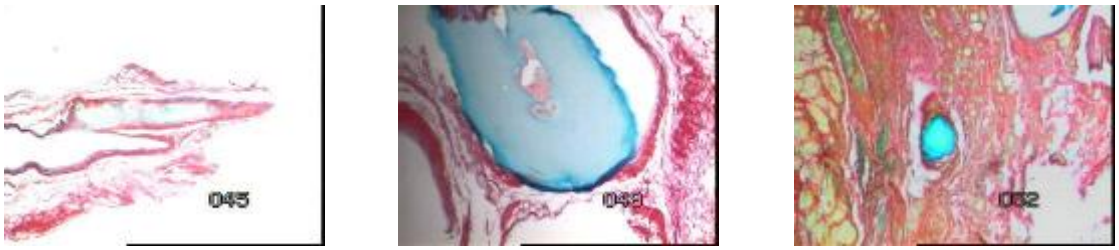
Populations of cells migrated through the collagen phase without directly contacting the HyA phase [Figure 10]. Unconstrained pure collagen gels are known to contract under the influence of cell-generated tensile forces; this did not occur in HyA-containing matrices. Passage of cells completely through the matrix was avoided in perfused cultures by turning the chamber over once a day. Such leakage of cells seemed more prominent in regions with sparse HyA fibers. While quantitative cell viability tests were not conducted, fewer cells survived on or in homogeneous collagen:HyA preparations compared to matrices containing pre-formed HyA or to tissue-culture plastic.

Figures 11a,b,c - *Implant at 3 weeks: neovascularization, collagenolysis*

In vivo implantation - Inflammation was minimal, with rapid capillary outgrowth into the matrix within 3 weeks, accompanied by evidence of collagenolysis [Figure 11].

Figures 12a,b,c - *Implant at 5 weeks: acute inflammation subsided*

At 5 weeks, acute inflammation had subsided, although chronic inflammatory cells were present [Figure 12].



Figures 13a,b,c - *Implant at 8 weeks: HyA component largely intact*

At 8 weeks, most of the original collagen had been replaced by fibrous connective tissue, while the HyA component was largely intact [Figure 13] vascularity was reduced.

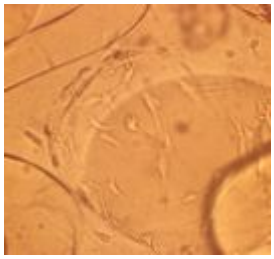


Figure 14 - *Cells enveloping individual HyA beads or strands*

As predicted from in vitro cell ingrowth experiments, migrating cells enveloped individual HyA beads or strands, and did not form a fibrous capsule around the entire implant [Figure 14].

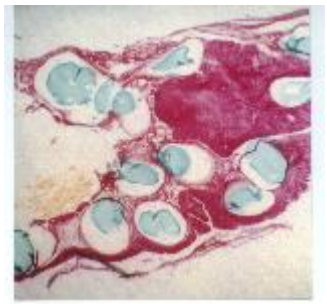


Figure 15a - *Vascular pedicle, cross section*

The collagen phase was nearly all resorbed by 8 weeks; in contrast, most HyA beads or strands were intact [Figure 15a]. Foreign body giant cells were present, but rare, adjacent to HyA surfaces. Individual HyA strands or beads became encapsulated by a 3 - 4 cell thick layer.

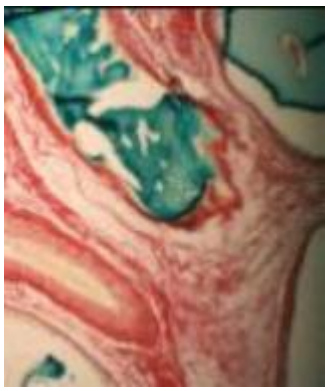


Figure 15b - *Vascular pedicle, longitudinal section*

The vascular pedical was evident in both cross [Figure 15a] and longitudinal section [Figure 15b]. The abdominal site did not provide a wound margin suitable for testing integration.

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Results 2

Mechanical Properties -

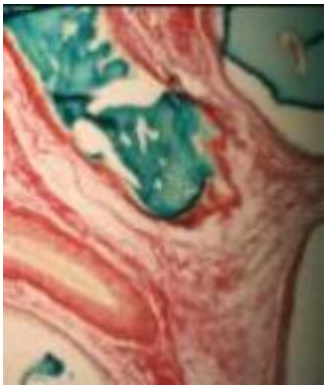

Figure 15b - *Vascular pedicle, longitudinal section*

Rate of indentation of HyA bead matrix revealed effects of surface dehydration on successive one hour tests, in that the initial run shows a steeper rate of penetration in the first millimeter than a second run, followed by 40-45 minutes of sinking at approximately the same rate. In neither test did the 1/8 inch sphere penetrate more than 2 mm, half its radius.

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Figure 16 - *Compression moduli of collagen:HyA*

The moduli derived from these data [Figure 16] can be best fit by a bimodal power curve in which E is proportional to t-0.94 and t-1.50 during the first 3 minutes of run 1 and 2, respectively, and proportional to t-0.38 and t-0.55 during the remainder of each run.

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Figure 17 - *Compression moduli for collagen gel without HyA*

Moduli for 6.5 mm thick samples of collagen gel without HyA using 1/8 spheres show good correllation between two runs with E proportional to t-0.75 and t-0.70 [Figure 17] despite differences in sample thickness.

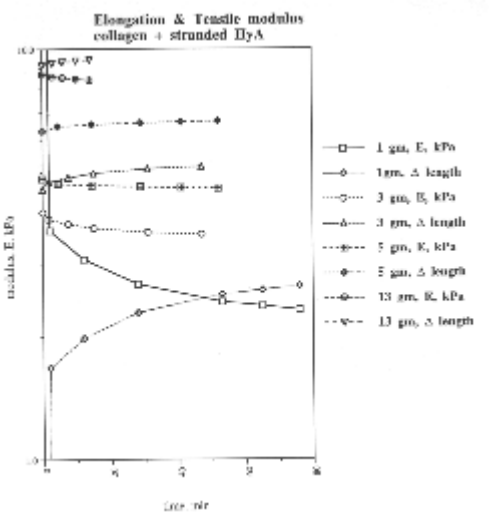

Figure 18 - *Tensile properties of stranded HyA:collagen*

The results for a second sample with 5/16 spheres show effects of bottoming, in which the curve for E flattens abruptly after 1 minute, having indented the sample by 0.7 mm or less. Moduli for rat muscle (¸ t-.55) and fat were nearly equal at 10 minutes (12 kPa). Tensile properties under stepwise increasing load revealed a gradual increase in apparent modulus [Figure 18].

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Discussion

Material properties of soft tissues tested as controls were comparable to published values

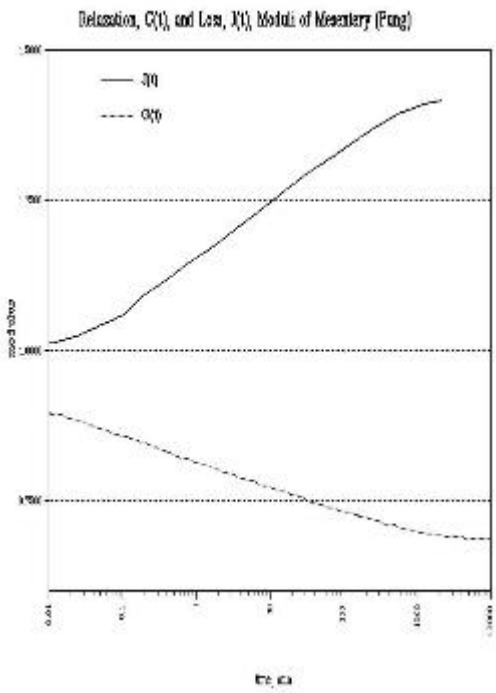

Figure 19 - *Creep & relaxation moduli of rabbit mesentery (Fung [23])*

for similar tissues (Fung[22,23]) gives tensile creep and relaxation moduli for rabbit mesentery, noting that a known pre-loading history is essential to derivation of repeatable elastic constants, and that over extremely long periods (¸103 minutes) soft tissues may exhibit unlimited strain [Figure 19].

This effect is evident in the approximation of indentation to an inverse power function with respect to time. In our specimens, the early (<2 minute) curve is most likely due to the collagen-rich surface of the collagen:HyA composite. Only after the stress field generated by the sinking sphere reaches the intermeshed HyA strands does this component alter the apparent modulus.

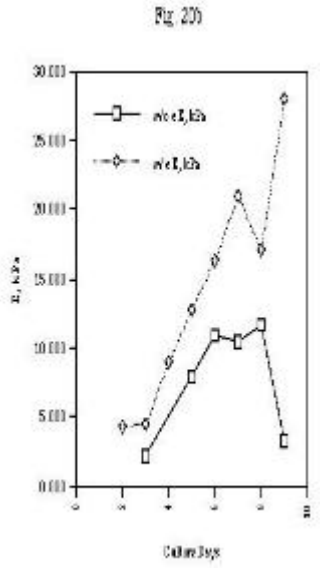

Figure 20 - *Tensile test of collagen with & without cells (Jain, et al., [24])*

In a study of cell-seeded collagen sponges, Jain, et al.,[24] conducted a tensile test of samples with and without cells. Stress/strain curves were comparable up to 50% strain [Figure 20].

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Healing of tissue is hypothesized to be accelerated by implantation of a synthetic tissue having the same chemical and structural properties as the lost tissue and inoculation of the implant with autologous cells. This concept is based on two premises: (1) that the human body lacks in maturity geometric and biochemical cell guidance information present in the embryo, which may be provided by an artificial extracellular matrix, and (2) that a completely inert biomaterial lacks the capacity to integrate with intact tissue and respond to functional demands, provided by a cellular component.

The general concept of facilitated regeneration through the use of composite artificial/cellular tissues has broad applications beyond the current clinical uses in skin repair and experimental use in peripheral nerve grafts. The pressure sore is a good model for this concept since it is localized, accessible without internal surgery and non-critical to immediate survival. The *in vitro* test is in fact a worst-case approximation of an avascular injury site, and if cell growth occurs as desired *in vitro,* growth should be as good or better *in vivo.*

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Acknowledgements

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