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WOUND HEALING OF TENDON—I. PHYSICAL, MECHANICAL AND METABOLIC CHANGES*

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Abstract – The wound healing process of New Zealand rabbit extensor digitorum communis tendon was studied from the fifth through thirteenth post-surgical day by concurrent observation of physical, mechanical, and biochemical properties. Mechanical testing was by isometric thermic denaturation followed by biochemical assay of tendon and the physiological saline bathing medium for collagen protein and acid mucopolysaccharides.

The changes observed in the physical properties showed an increased dry tendon weight and tendon cross-sectional area relative to intact tendon. Mechanical property changes resulted in a lower shrink temperature compared to intact tendon, increased tendon denaturation slope, and increased tension values with healing time. The changes in the biochemical parameters were increased concentration levels of soluble hydroxyproline, soluble protein, and insoluble hexosamine relative to intact tendon. Implications of the property changes to collagen structure are discussed.

INTRODUCTION

The physical, mechanical, and chemical properties of collagenous tissue are inherent in its basic structural organization. Mutual interaction between the systemized protein units and interaction between these units and the particular pattern of glycosaminoglycans which make up the ground substance maintain and stabilize the collagen structure (for reviews see Gustavson, 1956; Muir, 1964; Sinex, 1964; Steven, 1972; Gallop *et al.*, 1972; Nimni, 1975). During development and maturation collagen fibers have been found to exhibit changes that comprise increased crystalline orientation, increased density, decreased structurally bound water content, greater tensile strength, increased stiffness, resistance to digestion by collagenase, higher calcium binding capacity, and a change in the molecular arrangement that causes increased thermal contractility and decreased solvability (Lin and Sterling, 1968; Schubert and Hamerman, 1968; Viidik, 1969; Bihari-Varga and Biro, 1971).

In wound healing, architectural changes in the collagen matrix occur as a result of the intricate process of remodeling. Restoration of form and function depend on the extent of aggregation of the constituent polypeptides. The morphological observations of Fernando and Movat (1963) and Rokkanen and Vaino (1971) on regenerating rabbit tendon showed an initial haphazard extracellular polymerization of tropocollagen with abundant acid mucopolysaccharide. Evidence by Munro *et al.* (1970) demonstrated the interrelationship and interdependence of both cellular substances during healing. With growth, development, and maturation the fibrils increase in diameter, become highly organized largely due to the formation of hydrogen bonds, (Jackson, 1958; Ramachandran *et al.*, 1973) and further stabilized by intermolecular covalent crosslinks (Bailey *et al.*, 1974).

Coincident with the deposition and maturation of collagen in the healing wound is a rise in tensile strength (Harkness, 1968). Quantitative changes in acid mucopolysaccharides occur prior to and accompany the collagen and tensile strength change (Dunphy and Udupa, 1955). The best determinant of the gain in tensile strength was demonstrated by Bryant and Weeks (1967) to be the ratio of wound collagen to mucopolysaccharide and that alterations in the cohesive forces between collagen macrostructures were directly related to this ratio. Hence, the number of effective network chains per unit volume and not the total number of chains have the greatest influence on the load bearing structure (Milch, 1965).

Maximal tension during isometric hydrothermal denaturation has been employed on normal unwounded collagen tissue to assess structural stability related to maturation in aging (Verzar, 1964a). The fibers shorten in response to hydrogen bond destruction with maximal contraction a result of energy requirements to break covalent cross-links which increase with age (Gustavson, 1956; Verzar, 1969). Concomitant with thermal denaturation is the solubility of collagen, decreasing progressively with age

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due to the development of stabilizing covalent interchain bonds (Everitt *et al.*, 1970; Steele and Everitt, 1970).

The mechanical behavior of collagen tissue wounds has, however, been virtually limited to the measurement of tensile strength. This data is even clouded because of the failure to account for cross-sectional area dimensions in a great many of the studies. Concurrent biomechanical and biochemical wound healing studies have had limited documentation and used skin or cartilage specimens. Comparisons drawn from these experiments can only be qualitative as measurements were not from the same specimen.

The purpose of this investigation was to concurrently measure biomechanical and biochemical properties of tendon during wound healing to ascertain characteristics of how structural components of the regenerating tissue relate to increasing functional demand. The rate of collagen formation and the quality of crosslinking in wounded tendon were determined by physical measurements and mechanical testing utilizing isometric thermic denaturation with simultaneous assay of collagen and acid mucopolysaccharide levels.

MATERIALS AND METHODS

The subjects, 32 adult male New Zealand rabbits ranging in age between 20–24 weeks, weighing between 2.9 and 3.8 kg, were individually caged and maintained on a standard rabbit chow with water *ad libitum*. A two week acclimation period with intramuscular injection of Combiotic (200,000 units penicillin G, 250 mg streptomycin) given daily for the first seven days was provided prior to surgery. All animals were pre-anesthetized with intravenous chlorpromazine hydrochloride, 7.5 mg/kg, and the hair was clipped from the dorsal surface of both front paws. Subsequent anesthesia was with sodium pentobarbital through a 23 gauge infusion set inserted i.v. into the marginal vein of the left ear. These injections were in 10 mg doses, given to assure complete anesthesia during the operation. After confining the animal to the operating table, the skin was prepared with iodine solution and alcohol. Sterile technique was used throughout the procedure.

A U-shaped dorsal incision extending distally from the carpal joint was made in each paw such that a 2 cm exposure of the extensor digitorum communis tendon from phalanges 2–5 was made when the skin flap was retracted. Each tendon was carefully dissected and isolated from the surrounding facia and veins. A teflon splint, approximately 2 × 11 mm, 0.5 mm thick having four 0.6 mm dia holes spaced at 3 mm intervals, was sutured on the tendon dorsal surface of phalanges 2–4 using No. 6-0 Deknatel Tevdek II with 3/8 circle needle. The extensor tendon from the fifth phalanx was left intact to serve as the control. A No. 11 Bard–Parker surgical blade was used to sever the splinted tendons between the center two holes by carefully moving the blade between the tendon and the splint. The skin flaps were closed with interrupted horizontal mattress sutures using No. 4-0 silk thread. After surgery, both paws were cleaned with physiological saline, sprayed with anti-bacterial powder, bandaged with 1 ply gauze and elastic cotton, and cast with plaster in the neutral position.

The animals were assigned into five groups, each representing the number of days between surgery and sacrifice. Wound healing periods of 5, 7, 9, 11, and 13 days were investigated with animal group numbers of 8, 7, 6, 5, and 6, respectively.

An overdose of sodium pentobarbital was used to kill the animals. After the plaster cast and bandaging material were removed from each paw, the skin was excised and the extensor digitorum communis tendons carefully isolated and dissected out. Each tendon was cut to a standard length of 5.0 cm with a scalpel blade, splint facing upward. The teflon splint was carefully removed by cutting the sutures along a dorsal edge of the splint. Older wounds, 11 and 13 day, necessitated cutting either the most proximal or distal suture, folding the healed cuff outward and cutting the remaining sutures until the splint was removed. All tendons were kept moist with physiological saline to prevent desiccation. A maximum experimental time interval of three hours was allowed to complete testing of the animal tendons so as not to introduce time dependent mechanical changes (Matthews and Ellis, 1968).

Mechanical testing procedure

Cross-sectional area measurements were determined on the wounded and/or normal sections of each tendon using the polar shadow amplitude machine employed by Ellis (1969) with a 2.4 mm slit width. The specimen contours of both the distal and proximal ends of the control tendons and the center of the healed portion and the proximal end of the wounded tendons were later reconstructed as described by Ellis (1969).

Each tendon specimen was clamped in the isometric thermic denaturation testing machine, Fig. 1, with the proximal end of the tendon placed in the upper stainless steel clamp. In each of the clamps (*D*), the specimen was gripped between stainless steel collars backed with rubber and canvas and fitted over the raised serrated surface of the clamps. An eccentric cam located within a semicircular cutout behind each clamp tightened the collar against the tendon and the clamp. The interclamp distance was initially set at 1.4 cm. For the experimental (wounded) group, the material between the clamps consisted of healed tissue. A 25 × 100 mm culture tube filled with 25 ml, 40°C physiological saline was placed in the testing machine heater core (E) initially heated to 40°C. Immersion of the tendon was accomplished by raising the heater core platform utilizing a rack and pinion. The voltage to the heater core, 96 W resistive heater wire, was then raised to approximately 55 V such that the temperature of the bathing medium surrounding the tendon

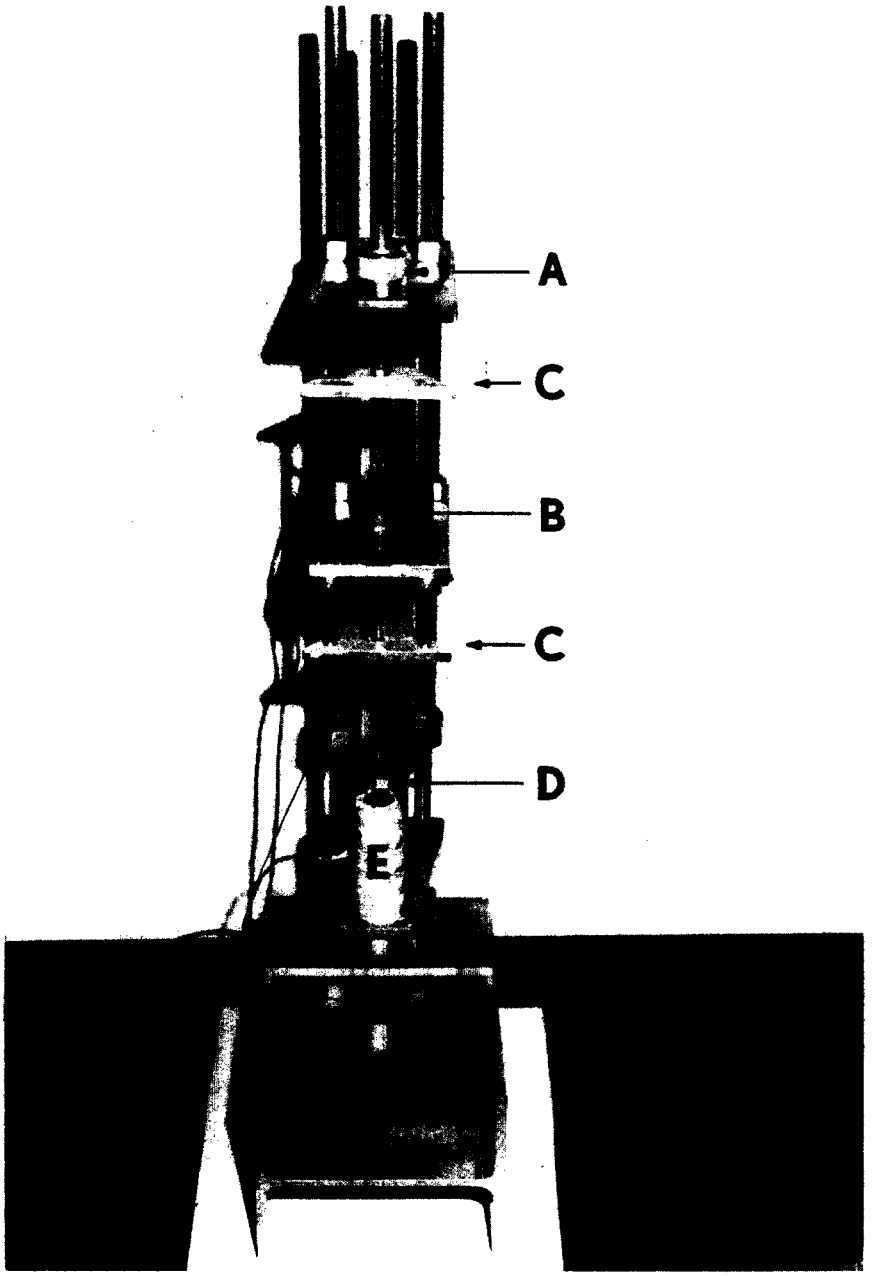

Fig. 1. Isometric thermic denaturation testing machine; (*a*) tension thumb screw, (*b*) load cell, (*c*) three pillared frame, (*d*) specimen clamps, (*e*) heater core.

would rise at a rate of 2°C/min to 65°C. Temperature was recorded by copper–constantan thermocouples attached at the back surface of the lower clamp above the grip serrations in a small hole lined with silicone adhesive, and within the heater core. The reference copper – constantan thermocouple was placed in a Dewar flask filled with ice water. Bathing medium temperature was recorded as lower clamp thermocouple voltage on a BLH electronic amplifier (model PR-601B6) with recording oscillograph (model BSA-670B). This thermocouple allowed continuous monitoring of the 25 ml bath temperature at the tendon site. Isometric contraction was measured by a strain gauge load cell (*B*), Statham load cell UC-3 with 0.5 lb adapter UL4-0.5, anchored to a rigid platform and in series with a fixed upper specimen clamp. An approximate 980 dyn load was initially applied to the tendon by lowering the movable lower clamp *via* a thumb-screw (*A*) located at the top platform of the test machine which interconnects through a two tier triangular three pillared frame (*C*). This small load was monitored and, if necessary, adjusted to maintain the tendon in tension as the bath temperature increased and until thermal contraction commenced. The experiment was concluded when an appreciable decrease in tension was recorded with increasing temperature past maximal tension. After the bathing medium cooled, the final volume was made up to 25.0 ml to compensate for evaporative loss, mixed, frozen on dry ice in (4) 6 ml aliquots, and stored at −20°C for later biochemical study. The tendon specimen was carefully removed, placed in a plastic tube, and dehydrated under vacuum. After one week, the dry tendon was weighed using a Mettler M5 microchemical balance and frozen at −20°C for later biochemical study.

Biochemical determinations

Biochemical assays conducted on the physiological saline bath of each tendon following isometric thermal denaturation utilized colorimetric techniques to detect the presence of metabolites dissolved in solution. The method of Lowry *et al.* (1951) was used to assess protein concentration levels, Kivirikko *et al.* (1967) for hydroxyproline, Bitter and Muir (1962) for uronic acid, and Blix (1948) for hexosamine determined at a photometric wavelength of 540mμm. The dry tendon was biochemically analyzed for hexosamine using the technique of Shetlar *et al.* (1972) prior to the Blix (1948) procedure. All absorbance values were determined in a Gilford model 2000 spectrophotometer.

Data analysis

Data from the tendons of both the left and right rabbit paws were pooled to yield mean control (intact) or experimental (wounded) tendon values for each animal. These individual animal mean values were again pooled to give 5, 7, 9, 11, and 13 day mean group values for the control and wounded animal tendons. Ratio values of experimental and control properties were determined utilizing complete data from each animal. Statistical significance ($p < 0.05$) between independent group means across the five healing periods were determined by least squares linear regression whereas differences within dependent groups from the same healing period were determined by paired t tests ($p < 0.05$).

RESULTS

Physical and mechanical measurements

During the first two week test period, union of tendon stump ends were effected by the progress of increased vascularization and tissue proliferation accompanied by swelling and edema. The transected ends retracted incrementally from 2 to 3 mm (gap region) with the regenerated tissue deposited over a length of about 1.5 cm. Cross-sectional area measurements were nearly 7 to 10 times greater within the gap region of the transected tendons compared to the average of the distal and proximal ends of the control group specimens throughout the five healing periods, Table 1. After desiccation, the extent of tissue bulbous measured by dry tendon weight, Table 2, was less, an increase of about twice its respective control. The wounded and control tendon group values for cross-sectional area and dry weight remained constant over 5 through 13 day healing periods. A comparison of the specimen dry weight and cross-sectional area, Figures 2 and 3, showed significant correlation with the control group ($r = 0.64$) and wounded group ($r = 0.66$) encompassing the five healing periods. The regression coefficients were 17.26 mg/mm^2 and 4.24 mg/mm^2 for the control group and the wounded group, respectively, indicating the wounded group increased four times in cross-sectional area for each gram of synthesized tissue dry weight when compared to the average control group over the time periods studied.

Figure 4 shows a typical thermic denaturation diagram for each tendon group. As the physiological saline bath was heated from 40°C with the tendon specimen held at a 980 dyn preload, a temperature was reached when the tendon commenced isometric contraction (shrink temperature, *Ts*). The contraction force developed slowly at first, followed by a rapid linear rise with increasing bath temperature. A plateau phase ensued for both groups where the rate of development of the contraction force decreased with increasing temperature followed by the failure of the control group tendon (maximum isometric thermic denaturation tension, *Lm*) and the initiation of a second contraction phase in the wounded group tendon. The intersection of the slope of the first plateau with the slope of the secondary contraction was considered a measure of the yield phenomenon (isometric thermic denaturation yield tension, *Ly*). As the bath temperature increased further, the contraction force of the wounded group tendon increased somewhat linearly again but at a lower rate than the initial linear rise. Later, a second plateau was reached whereupon the specimen began rupturing (maximum isometric ther-

Table 1. Tendon cross-sectional area between the average of the distal and proximal ends of the control group and the gap region of the experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days)		5		7		9		11		13	
Tendon specimen		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Cross-sectional area (mm^2)		0.34	2.13	0.42	3.15*	0.46	3.12*	0.41	3.92*	0.41	3.35*
	S.D.	0.13	0.60	0.10	1.01	0.09	1.24	0.10	1.54	0.08	1.48
	N	4	3	3	5	4	4	4	4	4	4
Ratio Exptl/Control		9.4		8.1		6.8		9.9		8.3	
	S.D.	—		1.2		2.3		3.9		3.6	
	N	1		3		4		4		4	

* Statistical significance ($p < 0.5$) between control and experimental group indicated.

Table 2. Dry tendon weight between control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days)		5		7		9		11		13	
Tendon specimen		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Dry weight (mg)		6.69	11.43*	8.21	15.14*	8.05	13.70*	7.34	14.27*	7.45	14.34*
	S.D.	1.02	2.53	1.36	2.87	1.38	2.62	1.10	4.20	0.88	3.33
	N	8	4	7	7	6	6	5	5	6	6
Ratio Exptl/Control		1.71		1.89		1.73		1.95		1.97	
	S.D.	0.30		0.45		0.36		0.49		0.65	
	N	4		7		6		5		6	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

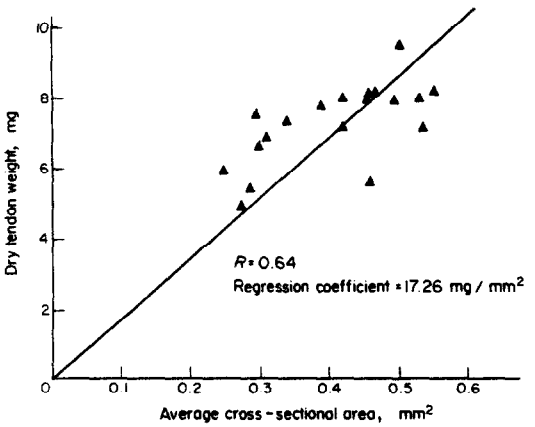

Fig. 2. The relationship of dry tendon weight to average cross-sectional area of the control tendons.

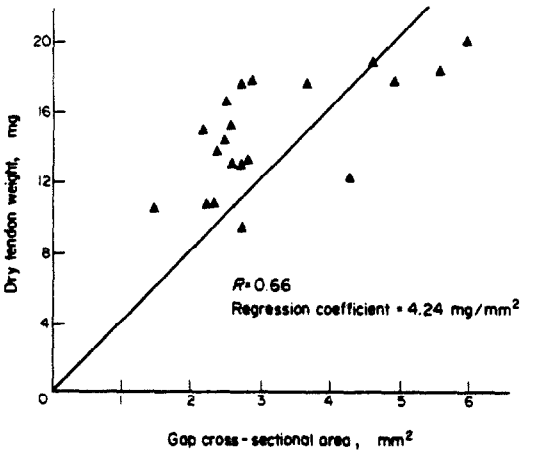

Fig. 3. The relationship of dry tendon weight to gap cross-sectional area of the experimental (wounded) tendons.

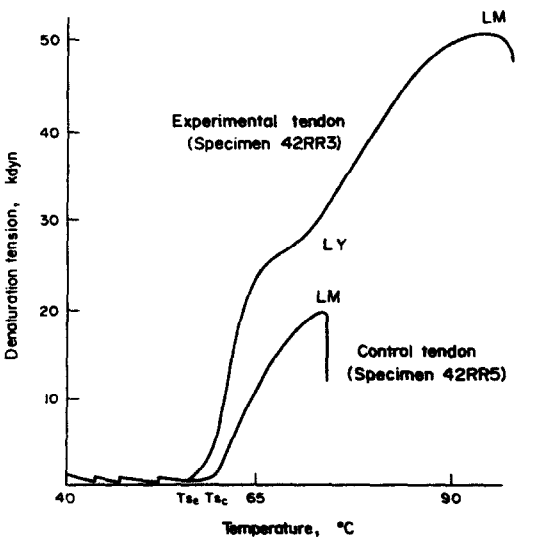

Fig. 4. Typical curves of isometric thermic denaturation tension with change in temperature.

mic denaturation tension, *Lm*). The distinguishing feature of the contraction force–temperature response of the wounded tendon group was the nature of its bimodal shape with much higher denaturation tensions.

Ts data, presented in Table 3, were lower in the wounded tendon group when compared to its corresponding control group at all healing periods, except day 5, with significant differences at days 7 and 13. No change was indicated in the control tendon group over the chosen time period.

The slope of the isometric thermic denaturation curve between the load range of 5 and 15 kdyn, Table 4, was significantly higher in the wounded tendon group at the 7, 9, 11, and 13 day healing intervals with the slope ratio of wounded to control increasing to day 11. No change was evident in the slope of the control tendon group throughout all healing periods. When the slope was normalized for dry tendon weight, slope', the wounded tendon group was less than its corresponding control prior to the ninth healing period, thereafter, the wounded group surpassed its control. No significant differences between the two groups were shown. When utilizing tendon cross-sectional area per unit dry weight, the adjusted slope' of the wounded tendon group was always less than its respective control and only approached 30% (1.17/4) of the control tendon group maximum value, i.e. between the 9 and 11 day healing periods.

Table 5 presents the compiled data for *Ly* of the wounded tendon groups with *Lm* of the corresponding control tendon groups. Significant least squares linear regression for the ratio *Ly/Lm* ($r = 0.73$) was determined from the fifth through the thirteenth healing period. A ratio greater than one which commenced at the ninth day was indicative of a higher wounded tendon group thermic yield tension compared to the respective control tendon group maximum thermic tension. There was no change in the control tendon group *Lm* over the healing periods studied. Normalization of *Ly* and *Lm* by dry tendon weight, *Ly'* and *Lm'* indicated significant least squares linear regression for the *Ly'/Lm'* ratio ($r = 0.72$) across the healing periods with a ratio less than one. Only at the 5 and 7 day healing periods were there significant group differences. Since the wounded tendon group was four times greater in cross-section/g of dry tissue than its corresponding control, the *Ly'/Lm'* ratio was further reduced and was only 22% (0.91/4) of the control group maximum thermic denaturation tension after 13 days.

A significant linear regression was apparent for the ratio of wounded group *Lm* to control group *Lm* ($r = 0.68$) throughout all healing periods, denoted in Table 6. The wounded tendon *Lm* was greater than its respective control after only 7 days of healing and significantly different from its respective control group at 9 and 13 days of healing. *Lm'* ratio also revealed significant least squares linear regression of wounded group to control group ($r = 0.70$) over the 5, 7, 9, 11,

Table 3. Shrink temperature, *Ts*, between control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days) / Tendon specimen		5 Control	5 Exptl	7 Control	7 Exptl	9 Control	9 Exptl	11 Control	11 Exptl	13 Control	13 Exptl
Ts (°C)		59.94	61.14	60.58	59.11*	59.38	59.23	59.42	58.60	59.32	57.52*
	S.D.	2.38	2.48	1.50	0.35	0.91	1.47	0.91	0.70	0.25	0.65
	N	8	4	7	7	6	6	5	5	6	5
Ratio Exptl/Control		1.04		0.98		1.00		0.99		0.97	
	S.D.	0.05		0.02		0.02		0.02		0.01	
	N	4		7		6		5		5	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

Table 4. Slope of the isometric thermic denaturation curve between a 5 to 15 kdyn load range for control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days) / Tendon specimen		5 Control	5 Exptl	7 Control	7 Exptl	9 Control	9 Exptl	11 Control	11 Exptl	13 Control	13 Exptl
Slope of denaturation curve (kdyn/°C)		1.67	1.78	1.96	2.74*	1.83	3.23*	1.87	3.94*	2.05	3.75*
	S.D.	0.48	1.13	0.59	0.49	0.72	1.03	0.77	0.92	0.89	0.91
	N	8	4	6	6	6	6	5	5	6	6
Ratio Exptl/Control		1.33		1.51		2.06		2.38		2.11	
	S.D.	0.81		0.50		0.99		1.01		1.05	
	N	4		6		6		5		6	
Slope of denaturation curve per dry tendon weight (kdyn/mg/°C)		0.250	0.156	0.253	0.179	0.221	0.241	0.256	0.280	0.274	0.261
	S.D.	0.054	0.104	0.069	0.042	0.057	0.091	0.093	0.036	0.111	0.034
	N	8	4	6	6	6	6	5	5	6	6
Ratio Exptl/Control		0.78		0.75		1.17		1.17		1.08	
	S.D.	0.50		0.25		0.51		0.31		0.39	
	N	4		6		6		5		6	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

Table 5. Maximum thermic denaturation tension, *Lm*, for the control group specimens and the thermic yield tension, *Ly*, for the experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days) / Tendon specimen		5		7		9		11		13	
		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Control *Lm* or Exptl *Ly* (kdyn)		21.47	8.28	25.94	15.64	23.46	24.66	23.54	24.88	20.58	30.38
	S.D.	5.16	8.59	9.00	6.62	9.01	6.74	13.19	5.56	7.26	6.36
	N	6	4	5	5	5	5	5	5	4	6
Ratio Exptl/Control		0.27		0.57		1.12		1.31		1.61	
	S.D.	0.39		0.25		0.35		0.61		0.64	
	N	3		4		4		5		4	
Control *Lm* or Exptl *Ly* per dry tendon weight (kdyn/mg)		3.34	0.67*	3.39	1.03*	2.96	1.73	3.23	1.72	2.81	2.16
	S.D.	0.52	0.68	1.19	0.36	0.83	0.40	1.63	0.27	0.92	0.40
	N	6	4	5	5	5	5	5	5	4	6
Ratio Exptl/Control		0.17		0.28		0.60		0.61		0.91	
	S.D.	0.26		0.08		0.18		0.20		0.45	
	N	3		4		4		5		4	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

Table 6. Maximum thermic denaturation tension, *Lm*, between control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days) / Tendon specimen		5		7		9		11		13	
		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Lm (kdyn)		21.47	12.68	25.94	25.70	23.46	37.24*	23.54	45.06	20.58	48.78*
	S.D.	5.16	13.92	9.00	11.28	9.01	6.15	13.19	13.90	7.26	15.55
	N	6	4	5	5	5	5	5	5	4	6
Ratio Exptl/Control		0.38		1.01		1.78		2.43		2.25	
	S.D.	0.58		0.59		0.61		1.28		0.49	
	N	3		4		4		5		4	
Lm per dry tendon weight (kdyn/mg)		3.34	1.01	3.39	1.62*	2.96	2.62	3.23	3.14	2.81	3.41
	S.D.	0.52	1.07	1.19	0.59	0.83	0.40	1.63	0.42	0.92	0.79
	N	6	4	5	5	5	5	5	5	4	6
Ratio Exptl/Control		0.24		0.48		0.95		1.16		1.32	
	S.D.	0.38		0.23		0.24		0.47		0.71	
	N	3		4		4		5		4	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

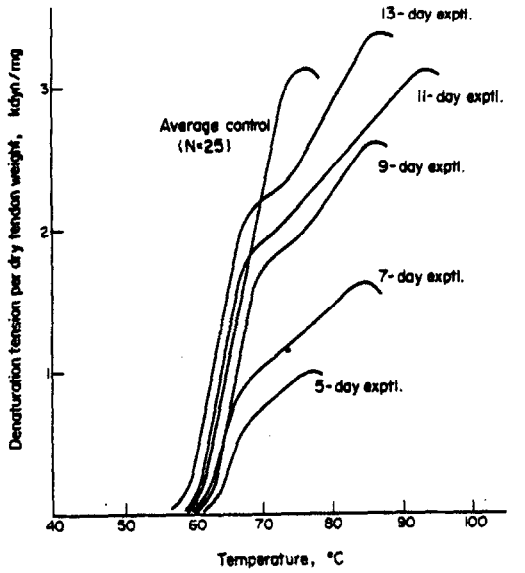

Fig. 5. Summary of the relationship of isometric thermic denaturation tension to temperature for the average control tendon group and the experimental (wounded) tendon groups at various time intervals.

and 13 day healing periods. The wounded tendon group *Lm'* was less than its respective control tendon group for the first 9 days of healing, and significantly different from its control at day 7. When tendon cross-sectional area per dry weight differences were applied, the adjusted wounded tendon group *Lm'* progressed from 6, 12, 23, 28, to 32% of its respective normalized control tendon group from 5 to 13 days of healing.

A summary of isometric thermic denaturation diagrams are presented in Fig. 5, expressed as load per dry weight.

Biochemical measurements

Protein determinations on the physiological saline bath disclosed significant differences between the two tendon groups at each healing period with the concentration of the wounded tendon group about three to four times greater than its respective control, Table 7. No change in the control tendon group protein level was evident throughout the five healing periods. Normalizing protein concentration by specimen dry weight lessened the increase of the wounded tendon group, up to about twice its respective control for all healing periods with significant differences at the healing periods of 7, 9, and 11 days.

Hydroxyproline concentration levels in the physiological saline bath increased in a similar manner with protein changes, Table 8, increasing three to five times in the wounded tendon group compared to its respective control with significant differences between the two groups at all healing periods. No change was found in hydroxyproline concentration for the control tendon groups at all healing periods studied. Utilizing dry tendon weight to normalize hydroxyproline levels moderated the increase of the wounded tendon group from about twice to three times its corresponding control tendon group. Significant differences remained between the two groups at 7, 9, and 11 days. The ratio of protein concentration to hydroxyproline concentration for the wounded tendon group compared to its respective control group was nearly 1 : 1 throughout all healing periods. When hydroxyproline concentration was multiplied by its molecular weight (131.13) and both hydroxyproline and protein concentration levels divided by dry tendon weight, the ratio was not significantly different from 7.46, the collagen equivalency factor, for both groups at all healing periods, except wounded tendon group at the fifth healing day.

Uronic acid and hexosamine determinations on the physiological saline bath indicated concentration levels below the sensitivity of the procedures, i.e. less than 0.022 μmole/ml uronic acid, 0.023 μmole/ml hexosamine.

Hexosamine levels within the individual tendons were about 2.5 times greater in the wounded tendon group compared to its respective control tendon group at all healing periods, Table 9. Significant differences between the two groups were evident throughout the healing periods with no change in the control tendon group hexosamine concentration. Hexosamine levels relative to dry tendon weight also indicated significant changes at all post-surgical healing periods with a consistent ratio of the wounded tendon group to its respective control tendon group of 1.4 : 1, somewhat less than the non-normalized ratio.

DISCUSSION

The wound healing process of rabbit extensor digitorum communis tendon was observed to regenerate in a manner described by Mason and Shearon (1932), Mason and Allen (1941), Fernando and Movat (1963) and Clayton *et al.* (1968). Splinting a tendon prior to transection effected: (a) a consistent initial gauge length, i.e. direct juxtaposition; (b) maintained end-to-end apposition with no overlap; (c) minimized retraction due to the softening of the tissues and diminution in holding power of the tendon for the suture within the first five days of healing observed by Mason and Allen (1941); and (d) provided direct measurement of mechanical properties of the healed tendon itself, not in combination with suture. The role of tension in inducing quantitative and qualitative changes in healing tissue has been reported (Forrester, 1973). Although the tension stimulus may have been somewhat minimized at the wound site by splinting when compared to sutural attachment, its effect was evident considering the gradual tendon end retraction from the fifth through thirteenth healing day caused by tonicity of the extensor *digitorum communis* muscle. Mechanical property development has been shown to occur even under more restricted closure methods (Williams and Harrison, 1977).

New tissue formed at the transection site of the wounded tendon group indicated a greater increase

Table 7. Protein concentration between control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days)		5		7		9		11		13	
Tendon specimen		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Protein concentration (μg/ml)		23.76	88.08*	24.93	90.53*	29.96	96.89*	25.30	99.85*	27.52	90.13*
	S.D.	8.69	4.08	10.03	13.75	13.56	31.17	9.71	21.37	5.82	28.40
	N	7	3	7	5	6	5	5	5	5	6
Ratio Exptl/Control		2.90		3.64		3.26		4.30		3.53	
	S.D.	0.18		1.25		1.02		1.43		1.16	
	N	2		5		5		5		5	
Protein concentration per dry tendon weight (mg/g/ml)		3.86	7.54	3.19	6.14*	3.78	7.09*	3.67	7.46*	3.70	7.07
	S.D.	1.86	1.58	1.31	0.44	1.49	2.42	2.10	2.26	0.61	3.31
	N	7	3	7	5	6	5	5	5	5	6
Ratio Exptl/Control		1.75		1.86		1.87		2.27		2.21	
	S.D.	0.29		0.59		0.69		0.72		1.39	
	N	2		5		5		5		5	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

Table 8. Hydroxyproline concentration between control group and experimental (wounded) group specimens removed at various healing periods after surgery

Healing period (days)		5		7		9		11		13	
Tendon specimen		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Hydroxyproline concentration (μmole/ml)		0.0247	0.1064*	0.0280	0.0979*	0.0302	0.1262*	0.0246	0.1194*	0.0312	0.1138*
	S.D.	0.0102	0.0023	0.0166	0.0434	0.0207	0.0573	0.0113	0.0384	0.0123	0.0586
	N	7	3	7	5	6	5	5	5	5	6
Ratio Exptl/Control		3.06		3.46		4.21		5.22		4.56	
	S.D.	0.47		1.68		1.81		1.96		3.15	
	N	2		5		5		5		5	
Hydroxyproline concentration per dry tendon weight (μmole/g/ml)		4.05	9.07	3.60	6.39*	3.82	9.25*	3.62	9.05*	4.18	9.07
	S.D.	2.19	1.63	2.12	2.25	2.33	4.37	2.46	3.78	1.38	6.02
	N	7	3	7	5	6	5	5	5	5	6
Ratio Exptl/Control		1.86		1.75		2.43		2.89		3.08	
	S.D.	0.48		0.76		1.15		1.39		3.06	
	N	2		5		5		5		5	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

Table 9. Hexosamine concentration within the individual tendon specimens between the control group and the experimental (wounded) group removed at various healing periods after surgery

Healing period (days) / Tendon specimen		5		7		9		11		13	
		Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl	Control	Exptl
Hexosamine concentration (μmole/ml)		0.0739	0.1800*	0.0887	0.2214*	0.0802	0.1998*	0.0820	0.2188*	0.0939	0.2361*
	S.D.	0.0098	0.0508	0.0165	0.0721	0.0154	0.0567	0.0204	0.0506	0.0106	0.0452
	N	7	4	7	6	5	6	5	5	5	6
Ratio Exptl/Control		2.44		2.73		2.48		2.83		2.79	
	S.D.	0.71		1.28		1.04		0.92		0.25	
	N	4		6		5		5		5	
Hexosamine concentration per dry tendon weight (μmole/g/ml)		11.50	15.63*	10.86	14.78*	10.37	14.68*	11.28	15.80*	12.20	17.74*
	S.D.	0.89	1.24	1.82	2.90	0.55	2.50	2.16	1.98	1.61	3.92
	N	7	4	7	6	5	6	5	5	5	6
Ratio Exptl/Control		1.40		1.41		1.41		1.43		1.59	
	S.D.	0.21		0.38		0.31		0.24		0.42	
	N	4		6		5		5		5	

* Statistical significance ($p < 0.05$) between control and experimental group indicated.

over its corresponding control when using cross-sectional area as the measurement compared to dry tendon weight. Tissue swelling due to edema enhanced the created bulbous cross-section, also reported by Sussman (1973), but was eliminated when the tendon tissue was dehydrated. This high water content accentuated the cross-sectional area measurement. Because cross-sectional area was so well correlated to dry tendon weight for both the wounded and control tendon groups, normalization of strength measurements to cross-sectional area was attainable to more accurately reflect the true physical state of the tendon.

The data showed the fifth healing day to be different from the other healing periods, inconsistent *Ts* considering the immaturity of the regenerated tissue, high mechanical property variability, and a tissue synthesis with protein and hydroxyproline in a non-collagenous ratio. Accordingly, day 5 may be the shortest possible healing test day using the tendon splint surgical technique. This is in agreement with the quiescent or latent period of healing reported in the literature (Harkness, 1968).

During the process of fiber formation, collagen molecules aggregate through hydrogen bonding, (Gustavson, 1956), largely between hydroxyl groups of hydroxyproline and keto-imide groups of adjacent helices. Thermal agitation disrupts these chemical bonds producing fiber shortening due to folding of the collagen chains and a rise in isometric thermic denaturation tension. Isometric thermic denaturation behavior beyond the shrink temperature is a reaction to disruption of high energy covalent bonds Verzar (1963, 1964a, 1969), which progressively form during fibrogenesis, Jackson (1958). Continued heating destroys all covalent bonds, whereby the fibers relax to their initial length or rupture (Verzar, 1963, 1964a, 1969).

The isometric contraction force developed in response to hydrothermal shortening for both wounded and control group tendons supported the documentation by Rigby (1964) on rat tail tendon heated just past the shrink temperature. Physical and mechanical property differences between the biphasic (normal and wounded) tissue sections of the wounded group tendons resulted in a biomodal isometric thermic denaturation curve. Because the wounded cross-section was edematose, containing large amounts of fluid, its mechanical behavior would be greatly influenced by the viscous component in rate dependent phenomenon like the process of thermal shrinkage. The normal tendon section was composed of ordered bundles of individual, white, non-branching fibrils orientated in parallel arrangement along its length; contrasted with the healed section comprised of a haphazard arrangement of fibrils, cells synthesizing collagen and acid mucopolysaccharide, cellular slough, and edema. Concomitant with the physical mass and dimensional differences between the wounded and control group tendons were disparities in the isometric thermic denaturation properties and the biochemical composition.

Lower shrink temperatures found for wound collagen were consistent with the random fiber meshwork observed in healing tendon using light and electron microscopy, Mason and Shearon (1932), Fernando and Movat (1963) and Rokkanen and Vainio (1971), and the lower effective number of hydrogen bonds associated with this immature unorganized structure. The data further supports the findings reported by Holm-Pedersen and Viidik (1972), and Viidik *et al.* (1972) on 8 and 21 day old rat skin wounds compared to adjacent skin using light microscopy to detect collagen fiber birefringence loss. Comparing the shrink temperatures of tendons from very old animals (greater than 1.5 years old) used in the preliminary work of this study with that of the control tendon group indicated a similar maturation relationship, old animal tendons had a greater denaturation temperature.

The slope of the isometric thermic denaturation curve was a measure of the highly accelerated rate of shrinkage following the melting of the hydrated crystallites. Wounded group tendons contracted at a slower rate than their respective control group tendons when comparisons were made relative to tendon cross-sectional area, indicative of thermal disruption of chemical bonds for a connective tissue of poor molecular arrangement. Although the energy of activation was higher in the control group tendon to initiate contraction, i.e. greater *Ts*, the increased fiber shortening rate in this tendon group resulted from a greater atomic and molecular collision frequency and an increased fraction of collisions that had proper orientation.

During the wound healing process increased numbers of intermolecular covalent bonds of the animo acid polymer chains develop resulting in stronger anatomical configuration and a change in the mechanical and chemical properties of the collagen fibers. Because covalent cross-links resist thermal denaturation, *Lm* was used as a measure of the number and strength of these newly formed chemical bonds. The increasing *Lm* with wound age found in the present investigation supported this concept. Aging in mammals has also been determined to involve the progressive formation of molecular covalent cross-linkages in collagen, Curtis (1963), Chvapil and Deyl (1964), Jackson and Steven (1969), and Bailey (1974), with a resulting increase of thermic denaturation tension with age (Boros-Farkas and Everitt, 1967; Takacs and Verzar, 1968; Verzar and Zs.-Nagy, 1970). Lower *Lm* for the wounded group tendons utilizing cross-sectional area to correct for hydration was further evidence of the immaturity of the healed tendon groups compared to their control.

An increase in the amount of cross-linking in collagen fibers found to be concurrent with maturation also resulted in tensile strength gains of aged wounds (Howes *et al.*, 1929; Dunphy and Udupa, 1955; Geever *et al.*, 1965; Hamilton *et al.*, 1970). The corresponding development of both maximum thermic denaturation

tension and tensile strength as a consequence of collagen maturation supported the use of the former as a measure of tendon load carrying capacity. Accordingly, since the wounded tendon group *Lm* reflecting adjustments for cross-section was always less than its corresponding control group, and only one-third that of the control group after 13 days of healing, the full load carrying capability of the wounded tendon group was never realized during the healing interval studied.

The release of the salt-soluble collagen extract during thermal shrinkage, measured as hydroxyproline and protein concentrations, increased with healing time in the wounded tendon group relative to the control group in a corresponding way with *Lm*. High levels of salt-soluble collagen were consistent with fibrogenesis in regenerating tendon, collagen fiber maturation gave support for increased denaturation tension values. Concomitantly, Dunphy and Udupa (1955) and Madden and Peacock (1971) reported good correlation between tensile strength gain and the appearance of skin wound collagen during the same healing interval. After three weeks of healing, however, Madden and Peacock (1971) reported stabilization of the wound collagen content with subsequent loss of the relationship between tensile strength and the quantity of scar collagen. The consistent salt-extractable collagen and maximum thermic denaturation tension of the control group tendons were indicative of the ultimate trend. Due to the progressive formation of intermolecular covalent bonds and the associated increased structural alignment of the collagen fibers during wound healing, it seems probable that the salt-extractable collagen would diminish in a similar fashion to aging collagen, Verzar (1964b), Takacs and Verzar (1968), Everitt *et al.* (1970), and asymptotically approach the low extractability level of uninjured animal collagen. An accumulation of insoluble collagen from mouse granulation tissue on the tenth and twelfth post-surgical days, Hosoda (1960), gives credence for this hypothesis.

Thermic denaturation of both the wounded and control tendon groups did not reveal any labile mucopolysaccharide, i.e. neutral salt-soluble mucopolysaccharide, unlike that found for collagen. No incidence of uronic acid or hexosamine were found in the physiological saline bath following the thermal test. Since the acid mucopolysaccharides found in tendon are macromolecular compounds made up of hexosamine and hexuronic acid moieties linked by glycosidic bonds, White *et al.*, 1968, this result was consistent with the structure and indicated that the bonds retaining the acid mucopolysaccharides within collagen were highly resistant to thermal agitation.

An increase of 40% in the hexosamine concentration level per unit of dry tendon weight was observed after 5 days of healing and gradually increased to an elevation of 59% after 13 days, not unlike that shown by Biro and Bihari-Varga (1972) on rabbit achilles tendon. The greatest amount of hexosamine seemed to be formed prior to the fifth healing day similar to Sadiq *et al.* (1973) in rabbit ear cartilage. Normalization of hexosamine concentration to dry tendon weight, however, probably underestimated the quantitative increase in the experimental tendon group relative to its corresponding control group since the biosynthesis of new tissue was deposited over a wound length of about 1.5 cm or 30% of the length of the excised wounded tendon. Correcting for wound length, the amount of hexosamine formed within the wounded tendon group would be nearly 5 times that of its respective control group, similar to Delaunay and Bazin (1964) who indicated in their review article that maximum hexosamine levels may reach four times that found in healthy tissue. The present data indicated that the increase level of acid mucopolysaccharides occurred concurrently with the high level of collagen, giving further support of their interaction to augment collagen tensile strength, as previously shown by Jackson (1953), Bryant and Weeks (1967), and Munro *et al.* (1970).

CONCLUSIONS

The tendon splint surgical procedure was successfully employed on regenerating rabbit tendon eliminating some difficulties previously associated with alignment, retention, and testing. The earliest healing test interval for mechanical property measurement using this technique was determined to be the fifth day. Denaturation characteristics reflected developmental changes in physical mass and dimension, fiber orientation and maturity, extracellular constituency, and viscous supporting medium. Maximal thermic denaturation tension, like maximum tensile strength, can be used as a measure of the effective number of newly formed chemical bonds within healing tissue. Normalization of strength measurement data to specimen cross-section must be utilized to more accurately reflect the load bearing characteristics.

Physical and mechanical property changes during the fifth through thirteenth post-surgical day were found to occur concurrently with the biosynthesis of collagen and mucopolysaccharides. The architectural changes in the collagen matrix were a direct result of progressive aggregation of the constituent polypeptides. The restoration of form and function ultimately being dependent on the total integration of all structural components.

Characterization of the wound healing process using the unique properties encompassed within the developing collagen structure to ascertain tissue mechanical properties might be a more descriptive measure of the rate and quality of remodeling. Correlation of the previous biochemical and biomechanical factors could lead to development of a mathematical representation of the healing process. This will be explored in a subsequent report.

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NOMENCLATURE

Ts	Shrink temperature, °C
slope	Denaturation curve slope, kdyn/°C
slope′	Normalized denaturation curve slope, kdyn/mg/°C
Ly	Thermic yield tension, kdyn
Ly′	Normalized thermic yield tension, kdyn/mg
Lm	Maximum thermic denaturation tension, kdyn
Lm′	Normalized maximum thermic denaturation tension, kdyn/mg.