

HEALING OF THE MEDIAL COLLATERAL LIGAMENT OF THE KNEE

A Morphological and Biochemical Assessment in Rabbits

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Complete midsubstance injuries of medial collateral ligaments of matched New Zealand white rabbits were allowed to heal without repair or immobilization for various lengths of time. Morphological and biochemical parameters were used to evaluate healing as compared with normal unoperated and sham operated ligaments. Results showed incomplete healing at the longest term follow-up (14 weeks) with significant biochemical abnormalities. Although there was a trend toward normal, recovery from injury was much slower than previously reported.

Key words: healing; ligament

Accepted 25.v.83

Collateral ligament healing has been studied in animal models for many years (Miltner & Hu 1933, Miltner et al. 1937, Jack 1950, O'Donoghue et al. 1961, Clayton et al. 1968, Booth & Tipton 1970, Fukubayashi et al. 1980, Vailas et al. 1981). Various animal species and methods of injury, treatment and rehabilitation have historically been assessed by a number of different methods. Morphological descriptions (Miltner & Hu 1933, Miltner et al. 1937, Jack 1950, O'Donoghue et al. 1961, Clayton et al. 1968), tensile testing (O'Donoghue et al. 1961, Clayton et al. 1968, Fukubayashi et al. 1980), and biochemical parameters (Tipton et al. 1978) have been used to study healing in the early months after injury, and have identified several factors which may affect the healing processes.

Increased awareness of the complex interactions which influence both normal and healing ligaments, however, have created important problems in interpreting previous data. Evidence

suggests that other factors must be more carefully controlled, including animal ages, weights and sexes (Booth & Tipton 1970, Tipton et al. 1978) in order to standardize results. Similarly, variables such as activity, immobilization (Akeson et al. 1980) and repair should not be mixed, since the possibility of additive, synergistic or antagonistic effects cannot be separated. In other words, there is a need to establish a model in which these proven variables are minimized to serve as a baseline for tests of treatment effectiveness at many post-injury intervals.

Our purpose in this paper is to document such a baseline, using gross morphology and biochemical indices of early ligament healing in a simple animal model. Indices were chosen according to those most drastically and consistently altered in the early phases of healing wounds (Dunphy & Udupa 1955) and those which would supply data on both cellular response and matrix composition.

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MATERIAL AND METHODS

Animal model

The model selected was the ruptured and non-repaired medial collateral ligament in the male New Zealand white rabbit. Twenty-four animals weighing from 3.0 to 3.5 kg were studied. Under general anesthesia and with sterile technique, the medial collateral ligaments (MCL's) of both hind limbs were exposed through longitudinal medial incisions in the skin and fascia. Fascial incisions were medial to the ligament to facilitate fascial covering for the ligament at the conclusion of the procedure. The right leg of each animal was designated as the experimental and its MCL ruptured by passing a 3-0 braided steel suture beneath it at the level of the suprameniscal recess (approximately 2 mm distal to a consistent transverse superficial vein on the ligament) and failing it at this point with a strong upward pull on both ends of the suture. Ligament ends were allowed to retract and fascia was allowed to cover the injury site. The fascial defect (medial to the covered ligament) was not closed surgically. Skin was approximated with 3-0 continuous nylon sutures supplemented with interrupted mid-incisional reinforcement. The medial collateral ligament in the left leg of each animal was exposed through identical skin and fascial approaches without injury to the ligament. The skin was closed in a similar fashion to the experimentals. The left legs of all operated animals were therefore designated as sham-operated controls.

Postoperatively, all animals were permitted normal and unrestricted activity in standard metal cages (60 cm × 60 cm × 50 cm) for various intervals until their sacrifice. Pairs of normal, unoperated animals were also placed in similar cages at the beginning of the experiment to serve as age, sex, and activity controls of all parameters at each post-injury interval. These pairs were weighed and sacrificed with each of four groups of six experimentals at 10 days, 21 days, 42 days and 98 days post injury.

The central 1 cm of each ligament (5 mm proximal and distal) centered at the injury site in the experimentals, and at comparable levels in shams and unoperated controls, was collected for biochemical analysis (Figure 1).

Biochemistry

a. Water content. The central healing site, as seen in Figure 1, of the medial collateral ligaments at the different healing intervals was quickly dissected. The samples were weighed and dried under P_2O_5 in vacuum to constant weight.

b. Total collagen. Total collagen was determined on the basis of hydroxyproline content (HP). Three to five mg of tissue were hydrolyzed without preliminary purification in 6N HCl for 3 h at 130°C. The acid hydrolysate was treated according to the procedure of

SAMPLE DISTRIBUTION

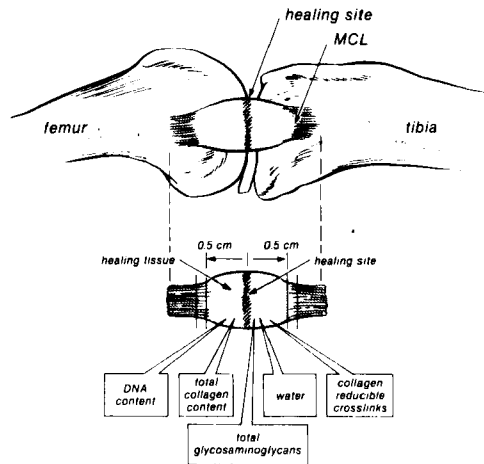


Figure 1. Schematic representation of the healing mid-portion of a medial collateral ligament (MCL) showing the distribution of tissue for analysis.

Woessner (1961), and the amount of HP determined spectrophotometrically at 557 m μ .

c. Collagen cross-linking. Twenty mg of lyophilized tissue from normal, experimental and sham ligaments were used for the detection of aldehydic cross-link precursors and the reducible Schiff base cross-links of collagen (Tanzer & Mechanic 1968, Mechanic & Balazs 1970, Akeson et al. 1977). The ratio of the intermolecular reducible Schiff base cross-links DHLNL to HLNL was used for comparative assessment of the healing and maturity of the tissue collagen formed during the repair (Bailey et al. 1973, Jackson & Mechanic 1974).

d. Total glycosaminoglycans. Total glycosaminoglycans were expressed on the basis of hexosamine content. Twenty mg of lyophilized tissue from normal, experimental and sham ligaments were hydrolyzed at 100°C in a boiling water bath using 6N HCl for 5 h. Hexosamine determinations were performed by the modified Elson Morgan reaction (Elson & Morgan 1933).

e. DNA concentration. Ten mg of lyophilized tissue from normal, experimental and sham ligaments were obtained for determination of DNA concentrations. Tissue cellularity was assessed by measuring DNA concentrations in accordance with a modified procedure of Bonting & Jones (1957). Results were expressed as μ g of DNA per mg of dry tissue. These measurements were used to evaluate changes in cellularity at each interval.

RESULTS

Gross appearance and morphology

The method of injury was consistently reproducible and animals tolerated the procedure well. All returned to normal cage activity within a few days of surgery. There were no definite complications in the animals in this series and all gained weight throughout the periods of observation.

At sacrifice, sham-operated ligaments at all intervals looked essentially normal, with the exception of slight fascial and superficial granulation adherence that gave them a slightly edematous appearance (Figures 2A, B). The tissue sampling technique as described in experimentals included a consistent amount of what could be called "old ligament" at either end, bridged by the healing "new ligament". The healing ligament

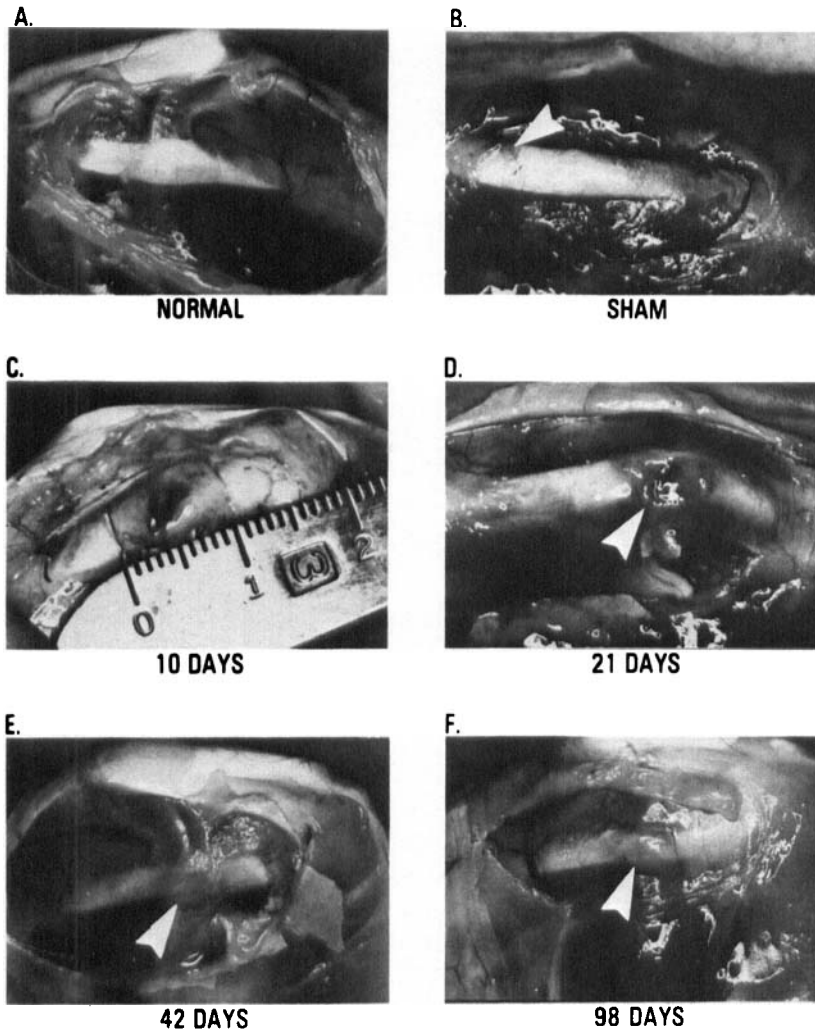


Figure 2. Medial collateral ligaments at various stages of healing. A. Normal unoperated ligament. B. 21 day sham operated ligament (arrow indicates typical adherent granulation tissue on edematous ligament). C. 10 day healing ligament with ruler showing the extent of the early healing response and the central area taken for biochemical analysis. D. 21 day healing ligament (arrow indicates large and diffuse bridging scar). E. 42 day healing ligament (arrow indicates scar area which is adherent to the surrounding capsule). F. 98 day healing ligament (arrow indicates area of scar which has remodelled to some extent, but still does not look completely normal).

mass was consistently larger than both shams and normal ligaments at all intervals, supplying adequate tissue for the number of tests outlined (Figure 2C).

Healing in this model could be subdivided into gross stages similar to those of wound healing, including: inflammation, proliferation and remodelling. Mixed inflammation and proliferation were seen at 10 days with edematous and hemorrhagic granulation tissue bridging the ligament defect and covering much of the "old ligament" (Figure 2C). At 21 days the scar mass appeared greatest but the translucency of the injury defect was still apparent (Figure 2D). Progressive reorganization then obscured the margins of in-

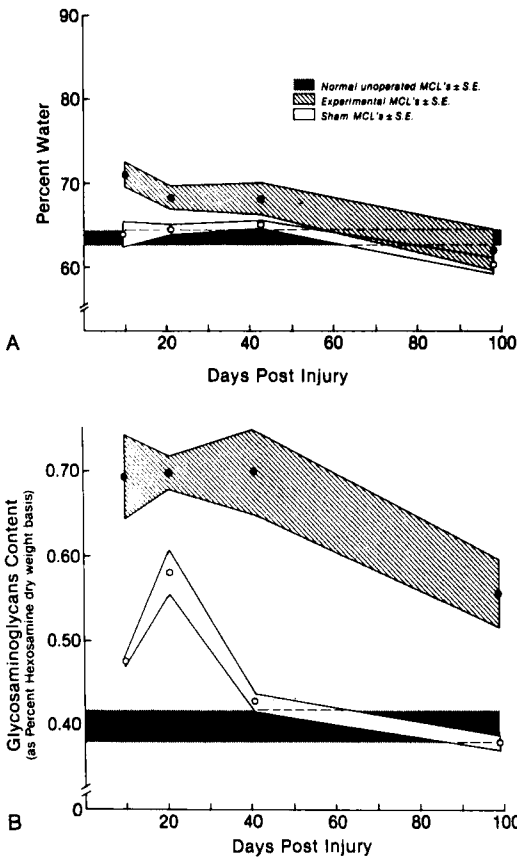


Figure 3. Biochemical results. A. Water content of central ligament site at various intervals, expressed on the basis of as received weight. B. Glycosaminoglycans content of ligaments at various intervals, expressed as percent of hexosamine.

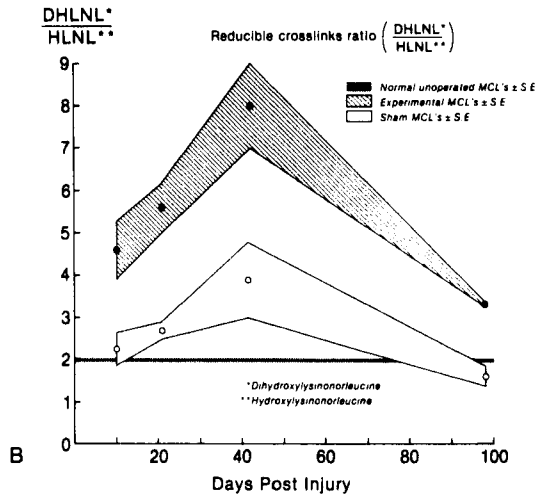
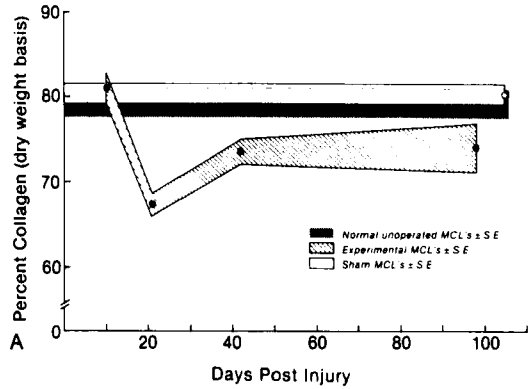


Figure 4. Biochemical results. A. Total collagen content of ligaments at various intervals as calculated by hydroxyproline content. B. Ratios of intermolecular reducible Schiff base cross-links of ligaments, as an index of collagen maturity.

jury within the scar and at 42 days a relatively uniform appearance was noted (Figure 2E). Ligament margins began to reappear as the diffuse superficial scar began to remodel. At 98 days this process had continued but the ligament still did not look completely normal (Figure 2F).

Biochemistry

Water content of the injury site was significantly elevated over shams and unoperated controls at 10 days ($P < 0.002$) and 21 days ($P < 0.02$). By

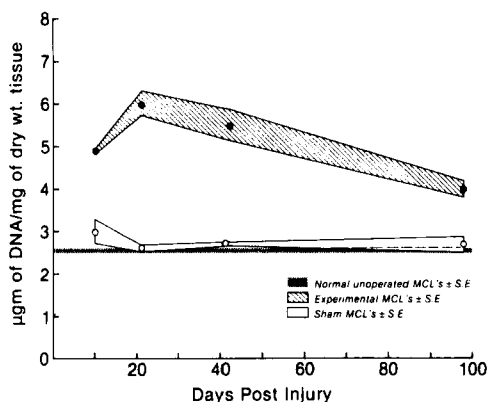


Figure 5. Concentration of DNA in ligaments at various intervals.

42 days, however, water content had returned to normal unoperated values (Figure 3A).

Hexosamine content was also highly significantly elevated early and at all post-injury intervals, with a trend toward normal at 98 days. There was a slight rise in content at 21 days in sham ligaments as well, consistent with the gross watery appearance as noted previously (Figure 3B). Collagen content of the healing site was within normal unoperated limits at 10 days, but had dropped significantly by 21 days ($P < 0.001$). Subsequent intervals revealed a slow return toward sham and normal limits (Figure 4A). Reducible cross-link ratios revealed significant alterations from 10 days to 98 days due to large increases in dihydroxylysinonorleucine (DHLNL). At 10 days, the ratio dihydroxylysinonorleucine to hydroxylysinonorleucine (DHLNL/HLNL) was increased statistically over sham and unoperated controls ($P < 0.05$). At 21 days, the ratio continued to increase, reaching a maximum at 42 days (Figure 4B). At 98 days, the ratio was returning toward normal limits, but remained significantly elevated ($P < 0.01$). Sham-operated animals also showed significant although smaller increases in these cross-link ratios at 21 days ($P < 0.01$) and 42 days ($P < 0.05$) versus unoperated activity and age controls.

Cellularity expressed as DNA concentration of the healing site was significantly elevated at all intervals, reaching a maximum at approximately

21 days (Figure 5). Cellularity remained elevated at 98 days post-injury ($P < 0.01$).

DISCUSSION

The early phases of collateral ligament healing in this model are similar in most respects to previous descriptions of experimental wound healing. While the early trends of gross and biochemical changes demonstrated by these results are similar to those described in skin (Dunphy & Udupa 1955, Jackson 1958), they clearly do not represent an end point, as values were still changing throughout the period of study. Water content is elevated in the early stages of ligament healing as part of the general processes of inflammation (Delaunay & Bazin 1964) and increased production of glycosaminoglycans. Proliferation of fibrovascular granulation tissue is seen grossly by 10 days after injury. This proliferation of healing tissue continues by gross estimation and increasing DNA values until 3–6 weeks. Increased cell numbers or size are still present in the healing site at 14 weeks (98 days) post-injury, however, with only a trend toward normal unoperated values.

Collagen maturation in the designated healing site, as measured by reducible cross-link ratio (Bailey et al. 1973), correlates with the observed cellular changes ($R = 0.78$). Collagen synthesis was apparently maximal at 3–6 weeks (highest cross-link ratios and DNA content) showing a subsequent slow decline toward normal.

Overall collagen content of the healing area shows a marked decrease until 21 days, at which time the trend is to return slowly toward normal limits. While collagen synthesis is elevated at this interval, as discussed above, there is apparently a simultaneous and greater increase in collagen degradation taking place. This balance has also been described in healing wounds (Delaunay & Bazin 1964). Although our study suggests that this increased turnover (synthesis and degradation) is maintained for at least 14 weeks, more specific quantitative techniques should be used to test this possibility.

Most importantly, we have established a multi-parameter baseline collateral ligament healing for a simple animal model. This baseline suggests

that, in a rabbit model, non-repaired and non-immobilized ligaments heal by diffuse and bridging scar formation that is remodeled with time. Synthesis of collagen necessary for scar remodelling combined with changes in "ground substance" are still taking place at 14 weeks, suggesting that these processes are much more prolonged in healing ligaments than initially thought (Miltner & Hu 1933, Miltner et al. 1937, Jack 1950, O'Donoghue 1955). The clinical implication of this observation is that, while the early period of ligament healing is clearly important, therapeutic manipulations (e.g. rehabilitation) may be necessary and effective for longer periods of time.

Another important observation is the possible influence of local inflammation, as induced by the sham operation, on ligament turnover. Increased reducible collagen cross-link (DHLNL/HLNL) ratios and increased glycosaminoglycans (GAG) contents in the sham at 21 days and 42 days suggest increased collagen and GAG synthesis at those intervals. Since collagen content is unchanged, however, simultaneous degradation is implied. This would suggest a somewhat surprising sensitivity of mechanically uninjured ligament tissue to local conditions (inflammation). Mechanisms and implications to mechanical and functional properties of this increased ligament turnover require further study.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Alberta Heritage Foundation for Medical Research, the Orthopaedic Research and Education Foundation and San Diego Veterans Administration Medical Center for their financial support. We would also like to thank Mr. F. L. Harwood, Mr. M. Furniss and Dr. J. Fronck for their technical assistance.

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