THE UNIVERSITY OF CALGARY

Biomechanical Creep of Rabbit Medial Collateral Ligament Autografts

by

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ABSTRACT

The "stretching out" of ligament grafts, otherwise known as graft creep appears to be a cause of clinical joint instability after at least 10-30% of ligament reconstructions. The short term objectives of this project were to test the hypotheses (in a rabbit medial collateral ligament (MCL) autograft model) that ligament grafts become increasingly susceptible to creep with healing time, and that immobilization of a joint after surgery decreases this susceptibility. The creep properties of these experimental grafts were determined at low physiological stress levels using a servo-hydraulic material testing system. Our results revealed that these autografts became more vulnerable to creep than normal MCLs after only 2 days ($p=0.0007$), and creep increased significantly between 2 days and 3 weeks ($p<0.05$). Immobilization further increased the susceptibility of the grafts to creep at 3 and 8 weeks ($p=0.0007$). All grafts were found to be less able to recover from creep strain than the normal MCL. These results could have significant implications to rehabilitation protocols after soft tissue reconstructive surgery.

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CHAPTER I

Background and Literature Review

Ligament Structure, Function, and Injury

Ligaments are organized bands of dense, specialized connective tissue which connect bones to bones. They function to guide and limit the motion of joints (41,54). Ligament structure has been found to be different to tendons, with a complex hierarchical pattern of collagen fibrils and bundles, which allow for progressive fiber recruitment with increasing loads (13,54,133). It is also well documented that different fiber bundles take up variable amounts of load at different joint positions, thus allowing the ligament to function throughout the range of motion of a joint (13,52,54). The collagen bundles themselves display a crimping pattern which is thought to give ligaments the ability to resist repetitive elongation without collagen fiber damage (41,49,86,140). Ligaments are most often exposed to repetitive loads well below their ultimate tensile strength (66). It has been estimated that ligaments carry loads ranging from 5-25% of their ultimate tensile failure strength during daily activities, and need only to resist higher loads during very strenuous activities (21,66,124,133,150).

Biochemically, the dry weight of ligaments is largely made up of collagen (49,51). Collagen is the primary molecule responsible for the tensile strength of a ligament, and it has a triple helical structure which forms fibrils in the extra-cellular matrix (6,54). The majority of the collagen in ligaments is type I collagen (6,52). There are many other important constituents of ligaments. Water makes up about 65-70% of their wet weight,

and proteoglycans, which absorb water, make up about 1-2% by dry weight (7,51,52). These components are very important for conferring time-dependent viscoelastic properties to ligaments, and are thought to have a role in allowing for sliding between collagen fibers (6,15,146). Minor, but functionally important components also include elastin (1-2%), actin, laminin,, and fibronectin (52). This complex matrix is maintained and replaced by resident fibroblast cells which are located between collagen fibers (52).

The Anterior Cruciate Ligament and Medial Collateral Ligament

The anterior cruciate ligament (ACL), is a knee ligament which connects the femur to the tibia (Figure 1), and which helps function in the guiding of the knee joint through a normal range of motion (112). Specifically, this intra-articular , extra-synovial ligament is a primary anterior stabilizer of the tibia with secondary rotatory stabilization functions (13,15,112). The tibial or medial collateral ligament (MCL) is an extra-articular ligament of the knee which is situated on the medial aspect of the joint (6). The primary function of this ligament is to resist valgus stress and rotation of the knee joint (6)

Injury to the ACL is very common with at least 36/100,000 people per year in the United States suffering disruption of this ligament(96), and this rate appears to be increasing (11). Unlike most other ligaments in the body such as the MCL, functional healing of this ligament is very rare (16,35,45,49,72,113). The ACL deficient knee can be permanently unstable, and has been shown in both animal models (1,113), as well as

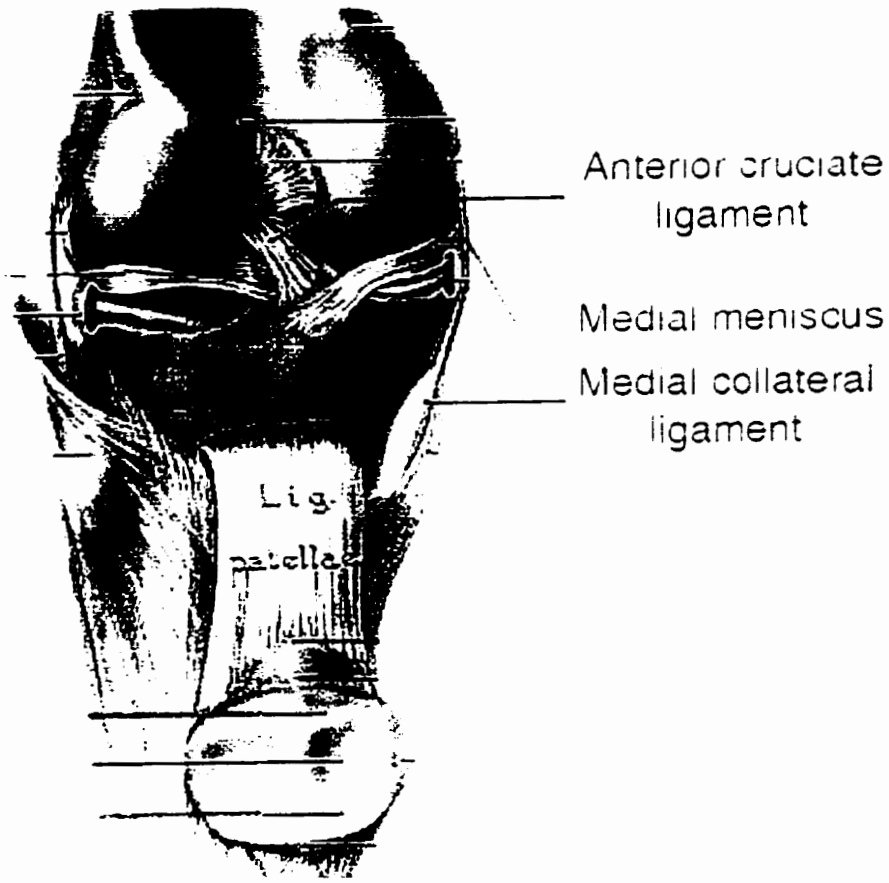

Figure 1. Anterior view of flexed knee joint, showing important ligamentous structures

clinically (12,35,47,109) to often go on to a progressive degenerative osteoarthritis. Orthopaedic surgeons have attempted to directly repair the ACL with little clinical success (18,41,113). The most commonly performed procedure currently for the treatment of the ACL deficient knee is reconstruction of the ACL using autogenous fascial or tendon grafts. It is estimated that 75,000 ACL reconstructions per year are done in North America (53). It is this ligament which is most often reconstructed and which has been most extensively studied, however the posterior cruciate ligament and to a lesser extent the lateral collateral ligament and medial collateral ligament ligaments of the knee are also being reconstructed with tendon and fascial grafts (68,106,125). Soft tissue grafts are also used about the shoulder (101), as well as the ankle (69). Although the literature seems to indicate that many patients have clinical improvement following the ACL reconstructive procedures (38,71,126) it is as yet unknown whether the progression of osteoarthritis is prevented (48). In fact there is literature which suggests that ACL reconstructed knees still progress to develop osteoarthritis (2,41,48,71). It is also well recognized that normal ligament ultra-structural anatomy and knee joint kinematics are rarely if ever restored with current reconstructive procedures (22,23,46,102,136).

Graft Elongation

A careful analysis of the literature suggests that ACL grafts are rarely failing by tearing apart, but instead seem to be failing by "stretching out" (2,3,11,17,20,21,23,24,30,46,57,59,71,75,76,94,97,120,128,136). This has been seen in both animal models (19,24,32,43,60,65,75,78,104,127,130,136,136,139,152), as well as

clinically (2,3,17,20,30,33,42,46,57,59,71,76,94,97,120,128). It is thus of great concern that graft elongation may be leading to recurrent knee instability and potentially to osteoarthritis. In goat ACL reconstruction models it has been shown that there is early knee joint laxity as well as an increase in the load-relaxation property of the grafts when compared to normal controls (104). Ng et al (1995) commented that some goat grafts appeared to be "lengthened" at one year post-op, and they hypothesized that "the uncontrolled early rehabilitation program and initial high load-relaxation in the grafts may account for the lengthening"(104). Similarly in the rabbit ACL autograft model, knee joint laxity greater than twice controls has been reported with graft stiffness reaching 25% of control (19). In dogs, graft elongation of up to 200% has been observed within the first three months post-operatively (152), and in goats elongation of up to 500% at just two weeks has been reported (65).

The clinical literature also indicates that between 10% and 30 % of ACL reconstructed knees become clinically lax (2,3,17,20,30,33,42,46,57,59,71,76,91,94,97,120,128).
This has been based largely on data from the "KT-1000", which is a validated arthrometer used to measure the anterior-posterior (AP) translation of the tibia with respect to the femur (40,64). This translation is primarily resisted by the cruciate ligaments (44). Normal people with no ligamentous injury have an average side to side measured difference (one knee compared to the other knee) of 1-2mm (40,64). Studies have shown that any values greater than 2-3mm of side to side difference as measured by the KT-1000 is abnormal, and the average side to side increase in translation of an ACL deficient knee over the contra-lateral normal knee ranges from 3-6mm, with 95% of patients with an

ACL-deficient knee having greater than 2mm of side to side difference (40,64,132). Importantly, it has been shown that despite the fact that 10-30% of ACL reconstructed knees have greater than 3mm of side to side difference on KT-1000 testing, and about 5-15% are greater than 5mm after variable amounts of follow-up time (2,3,71,94,119), the intra-operative KT-1000 values immediately after reconstruction range from -1.4mm (tighter than normal) to 0mm side to side difference (40). It has also been shown that the KT-1000 may under-estimate the knee joint laxity, since dynamic radiography and stereophotogrammetric analyses have shown even higher values of AP translation in ACL reconstructed knees (55,90,91). Furthermore, there is some evidence that the contra-lateral knee of ACL-injured patients has increased laxity compared to the normal population, which would give a falsely low estimate of the clinical instability of the ACL reconstructed knee, when side to side measured differences are utilized (44). Recent studies have also shown that the increase in AP translation after ACL reconstruction occurs progressively over the first 6 months and then seems to plateau over the ensuing follow-up (91). All this evidence suggests that ACL grafts are stretching-out or elongating over time, leading to recurrent joint laxity, and that this elongation may be occurring relatively rapidly. Although these measures of static AP laxity do not always correlate with patients' subjective assessment of their knee functional stability (some patients may have some dynamic muscular compensation) (40), this abnormal laxity and likely altered joint kinematics may be a major factor in the continued development of osteoarthritis, which seems to occur despite ACL reconstruction (2,41,48,71).

Biomechanical Properties of ACL Grafts

The majority of biomechanical studies performed to date on grafts have focused on graft strength over time of healing (19,31,34,37,65,78,95,113,127,139,144,152). In terms of ultimate tensile strength, grafts have been shown to become as weak as 13-17% of normal maximal stress at 8 weeks (31,78), and the majority of studies show a persistent graft weakness, even at 1-3 years (26,104,136).

The viscoelastic properties of grafts have received less attention in the literature. Given that the problem of joint laxity may be secondary to graft elongation, it seems that the graft viscoelastic properties may be of considerable clinical relevance. To date, the most common biomechanical test of graft viscoelastic behaviour has been stress-relaxation (104,122). In such a test, the tissue is stretched to a constant length and held there while it's stress decrease is measured over time. Experiments have shown that tendon grafts have increased load relaxation at early healing intervals (12-48 weeks) (80,104,122).

Another measure of the viscoelastic properties of tissues is creep. Creep is the elongation of a tissue under a constant, or cyclically repetitive load (56). This may be a more clinically relevant measure of the viscoelastic properties of grafts, as it may be the way that they are loaded *in vivo* (66,135). Creep behavior of ligament grafts over healing time has not been studied previously. Thornton et al. (1997) have shown recently that creep and load relaxation measurements do not correlate at low stresses in ligament structures (135). It has been speculated that load relaxation recruits a certain group of collagen fibers under a static elongation, while in creep testing there may be progressive

fiber recruitment with increasing length resulting in less creep than would be otherwise predicted (146).

Graft Remodeling

It has been shown in humans and in animal models that fascial, ligament, and tendon grafts which are used to replace ligaments are infiltrated by scar tissue, and some regular collagen bundles of the grafts are broken down (10,19,37,114,151). Grafts have been found to become hypocellular within days after transplantation due to necrosis of resident fibroblasts (10,78,83,151). The reason for this cellular necrosis is not entirely clear, as even grafts in which the blood supply has apparently been maintained, have also been shown to undergo a cellular necrosis, and diffusion studies have shown that nutrients are able to reach the center of grafts (31,83,84).

In any event it appears that hypocellular graft matrix becomes invaded by scar cells which begins at approximately 1-2 weeks, and has been seen to progress up to about 8-16 weeks after tranplantation (10,78,83,131,151). These cells have been shown to be mainly extrinsic mesenchymal cells which are derived from both the surrounding tissues as well as the vasculature (84). The grafts become hypercellular for several months and then the number of cells decrease closer to those in normal ligaments (10,19,105,115,131). Along with this cellular invasion, soft tissue grafts also undergo a re-vascularization (78,83,127,151).

The collagen scaffold of the graft is progressively degraded and replaced by scar tissue (78,151). In fact, during this "collagen formation" phase of healing (generally between 4-16 weeks) there is a gradual increase in the cross sectional area of the graft

(60,78,151). This new matrix is composed of small collagen fibers which has a higher proportion of type III collagen as opposed to the normal type I, and the fiber alignment has been shown with the scanning electron microscope to be randomly oriented, unlike the parallel arrangement of normal ligament fibers (78,151). However this scar tissue does remodel over time, with this process being described as "ligamentization", since the new tissue resembles that of normal ligament (8,9,78,123). More careful evaluation of this new material however, reveals that it is actually quite different from normal ligament, both biochemically as well as structurally (19,114,131). Even at 2-3 years post-grafting, it has been shown that the collagen fibril diameter profile is abnormal (104,115). These grafts, only become about 50-65% as strong as the native ligament and they often remain inferior to the original graft material (29,43,75,95,104,131). The scar tissue has also been shown to remain more viscous, as shown in load-relaxation experiments (104,122). It may also be that the grafts become more susceptible to reversible and irreversible creep as they are degraded and replaced by scar.

Factors Which Affect Graft Remodeling

There have been many studies which have investigated the effects of various biological and mechanical factors on the healing of soft tissue grafts (58,60,79-81,100,137,151,152). Stress shielding experiments have revealed that some load is beneficial to graft biomechanical properties (79,151). Grafts which carry no tensile load have been shown to have dramatically decreased failure strength, even at very early healing intervals (1 week) (93,151). On the other hand, loaded/functional grafts tend to be stronger, and they also tend to be larger, with the suggestion of increased scar tissue

production (60,81). Muneta et al. 1994 (98) were able to supply a known loading history via transcutaneous sutures to a patellar tendon graft implanted in the subcutaneous tissue on the back of a rabbit. Daily loading was found to increase the stiffness, strength, and cellularity of the grafts. The tension with which the graft is fixed at the time of surgery has also been investigated. Excessive tension has been shown to decrease the revascularization of grafts and lead to central areas of necrosis (152). Other studies however have shown less detrimental biomechanical consequences from "over tensioning", and in fact some studies have shown a viscoelastic stress relaxation of the graft shortly after transplantation. Thus high graft tension after fixation is a short lived phenomenon (58,80,152). The strain environment has also been shown to effect the elongation of grafts over time (136). Tohyama et al 1996 (136) showed in a dog model that grafts which underwent high strain with knee range of motion at the time of surgery resulted in permanent graft elongation over 18 months of healing. The grafts which had lower strains, elongated less after the same healing interval.

With regards to biological factors, studies have suggested that if extrinsic cells are prohibited from invading the graft, then there is less early post-operative biomechanical deterioration (137). It has also been widely speculated that different healing results will be attained from grafts in an intra-articular environment versus an extra-articular environment (80,137). The extra-articular environment has been considered to be more beneficial to graft healing because of the increased potential blood supply, increased availability/suitability of mesenchymal cells for graft re-population, lack of potentially harmful synovial fluid, and decreased load environment. Indeed, the structural strength

activities put the most strain on the graft. This group has advocated rehabilitation protocols which avoid the exercises which they have shown to produce the highest ACL strains. It is not known however, how much stress and when is optimal for graft healing. The ideal conditions would likely be ones in which the stresses promote scar remodeling that could resist creep, while at the same time not causing permanent stretch of the graft.

Effects of Immobilization on Joints and Healing

Immobilization is known to affect the structural properties of ligaments and scar (25,103,111). If one looks at ultimate load, energy absorption to failure, and stiffness, it has been shown that immobilization of ligaments and scars decreases these properties (25,103). Movement of healing scars, on the other hand, in animal models has been shown to decrease joint laxity and increase the strength of healing compared to immobilized healing scars (62). Immobilization of normal ligaments leads not only to a decline in the high load biomechanical properties of the matrix, but has also been shown to adversely affect the ligament insertion (111,149). Histologically there is a resorption of bone around the insertion site (111). Immobilization has also been shown to be deleterious to the whole joint (cartilage, bone, etc) (116,118). What has not been studied to date is what affect immobilization has on the creep properties of ligament or scar tissue. It has been shown in a rabbit model that the collagen fibers in immobilized scar tissue are arranged in parallel bundles sooner than that of scar from free moving joints (50). Further, it has been shown that immobilization of patellar tendons which have had the middle one third excised have less random collagen formation and a greater tangent modulus of elasticity than mobilized tendons (77). It might be hypothesized that parallel

arrangement of collagen bundles would be better able to resist the forces that cause creep, as maximal bundle recruitment would likely be achieved at a shorter length of stretch (146). In rabbits which have had the ACL and MCL transected, it has been found that immobilization leads to less laxity in the healing MCL. The non-immobilized MCL scars appear to have been over -loaded and subsequently they crept (27). Further, it has been shown that immobilization of rat MCLs which have been transected, leads to less scar production (138). If a ligament autograft had less scar infiltration due to immobilization, then it might be hypothesized that the graft would be better able to resist creep, since more of the native structure with well aligned collagen bundles would still exist. If this new scar material that replaced the original scaffold had more aligned collagen fibers, this too may decrease the susceptibility of the graft to creep.

RATIONALE

Recurrent joint laxity after ligament graft reconstruction procedures is a major clinical concern. The mechanisms responsible for this joint laxity are still not clear, however it is widely speculated that graft stretch is a major factor in causing this increased joint laxity, especially in animal ligament reconstruction models. Virtually all viscoelastic data to date on ligament and graft healing has been stress-relaxation data (104,122). However there is evidence to suggest that this may not be the most physiologically relevant measure of soft tissue viscoelasticity, since it is likely that *in vivo*, ligaments and ligament grafts are repetitively loaded to constant loads (creep), and are not repetitively elongated to the same length (load-relaxation) (66). Furthermore, recent studies have shown that soft tissue creep at low stresses cannot be predicted from

stress-relaxation experiments (135). The vulnerability of soft tissue grafts to creep over healing time has not been specifically studied, nor have factors which may effect this creep vulnerability. It is not known how much stress and when is optimal for graft healing, while at the same time not causing permanent graft stretch. Thus, it is currently not possible to optimize post-operative rehabilitation protocols. Through these experiments it is hoped that some insight can be gained into whether these soft tissue grafts become more susceptible to creep than normal ligaments, what the time frame for this increased susceptibility is, and how post-operative loading history can affect the creep of ligament grafts.

To begin to address the possibility that soft tissue grafts become inherently more vulnerable to creep than normal ligaments, an animal model was chosen for study. The rabbit knee is an accepted model for the investigation of knee ligaments (14). The MCL autograft model in particular has been shown to be relatively reproducible in the study of autograft healing, and the structure of the MCL is simple, well defined, and consistent in rabbit populations (14,80). This autograft model may represent the ideal conditions for ligament graft healing as it is extra-articular and the graft is placed in an anatomic and low stress environment (14,122). Furthermore, the biomechanical outcome of these grafts is one of the best reported in the literature (122). The extra-articular environment is normally well vascularized and thus cells involved in autograft repair are thought to be more abundant (80). Furthermore, the MCL autograft heals in a low stress environment (unlike most ACL grafts) and thus the intrinsic creep vulnerability of the grafts can be quantified under controlled *in vitro* conditions. ACL grafts may have already undergone creep *in vivo* before it can be specifically quantified, making these models less desirable

for this particular study. In the first part of the current study we intended to quantify the graft creep vulnerability over time in this model.

One of the most basic and controversial principles of post-operative treatment in all areas of orthopaedic surgery is whether or not to immobilize or move joints post-operatively, and how aggressive the rehabilitation should optimally be (22,50,92,108,111,121). These are also the factors which affect healing, that clinicians could potentially most easily manipulate. The effects of joint immobilization on soft tissue graft healing have never been investigated in a systematic way, and the effect of immobilization on graft creep over time has never been studied at all. There is evidence from animal models which suggest that joint immobilization may be detrimental to graft healing (62,118), but more interestingly joint immobilization may be beneficial in decreasing graft creep vulnerability by decreasing scar tissue in-growth into grafts and by allowing more creep resistant scar tissue to be formed (27,50,77,138).

PURPOSE

The purpose of this project therefore was to determine; 1) if rabbit MCL autografts become more vulnerable to creep than the normal MCL and, 2) when and how quickly this increased creep vulnerability may occur. It was also the purpose of this study to determine; 3) what effect knee immobilization would have on the creep of MCL autografts in the rabbit model at early healing intervals.

HYPOTHESES AND SPECIFIC AIMS

The ultimate goal of these experiments was to learn how mechanical conditions might be manipulated to minimize the "stretching out" of ligament grafts in the clinical setting. The following results were hypothesized; 1) Both immobilized and moved autografts would become increasingly susceptible to creep with time, as they are replaced by more scar tissue during healing, and 2) Immobilization would result in less biomechanical creep in the grafts. This was based on the extrapolation from wound healing studies in which immobilized rat MCL injuries produced less scar (138). If immobilization resulted in less scar infiltration into the grafts then more of the native MCL structure would be maintained. These well organized and densely arranged collagen bundles in the native graft structure would be better able to resist creep. Furthermore, immobilization may allow scar to form which has a more parallel arrangement of collagen bundles which are also better able to resist creep (27).

The specific aims of this project were:

1) To determine when, and how quickly after surgery that grafts become more susceptible to creep. Rabbit MCL autograft creep and creep recovery was measured in normal MCL controls, at time zero (immediately after grafting), 2 days, 3 weeks, and 8 weeks after transplantation.
2) To compare creep and unrecovered creep values at physiologically relevant stress levels, of immediately moved versus immobilized rabbit MCL autografts at 3 weeks, and 8 weeks of healing.

CHAPTER II

The Early Vulnerability of Ligament Autografts to Creep

INTRODUCTION

Soft tissue graft reconstruction has become the surgical treatment of choice for chronic joint laxity following ligament injury (38,71,113). Although patients can have improved function after these procedures for a number of years, it is generally recognized that the normal ligament structure and function are likely not being restored. After anterior cruciate ligament (ACL) reconstructions, clinically detectable recurrent joint laxity occurs in at least 10-30% of patients, despite the fact that no detectable laxity is present immediately after surgery (2,3,17,20,30,33,42,76,91,120,128). Furthermore, there is evidence which suggests that osteoarthritis can still be the ultimate fate of these reconstructed joints (12,35,47,109). This evidence suggests that the grafts are either stretching out or failing with time (21). Animal models of soft tissue ligament reconstructions have revealed an even more dramatic development of joint laxity which occurs within weeks, and which is widely thought to be related to overloading and stretching of grafts in the early post-operative period (19,32,43,60,65,75,78,104,127,130,136,139,152).

Biomechanical studies have shown that soft tissue grafts have significantly decreased ultimate tensile strength within weeks of transplantation, however the vulnerability of these grafts to elongation with time has not been well studied, especially at the early healing intervals. Studies have revealed increased stress-relaxation of

ligament grafts in goats at 3 weeks post-operatively, and this increase has been shown to persist up until 53 weeks (104). However, stress relaxation of ligament grafts may not be the most physiologically relevant measure of the viscoelastic properties (66,135). It is likely that *in vivo,* ligaments are repetitively loaded to a constant stress during ambulation, and not necessarily loaded to a repeated elongation (66). Therefore the more relevant viscoelastic property is likely creep (deformation under a constant or cyclically repeated load), not stress relaxation (reduction in stress under a constant or cyclically repeated elongation). Furthermore, recent evidence has revealed that stress relaxation data cannot be used to predict creep in soft tissues (135).

It was the purpose of this study therefore, to determine the susceptibility of a ligament autograft to creep under low physiological loads, at various early healing times. The rabbit bone-medial collateral ligament (MCL)-bone extra-articular autograft model was used, as it has been studied previously and it has been shown to have favorable biomechanical outcomes (122). Thus, it may represent the "best case scenario" for graft healing (122). The graft in this model also heals under what has been estimated to be a low stress environment (unlike ACL grafts), and therefore the intrinsic vulnerability of the grafts to creep at various healing intervals can be quantified under controlled conditions since there is likely no *in vivo* creep occurring prior to testing. The elongation of ACL grafts which occurs *in vivo* prior to mechanical testing makes them a poor model for this specific *in vitro* quantification of creep vulnerability. Because of the relatively rapid time course of increased joint laxity, especially in most animal reconstruction models, this study focused on the very early healing intervals (2 days to 8 weeks). We

hypothesized that these grafts would become increasingly more susceptible to creep with healing time over this interval.

METHODS AND MATERIALS

Model

Thirty eight skeletally mature (>12 months), female New Zealand white rabbits (weighing 4.5-6 kg) had a standardized orthotopic medial collateral ligament (MCL) autograft procedure to the right hindlimb, under halothane general anesthetic, as described previously (80,122). The MCL was harvested with femoral and tibial bone plugs, which had been pre-drilled and pre-tapped before graft removal. The graft was removed, washed in saline, and replaced immediately with screw fixation in an anatomical position (Figure 2)(122). The animals were allowed normal activities in a 65x45x30cm cage, and they received a standard diet and water *ad libitum.* The animals were handled according to established ethical guidelines approved by the local animal care committee, and were sacrificed with a phenobarbitol overdose (Euthanyl 275mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) at time zero(n=7), 2 days (n=11), 3 weeks (n=10), or 8 weeks (n=10) post-operatively. Eight normal control rabbits were sacrificed similarly. The hindlimbs were harvested, and frozen with the skin intact, in a sealed plastic bag at -70° C. On the morning of testing, the samples were thawed at room temperature and all soft tissues were removed from the knee except for the collateral ligaments, the cruciate ligaments, and the menisci. The bones were then cut with a saw approximately 5 cm from the joint line.

Biomechanics

The dissected samples were potted in polymethylmethacrylate, and mounted on a specialized closed loop, servohydraulic material testing system (MTS, Systems Corporation, Minneapolis, Minnesota) for creep testing. After mounting the tibia of the specimen in series with a load cell to the actuator cross-head, and aligning the length of the graft with the load axis of the testing machine, the femur was lowered into a second pot and fixed similarly with cement at a joint angle of 70° flexion. Both the femoral and tibial graft bone plugs were incorporated into the cement to avoid creep at the fixation

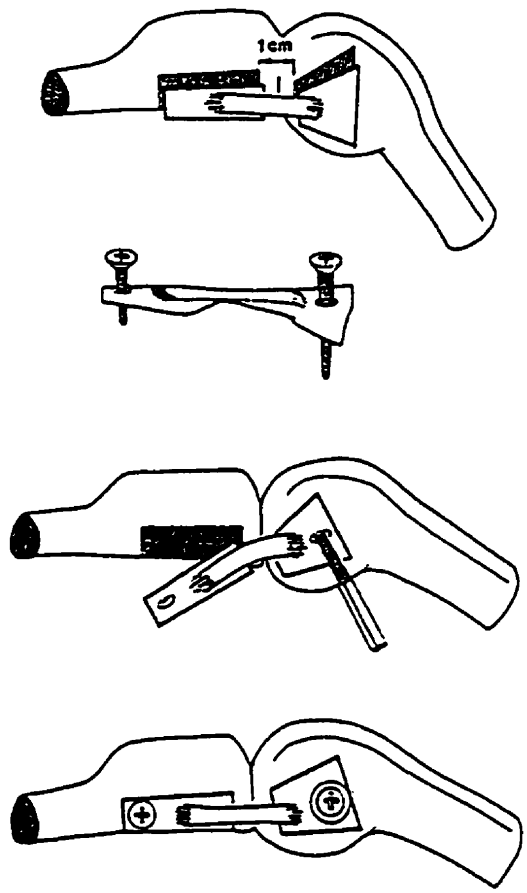

Figure 2. Schematic of medial collateral ligament (MCL) autograft procedure. MCL removed on tibial and femoral bone fragments, washed in saline, then immediately re-implanted in anatomic position with screw fixation.

sites. The cement was still kept away from the graft, thus avoiding any thermal damage. During the mounting procedure, the grafts were kept moist by frequent irrigation with 0.9% phosphate buffered saline.

In order to obtain a measure of joint laxity the specimens were cycled between 2.5 N and -5 N. The laxity was defined as the displacement of the joint from 0.1N compressive load, and 0.1N tensile load. After obtaining a "whole joint laxity" measure, the lateral collateral ligament, the cruciate ligaments and the menisci were removed , leaving just the femur-MCL-tibia complex. This complex was then cycled in order to find "ligament zero". "Ligament zero" was identified as the cross head position at which the ligament just began to take up a detectable load (0.1N) (Figure 3). The ligament length was measured at this position in a standard way using digital calipers. The medial femoral condyle was then carefully removed and specialized calipers were used to measure the cross sectional area of the grafts at the joint line (accurate to +/- 5%) (129). The specimens were then enclosed in a humidity chamber (relative humidity 99%) at 37°C, to provide a constant, moist environment during creep testing (142) (Figure 4).

The creep protocol (Figure 5) involved 30 cycles of cyclical loading at 1 Hz. to a constant stress level of 4.1 MPa, followed immediately by a 20 minute static creep, at the same stress level. This stress level is approximately 5% of the failure stress of a normal rabbit MCL, and represents the estimated normal tensile loads carried by the rabbit medial collateral ligament *in vivo* (21,124,150). The stress level for each ligament graft was calculated based on the cross-sectional area measurements at the joint line. The resulting elongation of each graft was measured by cross-head displacement and stored on

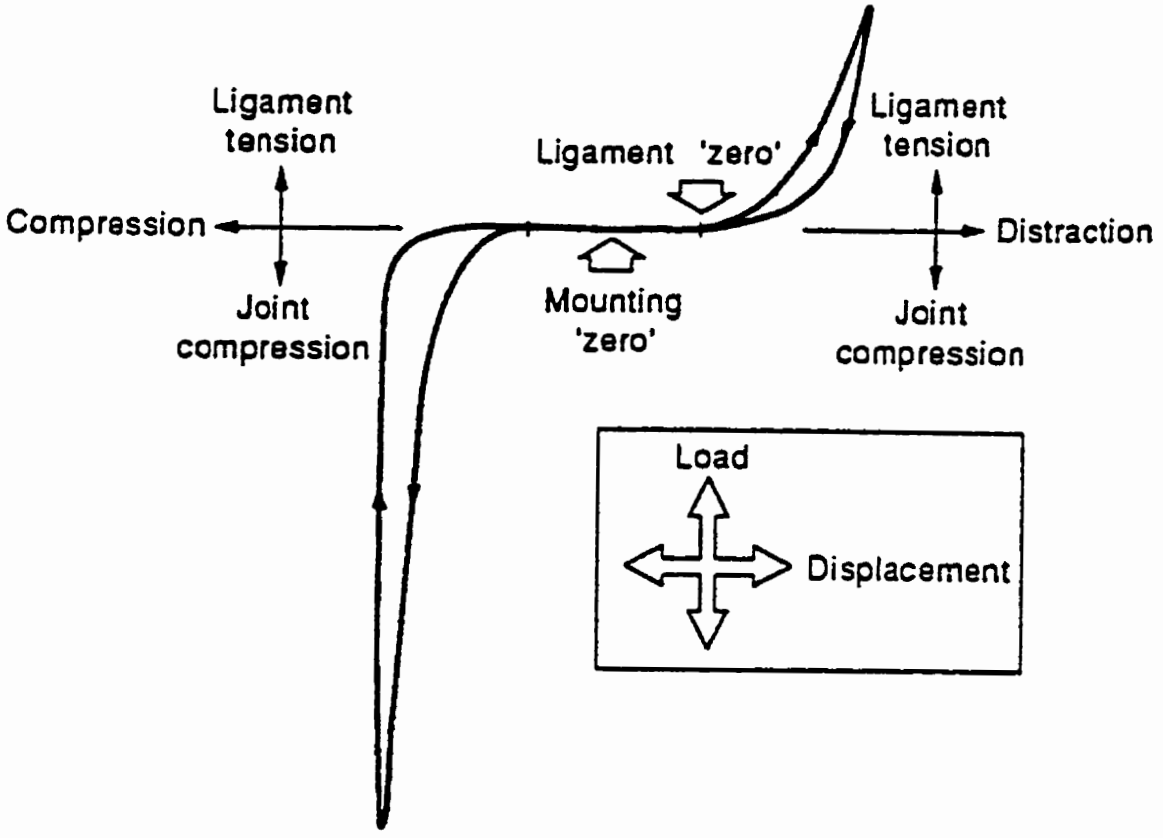

Figure 3. Schematic of the compression-tension protocol for establishing ligament zero. The displacement at which the ligament carries 0.1N of load is defined (and set) as "ligament zero", from which creep testing is carried out.

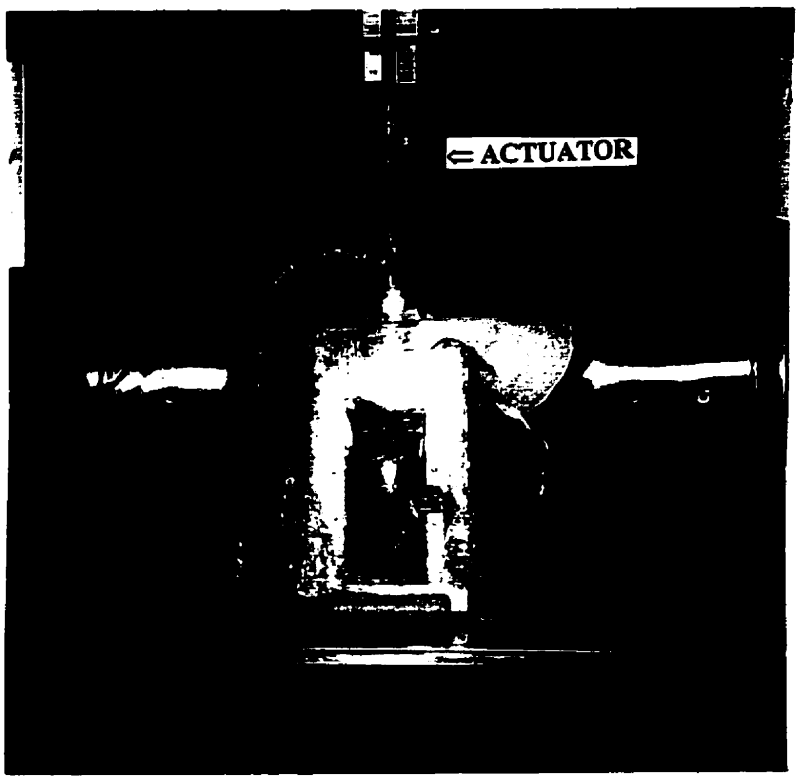

Figure 4. Photograph of the humidity chamber installed around the specimen, mounted on the MTS. Temperature control feed-back loop allows the testing environment to be controlled within +/-0.5°C at 99% relative humidity.

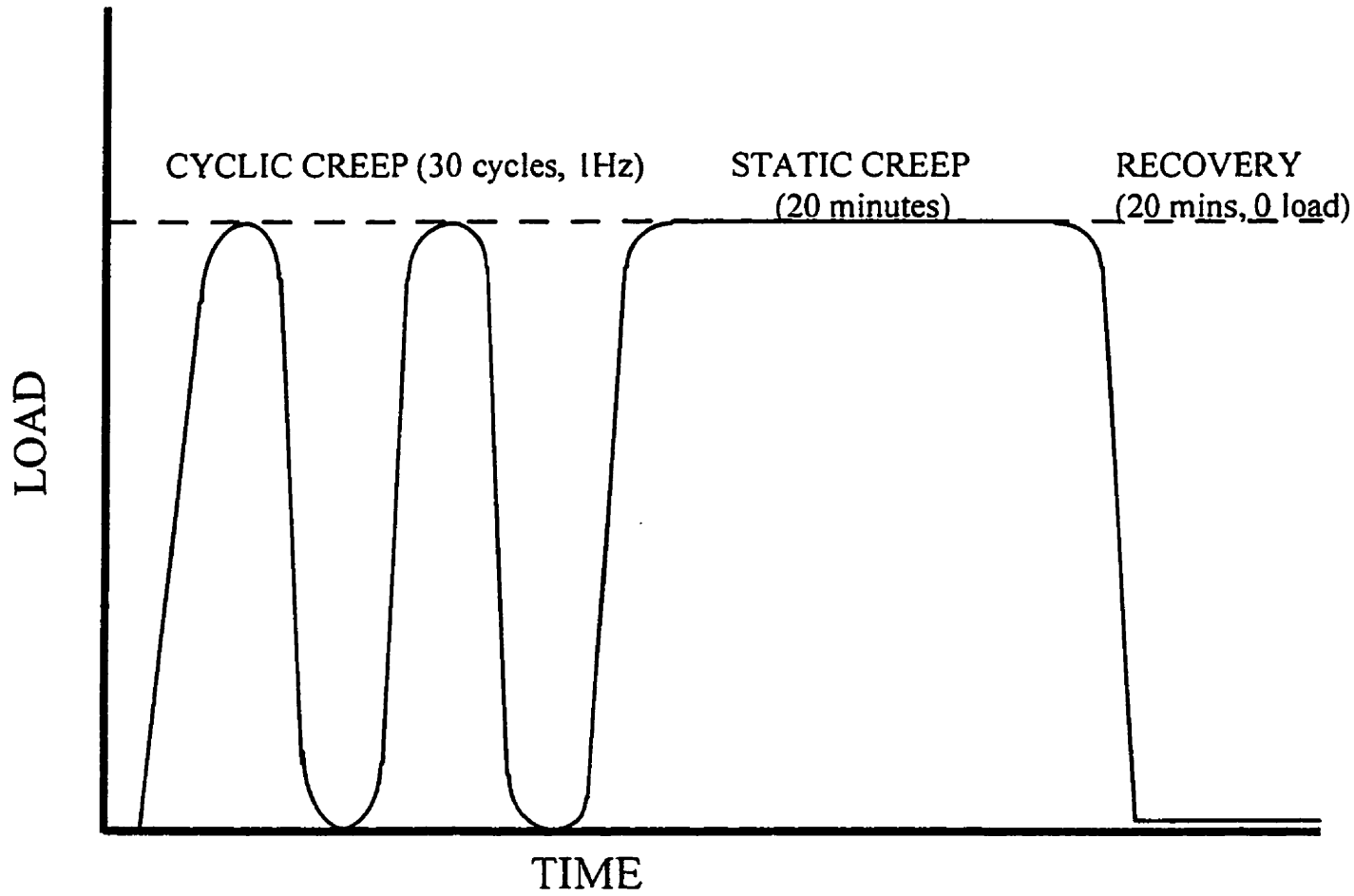

Figure 5. Schematic of creep protocol. Grafts were repetitively loaded to a constant stress level (4.1 MPa.) for 30 cycles, and the resulting deformation (and strain) was measured (cyclic creep). The grafts were then immediately held at this same stress level for 20 minutes (static creep), and then allowed to recover for 20 minutes at zero load. The remaining strain was defined as unrecovered creep.

a computer data file (Compaq 486). Tensile strain was defined as this measured graft deformation in millimeters, divided by the "ligament zero" length. Cyclic creep strain was defined as the graft strain at the peak of the thirtieth cycle minus the strain at the peak of the first cycle. Static creep strain was defined as the strain at the end of the 20 minutes minus the strain at the beginning of the 20 minutes (no recovery was allowed between the cyclic and static tests). Total creep strain (creep strain resulting from both the cyclic and static creep tests) was defined as the strain at the end of the static creep test minus the strain at the peak of the first cycle of cyclic creep. Following the creep testing, the ligaments were allowed to recover at zero load for 20 minutes. The change in the strain over this time period was defined as recovered creep, while the unrecovered residual strain after this 20 minute period was defined as unrecovered creep.

It is important to note that the strain of the ligaments was determined by dividing the cross-head displacement by the initial "ligament zero" length. This method of measuring tensile strain clearly results in higher values than if methods such as the video dimension analyzer (VDA) is used (150), since cross-head displacement represents strain of the entire structure, where as the VDA measures only the strain of the marked ligament substance. Of particular concern at the outset of this experiment was whether or not creep could occur at the bone fixation sites of the grafts, particularly at two days, where no bony healing would have occurred. Magnified video images of creep tests on grafts on which a thin line was drawn which spanned from the insertional bone island to the adjacent bone, showed no movement of the bone graft. We also incorporated the bone grafts into the cement during potting, and thus re-enforcing their fixation. Finally, time-zero ligament grafts were creep tested, and found not to be different to normal ligament

controls. From these results we were confident that no creep was occurring at the bone graft fixation sites during testing.

Outcomes were analyzed statistically using a one-way ANOVA, with time as the variable (SAS software version 6.12). Groups were then compared using Tukey's test for multiple comparisons (student standardized range). Grafts were compared to normal MCLs and time zero control grafts using a student t-test using transformed data. Data was transformed in order to stabilize the unequal variance between grafts and normal controls. A significance level of 0.05 was used in all tests.

RESULTS

Gross Morphology

Even after only 2 days post-transplantation, the MCL autografts were visibly different than normal MCLs. The grafts had a fibrovascular scar material adherent to their surface. The graft fixation sites remained solidly fixed with the screw fixation. After 3 weeks of healing there was abundant new material encasing the MCL autograft and the knee joint. This scar had a fibrous quality to it. Similar scarring was apparent at the 8 week healing interval as well. At both 3 and 8 weeks the graft fixation sites showed evidence of substantial bony healing. Subjective assessment of the range of motion (ROM) indicated that joints at all healing intervals had a full ROM. Cross-sectional area measurements revealed that the grafts became larger with time (Figure 6). The 2 day grafts were statistically significantly larger than both the time zero grafts and normal MCL controls (t-test; $p= 0.001$, $p= 0.006$ respectively). ANOVA revealed a statistically

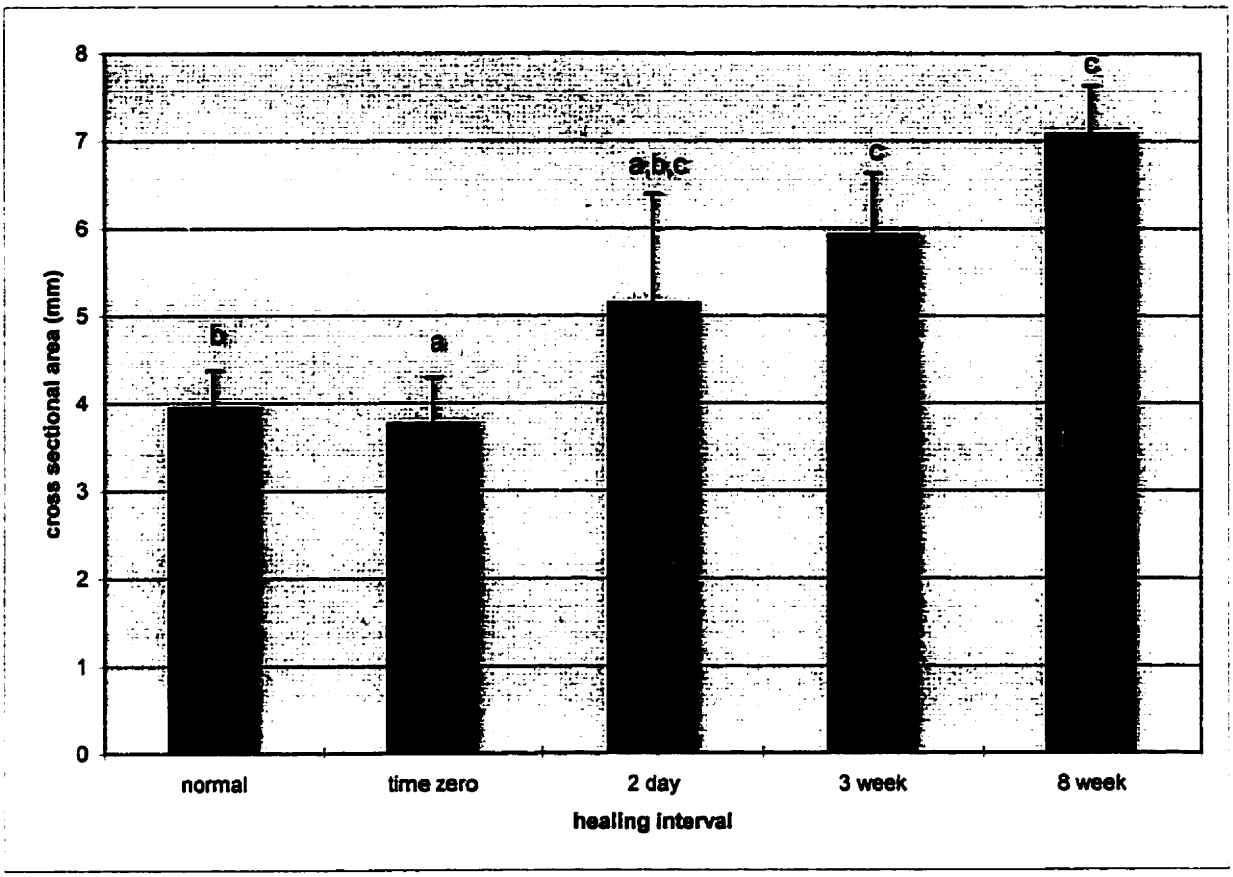

Figure 6 : Cross sectional area measurement of grafts with time. Group means with standard deviations. 2 day grafts are statistically significantly larger than time zero (a)and normals (b) (p=0.002, p=0.006), and there was a statistically significant increase with time from 2 days to 8 weeks (c) (ANOVA, p=0.0002). n=7-11 per group.

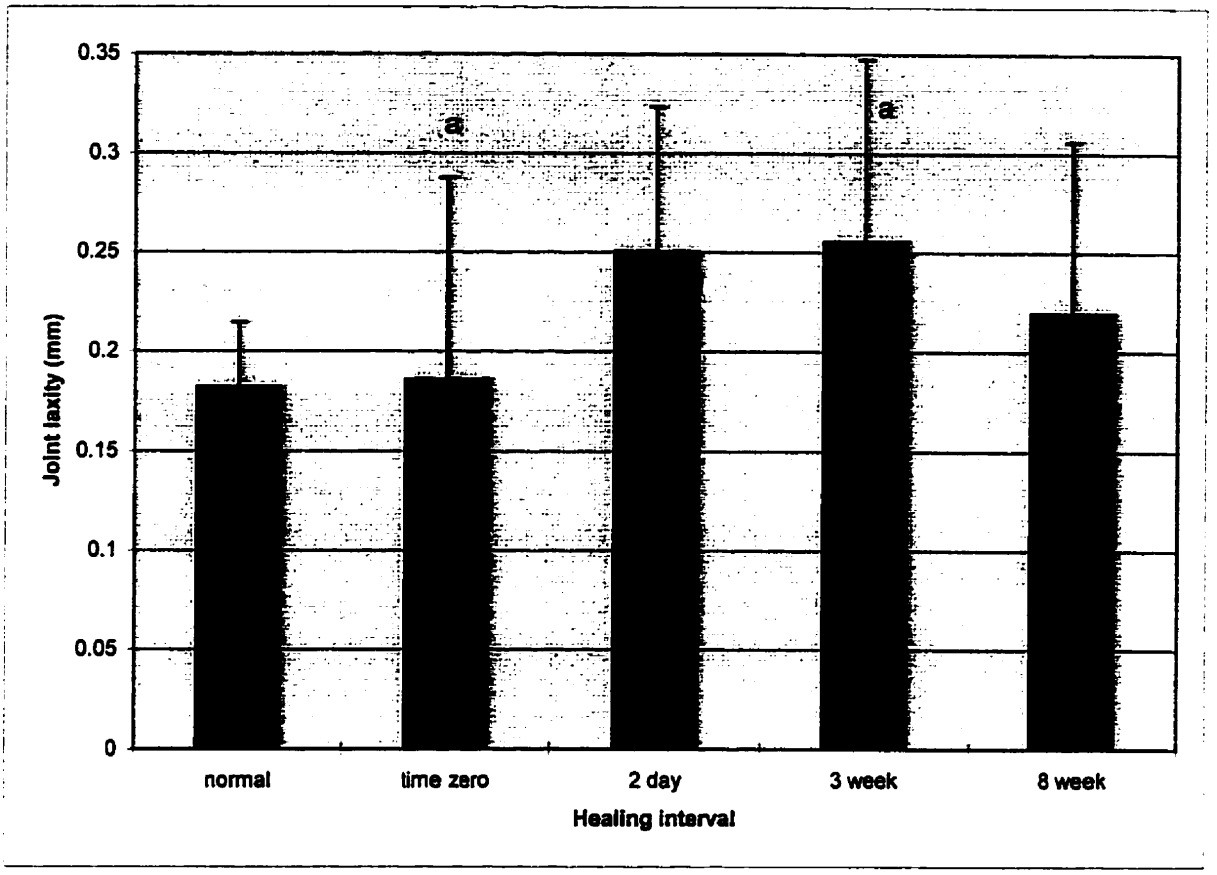

Figure 7: Joint laxity. Group means and standard deviations. Statistically significant increase in joint laxity between time zero and 3 week grafts (a) (p=0.0001). No statistical difference between 2 days and time zero, nor between 3 weeks and 8 weeks. n=7-11 per group.

significant increase in graft cross-sectional area with time ($p=0.0002$), however Tukey's test showed that the statistically significant increase in graft size was between 3 weeks and 8 weeks, with no statistically significant increase between the 2 day grafts and the 3 week grafts.

The 2 day grafted joints were not statistically significantly more lax than the time-zero controls ($p=0.33$), however the 3 week grafts were ($p=0.0001$) (Figure 7). There was no further increase in mean joint laxity observed between 3 and 8 weeks ($p>0.05$). In fact, there was an apparent, non-statistically significant decline in joint laxity between these intervals (Figure 7).

Biomechanics

Table 1 summarizes the creep results. After 2 days of healing the MCL autografts crept on cyclic creep testing statistically significantly more than the normal MCLs, and the time-zero control grafts (t-test; $p=0.004$, $p=0.0003$ respectively)(Figure 8). There was a further statistically significant increase in mean graft cyclic creep strain with time (ANOVA, $p= 0.01$) (Figure 8). However, Tukey's multiple comparison test found that at the 0.05 significance level that the inter-group differences were between 2 days and 8 weeks. No difference was found between the time-zero control grafts and the normal MCLs ($p=0.86$) (Figure 8).

Figure 9 shows the static creep strain results, which again show that the 2 day grafts crept more than the time-zero grafts ($p=0.02$) and the normal MCL controls ($p=0.05$). There was a further increase in the vulnerability of the grafts to static creep at the same stress level between 2 days and 3 weeks ($p<0.05$). However, there was a plateau

Table 1. Summary of creep results. Group means +/- standard deviations.

GROUP	CYCLIC CREEP STRAIN %	STATIC CREEP STRAIN %	TOTAL CREEP STRAIN %	UN-RECOVERED CREEP STRAIN %
Normal n=8	0.17+/-0.17	0.71+/-0.13	0.97+/-0.20	0.20+/-0.12
Time-zero n=7	0.20+/-0.03	0.69+/-0.10	1.05+/-0.10	0.44+/-0.38
2 day graft n=11	0.417+/-0.15 a,b	0.86+/-0.19 a,b	1.29+/-0.43 a,b	1.12+/-0.29 a,b
3 week graft n=10	0.510+/-0.11 a,b	1.00+/-0.20 a,b,c	1.63+/-0.32 a,b,c	1.25+/-0.27 a,b
8 week graft n=10	0.59+/-0.11 a,b,c	1.02+/-0.16 a,b,c	1.74+/-0.25 a,b,c	1.22+/-0.23 a,b

"a" = statistically significantly different to normal MCL
"b"= statistically significantly different to time-zero control
"c"= statistically significantly different to 2 day grafts
"d"= statistically significantly different to 3 week grafts

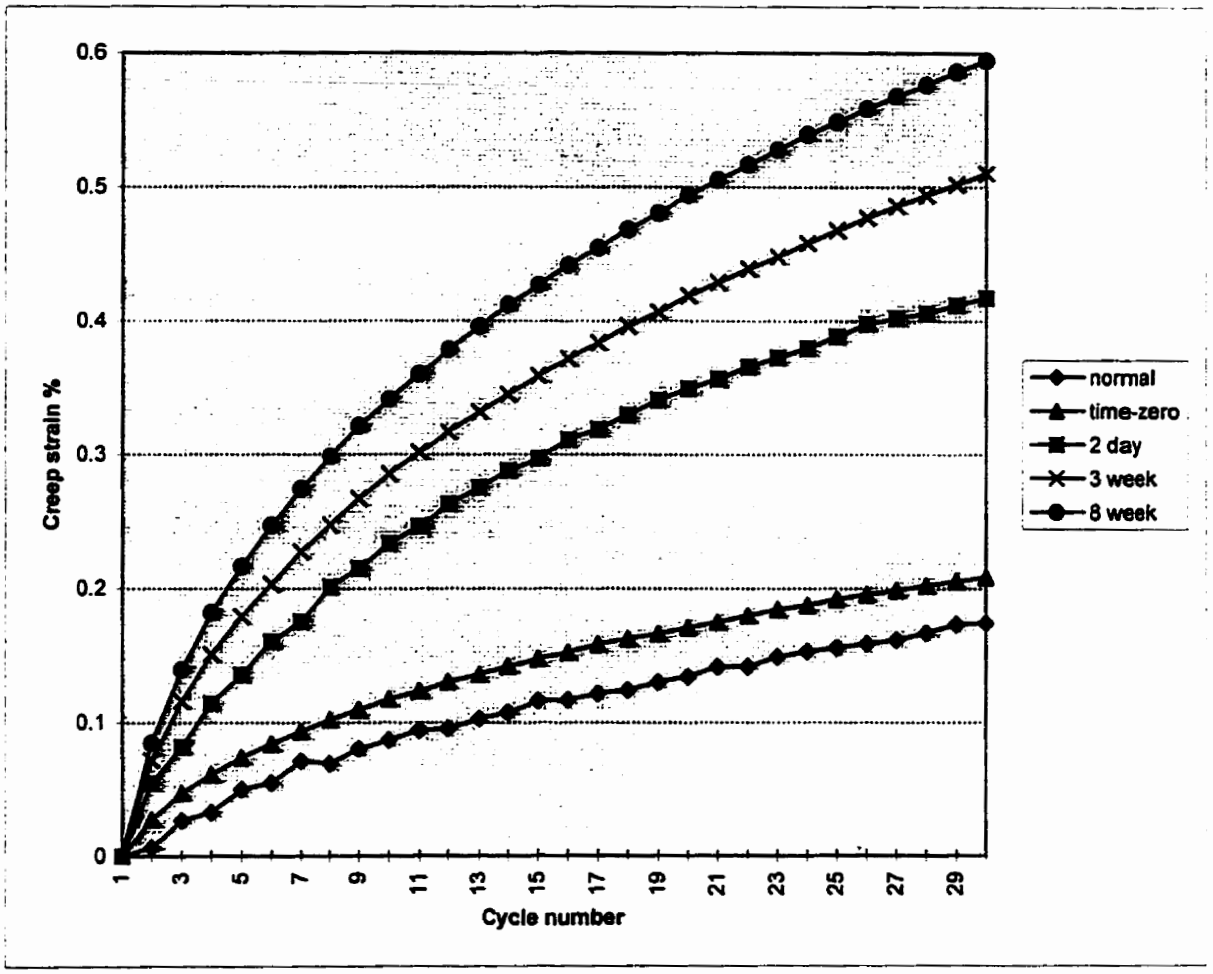

Figure 8: Cyclic creep strain %. Group means for each cycle. Grafts repetitively stressed to 4 MPa at frequency of 1 Hz. 2 day grafts crept significantly more than both normal MCL and time-zero grafts (p=0.004. and p=0.0003). Note the significant increase in cyclic creep at the same stress with healing time (ANOVA, p=0.01). n=7-11 per group.

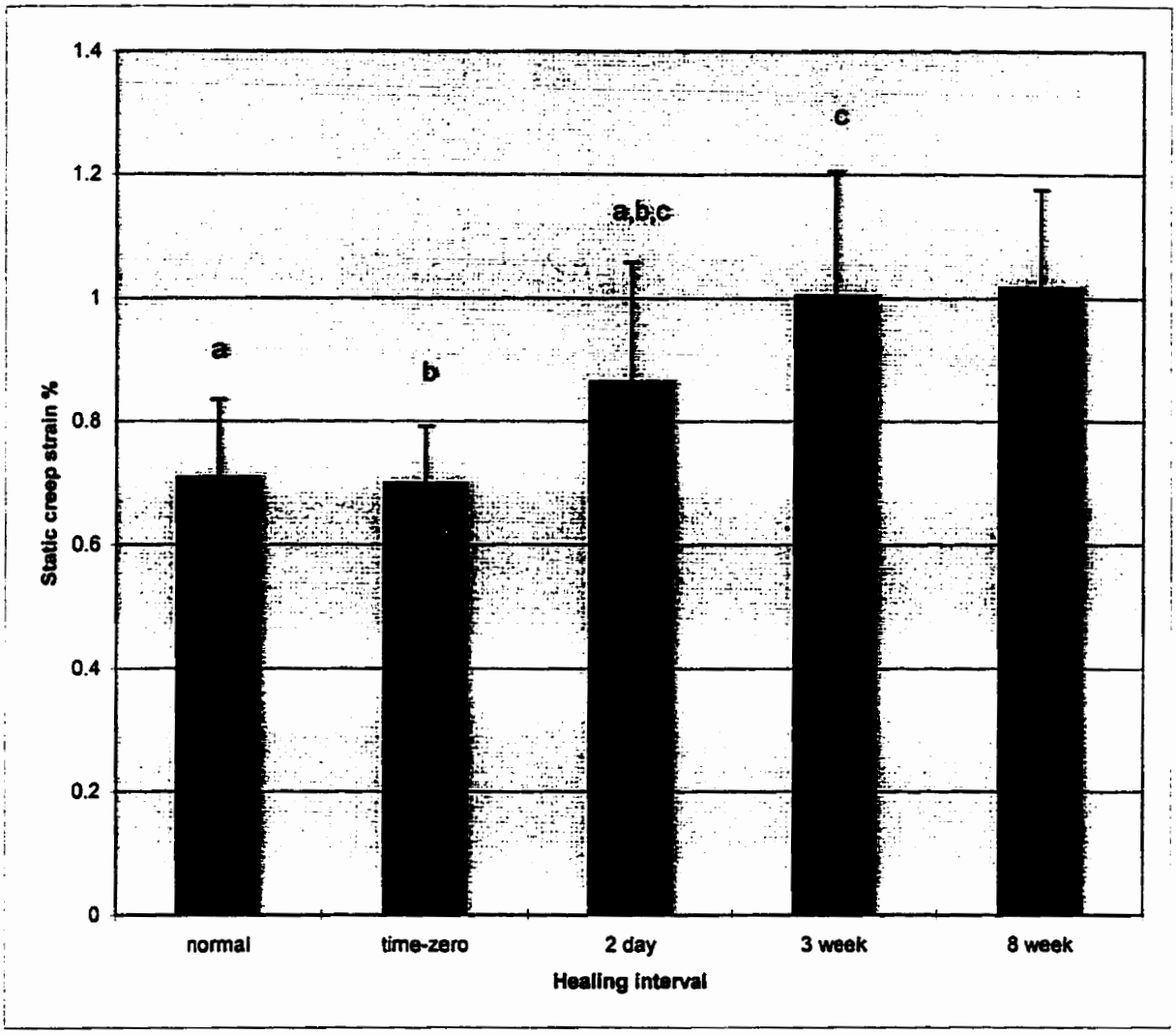

Figure 9: Static creep strain %. Group means and standard deviations. Statistically significant in static creep between 2 day grafts and normal MCLs (a)($p=0.05$) and 2 day grafts and time zero control grafts (b) ($p=0.02$). A further statisticaly significant increase was found between 2 days and 3 weeks (c) ($p<0.05$), but not between 3 weeks and 8 weeks n=7-11 per group.

between 3 and 8 weeks, with no further increase in the susceptibility of the grafts to static creep ($p>0.05$)(Figure 9).

The total creep of the grafts represents the combined creep strain which occurred in both the cyclic and static creep tests. The 2 day grafts crept statistically significantly more than the normal MCLs ($p=0.008$) and the time zero controls ($p=0.02$) (Figure 10). Again there was a significant increase in graft creep in the 3 week grafts, as compared to the 2 day grafts ($p<0.05$), while only a modest, non-statistically significant increase ($p>0.05$) in graft creep between 3 and 8 weeks (Figure 10). No difference was found in the total creep strain between normal MCLs and time zero controls ($p=0.49$).

The unrecovered creep strain after the 20 minute recovery period is shown in Figure 11. At all healing intervals (2 days, 3 weeks, and 8 weeks), grafts were found to have more unrecovered creep strain than the normal MCLs ($p=0.01, p<0.05, p<0.05,$) or the time-zero control grafts ($p=0.002, p<0.05, p<0.05$). No statistically significant differences could be detected in the unrecovered creep strain between the different healing intervals. Under the test conditions used, the normal MCLs, and the time-zero control grafts did not return to zero strain (Figure 11).

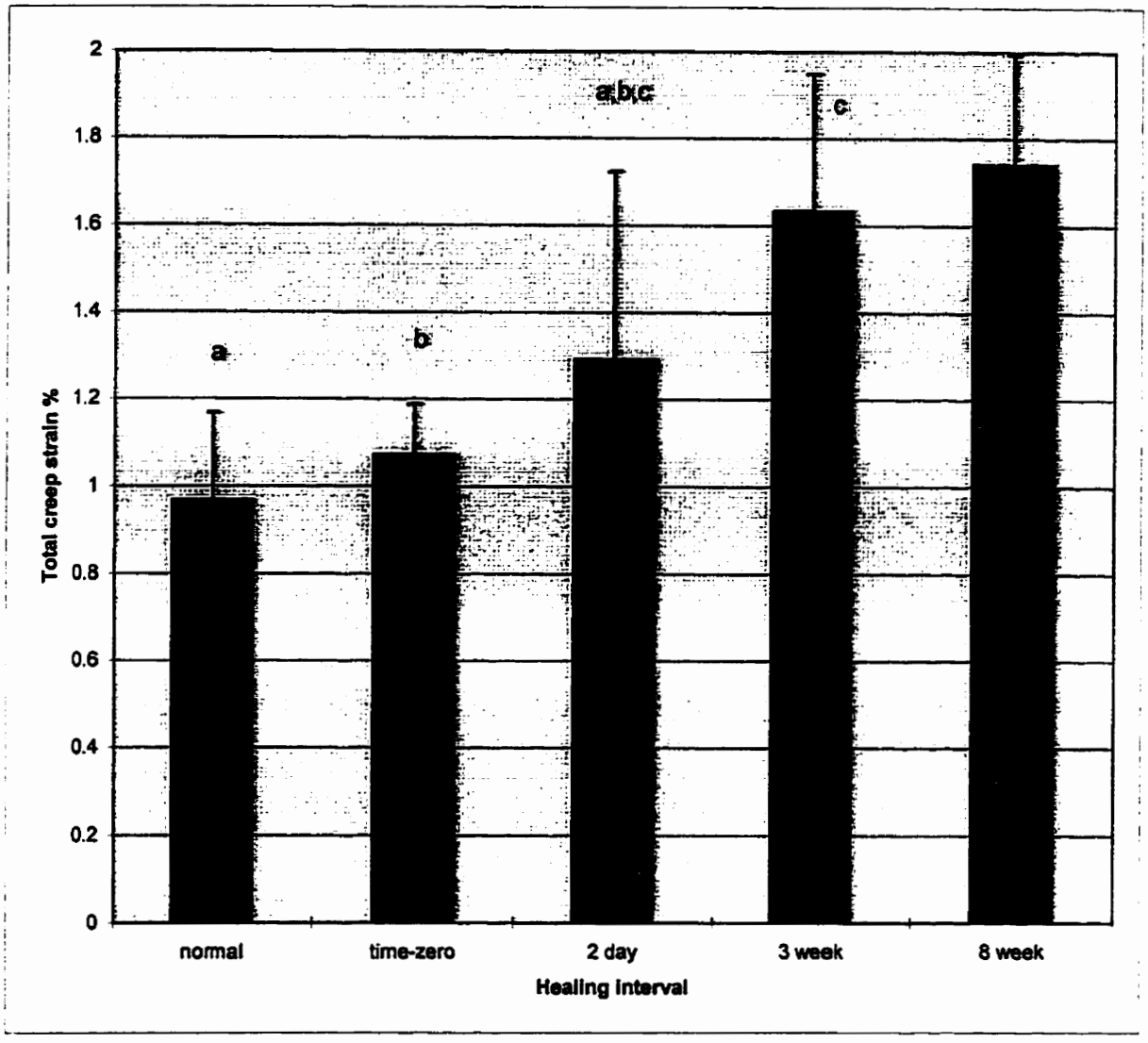

Figure 10: Total creep strain %. Group means and standard deviations. Total creep strain represents the cyclic creep strain plus the static creep strain. 2 day grafts crept significantly more than the normal MCLs (a)($p=0.008$), and the time zero control grafts (b) ($p=0.02$). Graft creep significantly increased between 2 days and 3 weeks (c), ($p<0.05$), but not between 3 weeks and 8 weeks. n=7-11 per group.

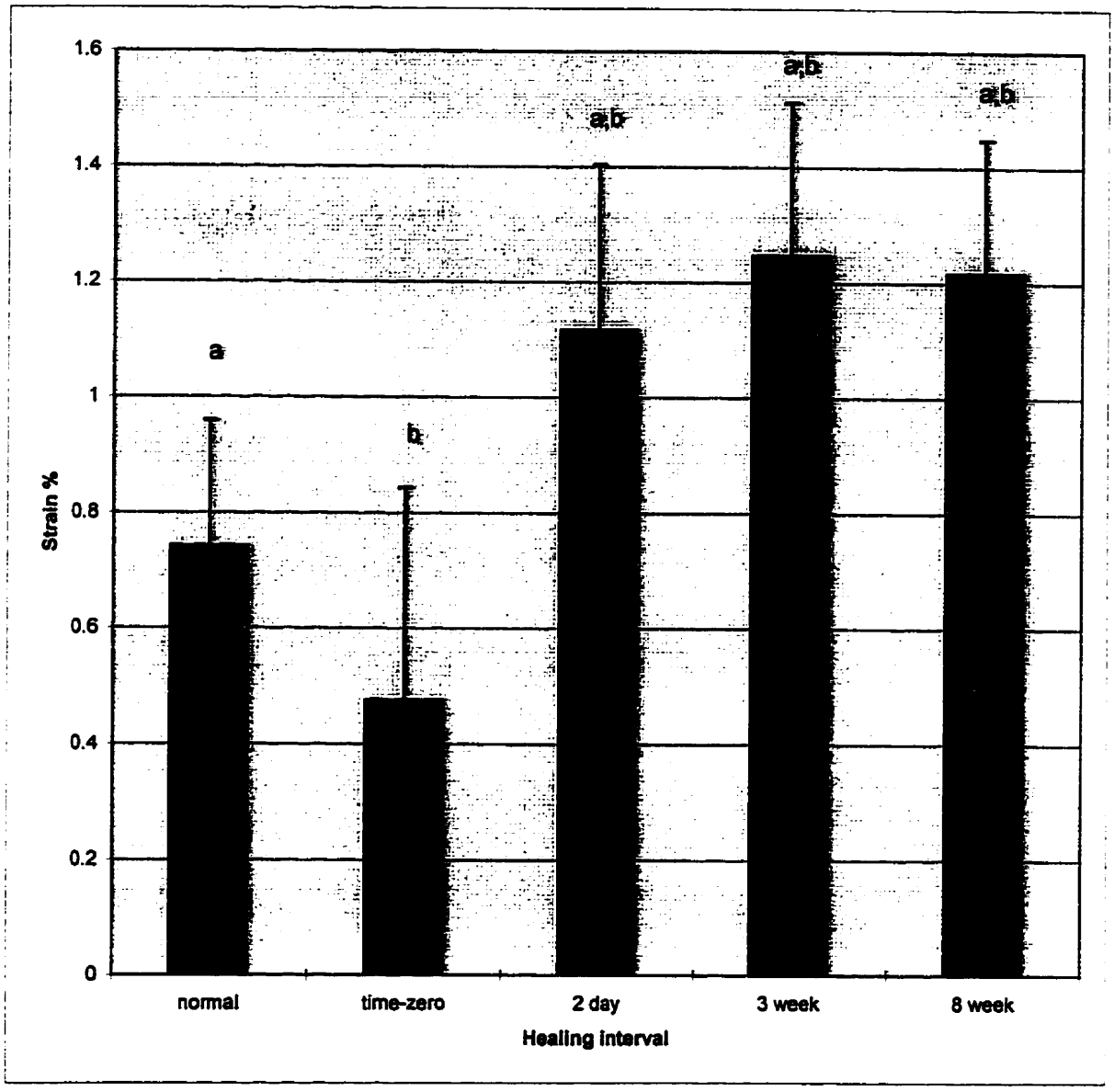

Figure 11: Unrecovered creep strain % following 20 minutes of recovery time at zero load. Statistically significant increase in un-recovered creep strain in the 2 day, 3 week, and 8 week grafts, compared to normal MCLs (a) and time zero controls (b) ($p<0.05$) n=7-11 per group.

DISCUSSION

This study has revealed a very early vulnerability of fresh, anatomically placed, extra-articular rabbit MCL autografts to increased creep. After only two days post-operation, a significant increase in the cyclic, static, and total creep strain was seen, as compared to time-zero grafts and normal MCL controls. The creep vulnerability increased up to about 1.66 times normal by the three week healing interval. These results were seen even under this relatively non-provocative mechanical test of short duration (less than 30 minutes of total loading time), and under what can be estimated to be low physiological loads. The results show that the first three weeks are most critical to the observed graft biomechanical deterioration, as there was no statistically significant subsequent increase in the total creep strain of the 8 week grafts compared to the 3 week grafts. Furthermore, all grafts were less able to recover their original length than normal MCLs, under these test conditions. Because of this early increased susceptibility to creep, and an apparent inability of grafts to completely recover, we speculate that permanent stretch of such grafts could occur *in vivo*, particularly if the grafts were exposed to higher loads, over longer periods of time.

Little has been reported previously about the biomechanical properties of ligament grafts in the very early phases of healing, likely because the majority of biomechanical testing has focused on their high load properties. During the early phases of healing, the weak link for structural failure has been at the graft fixation site, and thus the ligament substance could not be well characterized (22,80,85). In fact, our review of the literature revealed no biomechanical data on ligament grafts at less than 1 week post-operatively.

We had hypothesized that the MCL autografts would become relatively slowly more susceptible to creep with time (over weeks to months), due to what we have seen previously in this model, a progressive infiltration of scar tissue into the graft matrix. Instead we found an early increase in the susceptibility of the grafts at the 2 day to three week healing intervals, prior to any detectable scar tissue infiltration,

Interestingly, a critical analysis of the literature reveals that the majority of ligament reconstruction models in animals, most often involving the ACL, have also shown relatively rapid increases in post-operative joint laxity (usually within weeks) (65,73,104,136,139,152). This result has been explained by slippage of graft fixation, graft failure, secondary whole joint changes, or graft elongation. Ng et al, 1995 (104), reported that the large increase in observed joint laxity in a goat anterior cruciate ligament reconstruction model just three weeks after surgery, may be due to graft elongation secondary to an observed increase in the load relaxation behaviour of the graft, and simultaneous overloading of the graft by the animal. Although load relaxation is a measure of viscoelastic behaviour of soft tissues, it is likely not involved in *in vivo* graft elongation (66). Our results would support the concept that such ACL grafts could elongate in the early post-operative period, but via a mechanism of creep. Clinically, the increased joint laxity which occurs post ACL reconstruction appears not to be as rapid, nor as extensive as that seen in animal models perhaps due to stress differences, since patients likely protect their grafts in the early post-operative period. However the concern expressed in the literature about the current trend of early and aggressive rehabilitation after ligament reconstructions (21,23,73) may have some validity, if stresses are actually being carried by grafts as Beynnon et al have suggested (23).

It is likely however that the intra-articular ACL graft carries more load than the extra-articular MCL graft used in this experiment. The best estimates of *in vivo* stresses in the rabbit MCL is about 2-5 MPa (150). Because of this low stress environment we hypothesized that unlike the ACL grafts, the MCL autografts do not elongate to any great extent *in vivo*. This hypothesis was supported by our data, as there was no increase in joint laxity between 3 weeks and 8 weeks (Figure 7). Nevertheless, we have shown that if this soft tissue graft were exposed to increased stress, it would be vulnerable to creep.

There are several possible explanations which could be used to explain these creep results. The increased early vulnerability of the grafts to creep could be to due any, or all of three mechanisms; Increased graft water content, graft degradation by inflammatory enzymes, or scar tissue infiltration into the grafts.

Water content has been shown to be very important in soft tissue biomechanics, particularly as it relates to viscoelastic properties (63,146), and therefore represents the first possible mechanism by which graft creep may be increased. It has been speculated that interstitial water allows for decreased friction between collagen fibers and thus allows for the easier inter-fibrilar sliding with tissue elongation (146). The movement of water is also important in the time-dependent creep behaviour of tendons and ligaments(61,89,146). Water makes the tissue less stiff as well as more viscoelastic (61,87,89,146). In this study the two day grafts were found to have larger cross-sectional areas than normal MCLs (Figure 6). This increase in size, in such a short period of time, may be due in part, to tissue edema (ie. increased water content) . This swelling may also have contributed to the results seen at 3 weeks and 8 weeks.

It should be clearly pointed out that the increased cross-sectional area of the 2 day grafts over the normal MCL controls and the time-zero grafts required an increase in the applied loads, which were necessary to achieve the standard testing stress level of 4.1MPa in each specimen. The average load carried by the 2 day grafts was 21.05 +/- 5.17 N, while the normal MCL controls carried 16.1 +/- 1.8 N, and the time-zero grafts carried 15.4+/-2.2 N. The differences in load are small, however they were statistically different in t-test comparisons ($p=0.006$, $p=0.003$, respectively). Although this difference in load may have been implicated in the increased creep seen in the 2 day grafts over the control groups, data from our lab has conversely shown that there is no statistical difference in the creep of normal rabbit MCLs between stress levels of 4MPa and 7MPa (This range of stresses actually represent larger differences in load on the ligaments than in this experiment) (134). In other words, it is highly unlikely that the load difference that we utilized in order to apply the same stresses to each ligament was responsible for the creep difference measured.

It seems likely that if simple swelling were solely responsible for the increased creep of the 2 day grafts, that this water would have been easily expelled from the tissue during the cyclic creep component of the test and that no difference would be seen in the static creep test between the grafts and the controls. We found however, that the 2 day grafts crept more in the static creep test than the normal MCLs and time zero control grafts. Graft degradation therefore represents a second possible important explanation for our results. A very rapid degradation of the collagenous scaffold, might have been largely responsible for decreasing the grafts' ability to resist creep forces at the 2day healing interval (and perhaps even more importantly at 3 and 8 weeks). It is possible that very

early degradation of the collagen matrix may also have contributed to the swelling of the 2 day grafts, similar to swelling seen in early arthritic cartilage in which the collagen type II (which resists swelling pressure) has been shown to be damaged (67). In support of this hypothesis of early degradation, it has been shown in rat Achilles tendon grafts that there is a rapid turnover of collagen (degradation and synthesis), with approximately 50% of the collagen being degraded within the first month (82). We are currently addressing the possibility that enzymatic degradation can alter creep vulnerability in this MCL autograft model.

The third possibility is that the grafts are being infiltrated and replaced by scar tissue (10,19,114). Biopsies of human soft tissue grafts (115), as well as animal ligament reconstruction models (19), have shown that there is an almost complete cellular necrosis of the graft fibroblasts, which is followed by new blood vessel and scar tissue infiltration into the graft matrix. This would clearly become increasingly more of a factor at the 3 week and 8 week healing intervals. Recent work has shown that scar tissue creeps more than normal ligament tissue (134). Therefore, the replacement of the degraded graft collagen scaffold by scar tissue would theoretically make the structure more susceptible to creep, even if the overall collagen balance is not negative, as has been previously shown (82).

The reasons for the increased creep vulnerability of scar tissue has not yet been studied at the ultra-structural level; however, collagen fiber recruitment is generally considered to be important to a tissue's ability to resist creep forces (56,146,150). As ligament tissue elongates, collagen fibers are progressively recruited to take up load (146,150). Thus, well aligned collagen fibers resist creep better than disorganized fibers,

because they are fully recruited at a shorter length of stretch. Scar tissue collagen has been shown with scanning electron micrographs to have very disorganized collagen fibers, especially in the first 8 weeks of healing (27,115). Therefore, based on the theory of fiber recruitment, scar tissue would be expected to creep more than normal ligament tissue, which has well aligned collagen fibers. Other possible explanations for the increased creep of scar tissue in grafts, could also be proposed. For instance, scar collagen fibers are smaller in diameter than normal ligament fibers (115). Because fewer of these small fibers are initially recruited to resist load when scar is exposed to creep forces, these fibers could be failing, and thus allowing for increased tissue elongation. This fiber failure may be one possible explanation for the decreased ability of the grafts to recover from creep strain (ie. a mechanism for their plastic deformation) (Figure 11).

The last interesting result of this study was the failure of the normal ligaments to completely recover their original length under our test conditions, following the creep testing and an equal period of time of recovery at zero load. Clearly a normal ligament would be expected to be able to completely recover its length fairly quickly following loading *in vivo*. Several explanations could account for this incomplete creep recovery; 1) potential vascular contributions to tissue fluid and recovery, 2) increased time for recovery *in vivo*, 3) lower stresses *in vivo*. It is possible that 4.1 MPa is larger than normal stress for the rabbit MCL, and thus fiber damage and plastic deformation resulted. Also the distribution of the stress within the ligaments was likely not equal, as some collagen fibers may have been damaged from carrying much higher stresses. Despite these potential differences to the *in vivo* situation, we found that under these test conditions, the grafts were less able to recover from the creep test. This suggests that

there were changes within the graft matrix which allowed for either a slower recovery, or a permanent plastic deformation of the grafts (or both).

In conclusion we have shown that rabbit MCL autografts become more vulnerable to creep under low loads as compared to the normal MCL, and this vulnerability develops within days after transplantation. This susceptibility increases up to three weeks of healing, and subsequently plateaus somewhat between three and eight weeks. It appears that this first three weeks is critical to the deterioration of these fresh anatomically placed autografts with respect to their ability to both resist and recover from creep forces. This increased vulnerability to creep would almost certainly have significant functional consequences to a reconstructed joint, if the graft were to subjected to sufficient loads to cause progressive elongation (creep) over time. Future investigations will be required determine whether a similar creep vulnerability is found in intra-articular ACL grafts.

CHAPTER III

Knee Immobilization Increases the Biomechanical Creep of Rabbit Medial Collateral Ligament Autografts

INTRODUCTION

Ligament injuries are a significant cause of morbidity, especially among active individuals (96,110). Many of these ligament injuries are capable of healing with scar tissue, and restoring normal or close to normal function; however, there are a large number of clinical situations in which functional healing does not occur (45,69,101,113). This can result in chronic joint laxity, and subsequent osteoarthritis (12,45,71,109,110,113). The most widely accepted surgical treatment for these chronically lax post-traumatic joints is a ligament reconstruction with a soft tissue graft. Many patients have clinical improvement following these graft procedures, however there is literature which suggests that these reconstructions do not always prevent the progression of osteoarthritis, and normal joint kinematics are not restored generally (23,41,48,136).

It is now being recognized increasingly that one of the reasons for failure of such soft tissue reconstructions is a "stretching out", or elongation over time (2,3,17,21,22,30,33,71,76,94,104,128). Careful analysis of the clinical literature on anterior cruciate ligament ACL reconstructions of the knee, reveals at least a 30% incidence of recurrent post-operative joint laxity

(2,3,17,20,30,33,46,57,59,71,76,91,94,97,120,128). Similarly in animal ligament reconstruction models, significant joint laxity and osteoarthritis often develops (19,24,32,43,60,65,75,78,104,127,130,136,139,152).

The appropriate engineering definition of this stretch phenomenon is creep, which is defined as a deformation (elongation) under a constant or cyclically repetitive load (56). Creep properties of ligament grafts over time have never been studied, and recent evidence reveals that stress/relaxation (a previously measured viscoelastic property of grafts) cannot be used to predict or model creep at low stresses in ligament tissue (135). Further evidence also suggests tissues are likely loaded repetitively *in vivo,* and not deformed repetitively to a fixed deformation, making creep testing a potentially more relevant measure of graft viscoelastic behavior than that of relaxation(66).

The current trend in rehabilitation after anterior cruciate ligament reconstructions, is early and aggressive physiotherapy (108,126), and there is a great deal of concern expressed in the literature that excessive loading could be contributing to the elongation of ligament grafts (21,22,104). After implantation, these grafts have been shown in both humans and animal models, to be infiltrated by scar tissue and weaken (10,19,29,43,75,78,95,104,115,131,151). The majority of biomechanical studies to date have studied this decrease in graft ultimate tensile strength, however the viscoelastic properties, related to graft elongation have received relatively little attention. Further, the effects of different rehabilitation methods on the biomechanical outcome of grafts are controversial (102).

Based on the results of ligament gap healing models in rats which showed that immobilization resulted in less scar tissue formation, and that immobilization of the

rabbit knee in which the MCL had been injured resulted in earlier formation of well aligned collagen fibers in the MCL scar (27,138), we hypothesized that knee immobilization would decrease the creep potential of ligament grafts. The purpose of this study was to test this hypothesis in an animal model, the rabbit bone-medial collateral ligament-bone autograft.

METHODS AND MATERIALS

Model

Thirty nine skeletally mature (>12 months), female New Zealand white rabbits (weighing 4.5-6 kg) had a standardized orthotopic medial collateral ligament (MCL) autograft procedure to the right hindlimb, under halothane general anesthetic, as described previously (80,122). The MCL was harvested with femoral and tibial bone plugs, which had been pre-drilled and pre-tapped before graft removal. The graft was removed, washed in saline, and immediately replaced with screw fixation in an anatomical position (Chp. 2, Figure 2)(122). In order to properly test our hypothesis, a relatively rigid immobilization method was required. This was achieved by using a previously validated method of Akeson et al (4). This involved immobilizing the animals with an internal fixation pinning, in which a 1.6mm stainless steel wire was passed through the tibia and hooked around the mid-shaft of the femur, thus rigidly immobilizing the knee in a flexed position, but avoiding further trauma to the joint itself (Figure 12) (4). The non-immobilized animals were allowed normal activities in a 65x45x30cm cage. Animals received a standard diet and water *ad libitum*. The animals were handled according to established ethical guidelines approved by the local animal care committee, and were

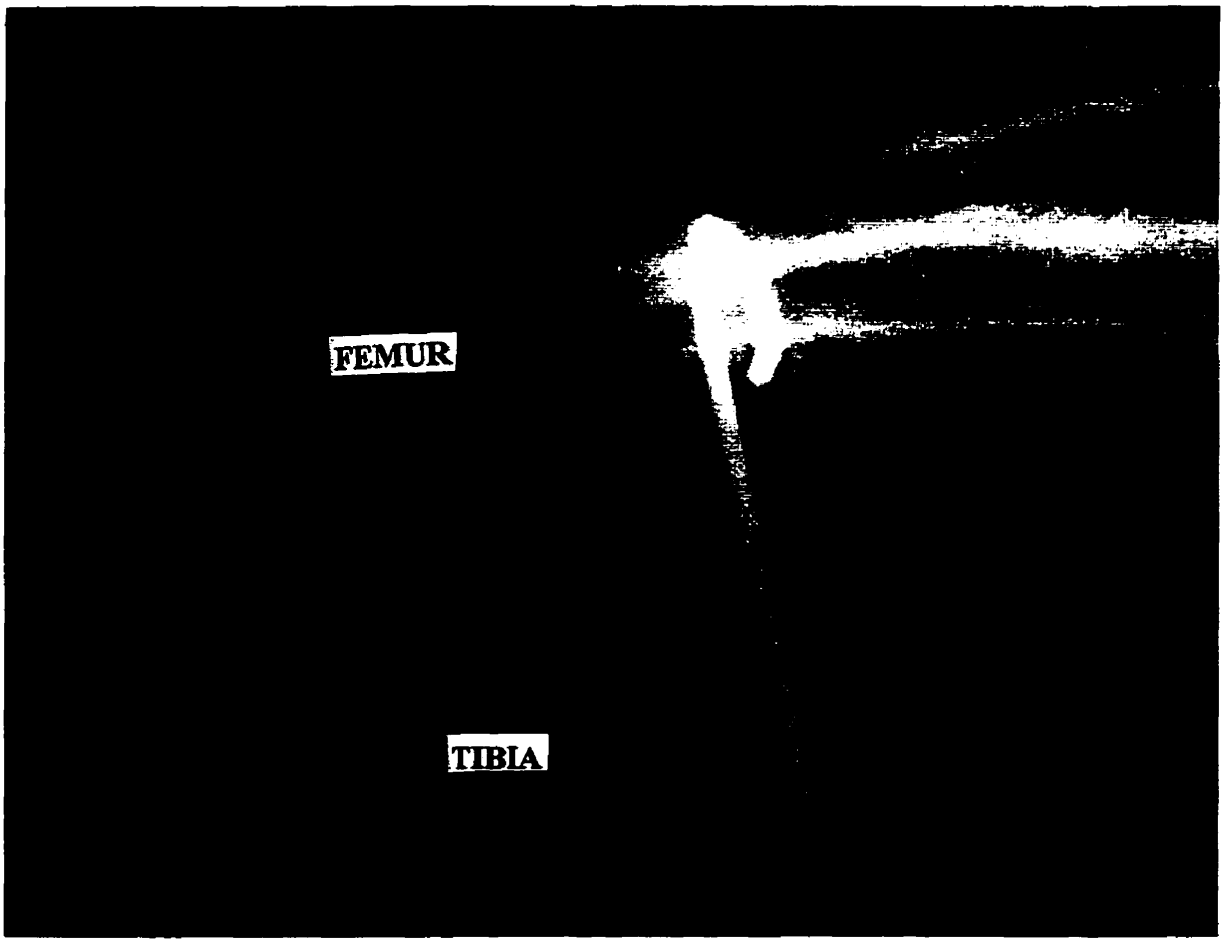

Figure 12. X-ray picture of pin immobilized rabbit hind-limb. Kirschner wire placed through shaft of tibia, and then hooked around the femur with extremity in flexion. Wire is tunneled laterally through soft tissue. (Note: Distal third of the shafts of both tibia and femur obscured by X-ray over-exposure.)

sacrificed with a phenobarbitol overdose (Euthanyl 275mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) at 3 weeks (moved; n=10, immobilized; n=8), or 8 weeks (moved; n=10, immobilized; n=11) post-operatively. Eight normal control rabbits were sacrificed similarly. The hindlimbs were harvested, and frozen with the skin intact, in a sealed plastic bag at -70° C. On the morning of testing, the samples were thawed at room temperature and all soft tissues were removed except for the collateral ligaments, the cruciate ligaments, and the menisci. The bones were then cut with a saw approximately 5 cm from the joint line.

Biomechanics

The dissected samples were potted in polymethylmethacrylate, and mounted on a specialized closed loop, servohydraulic material testing system (MTS, Systems Corporation, Minneapolis, Minnesota) for creep testing. After mounting the tibia of the specimen, in series with a load cell (Model #661.12A-05), to the actuator cross-head, and aligning the length of the graft with the load axis of the testing machine, the femur was lowered into a second pot and similarly fixed with cement at a joint angle of 70° flexion (88). During the mounting procedure, the grafts were kept moist by frequent irrigation with 0.9% phosphate buffered saline, pH 7.4. In order to obtain a measure of joint laxity the specimens were cycled between 2.5 N and -5 N at 1mm/min. The laxity was defined as the displacement of the joint from 0.1N compressive load, and 0.1N tensile load. After obtaining a "whole joint laxity" measure, the lateral collateral ligament, the cruciate ligament and the menisci were removed , leaving just the femur-MCL-tibia complex. This complex was similarly cycled to gain a measure of "MCL" laxity. Also during this procedure, "ligament zero" was identified as the cross head position at which the

ligament just began to take up load (0.1N) (Chp.2, Figure 3). The ligament length was measured at this position in a standard way, using digital calipers. The medial femoral condyle was then removed carefully and specialized calipers were used to measure the cross sectional area of the grafts at the joint line (accurate to +/- 5%) (129). The specimens were then enclosed in a humidity chamber (relative humidity 99%) at 37°C, to provide a constant environment during creep testing (Chp. 2, Figure 4)(142). Both temperature and test environment have been shown to have significant effects on the biomechanical behavior of soft tissues (36,63,147).

The creep protocol (Chp 2., Figure 5) involved 30 cycles of cyclical loading at 1 Hz. to a constant stress level of 4.1 MPa, followed immediately by a 20 minute static creep, at the same stress level. This stress level is approximately 5% of the failure stress of a normal rabbit MCL (within "toe" region of stress/strain curve), and represents the estimated normal tensile loads carried by the rabbit medial collateral ligament *in vivo* (150). The stress level for each ligament graft was calculated based on the cross-sectional area measurement at the joint line. The resulting creep of the grafts was measured by cross-head displacement (linear variable differential transformer) and stored on a computer data file (Compaq 486). Tensile strain was defined as this measured graft deformation in millimeters, divided by the "ligament zero" length. Cyclic creep strain was defined as the graft strain at the peak of the thirtieth cycle, minus the strain at the peak of the first cycle. Static creep strain was defined as the strain at the end of the 20 minutes, minus the strain at the beginning of the 20 minutes (no recovery was allowed between the cyclic and static tests). Total creep strain (creep strain resulting from both the cyclic and static creep tests) was defined as the strain at the end of the static creep

test, minus the strain at the peak of the first cycle of cyclic creep. Following the creep testing, the ligaments were allowed to recover at zero load for 20 minutes. The residual strain after this 20 minute period was defined as un-recovered creep.

Outcomes were analyzed statistically using a two-way ANOVA, with time and treatment (immobilization) as the two variables (SAS software version 6.12). Groups were then compared using the Tukey's test for multiple comparisons (student standardized range). If the two-way analysis showed a significant interaction between time and treatment, then outcomes were compared with a one-way ANOVA analysis, using a combined factor. Again multiple comparisons were made using Tukey's test. Grafts were compared to normal MCLs using a student t-test using transformed data. Data were transformed in order to stabilize the unequal variance between grafts and normal controls. A significance level of 0.05 was used in all tests.

RESULTS

Gross Observations

At three weeks there was abundant fibrous scar tissue encasing the MCL autografts, and the entire medial side, and the intra-articular areas of the knee joint. This was more prevalent in the immobilized group, as the knees were heavily scarred into flexion (Figure 13), and they could not easily be straightened into full extension, until all scar tissue had been dissected (Figure 14). A similar scar response was seen at the eight week healing interval. At both 3 weeks and 8 weeks, all grafts appeared larger than a normal MCL. There was extensive bony healing at the fixation sites at the three and eight week intervals.

Graft size and joint laxity

The cross-sectional area of grafts increased with time in the non-immobilized groups (Figure 15)(ANOVA, $p=0.0002$). The immobilized animals showed an increase in cross-sectional area after 3 weeks of healing (t-test, $p=0.0001$), but no subsequent increase between 3 and 8 weeks (Tukey's, $p>0.05$). At the 8 week healing interval the non-immobilized grafts seemed larger than the immobilized grafts but this difference did not reach statistical significance when compared directly (Figure 15). However, as mentioned the ANOVA analysis revealed that there was a statistically significant increase in graft size between 3 weeks and 8 weeks in the non-immobilized group but not in the immobilized group, suggesting that immobilization did limit the increase in graft cross-sectional area.

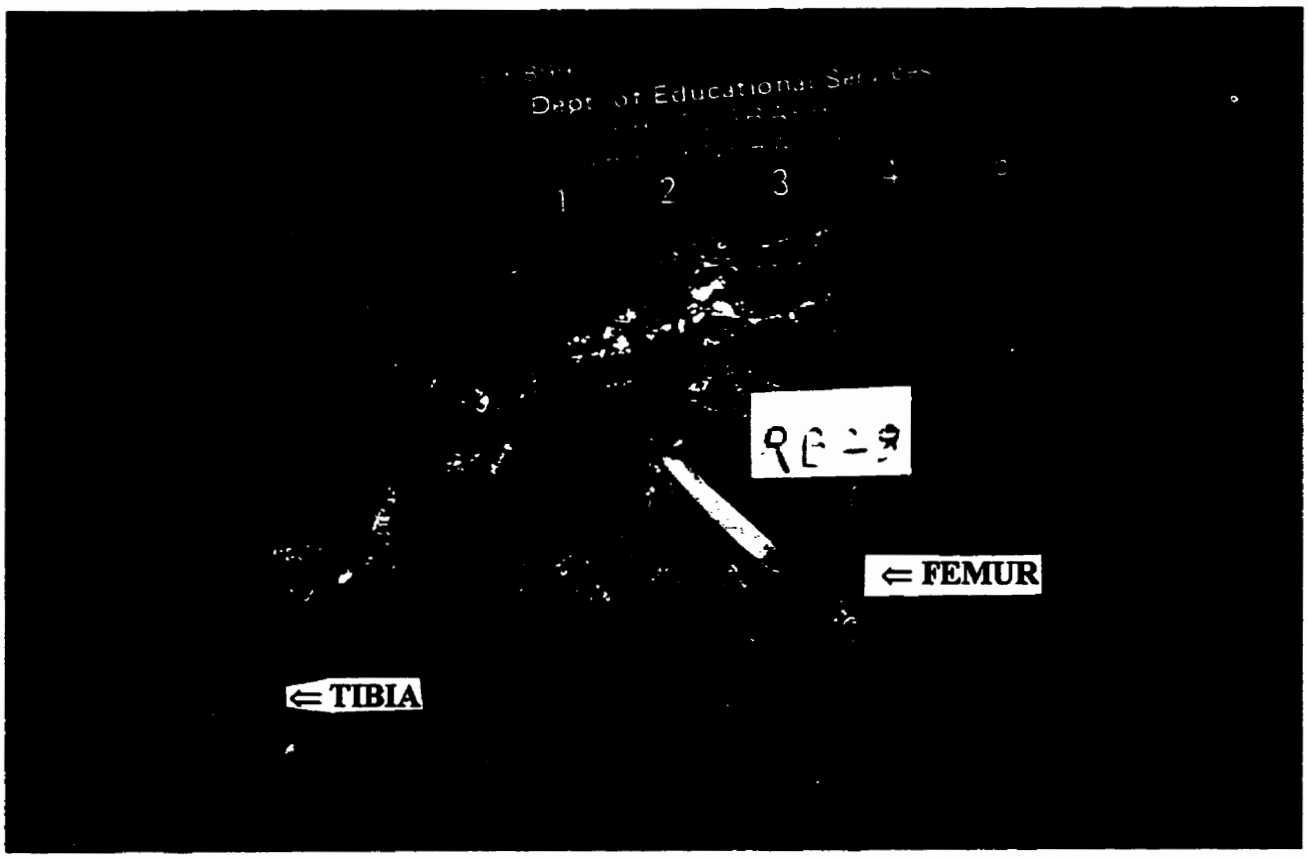

Figure 13. Gross photograph of 3 week immobilized MCL autografted hind-limb. There is a large amount of scar tissue encasing the graft and the entire medial side of the knee joint. Wire can be seen holding limb in flexed position. Similar findings were seen in 8 week immobilized grafts

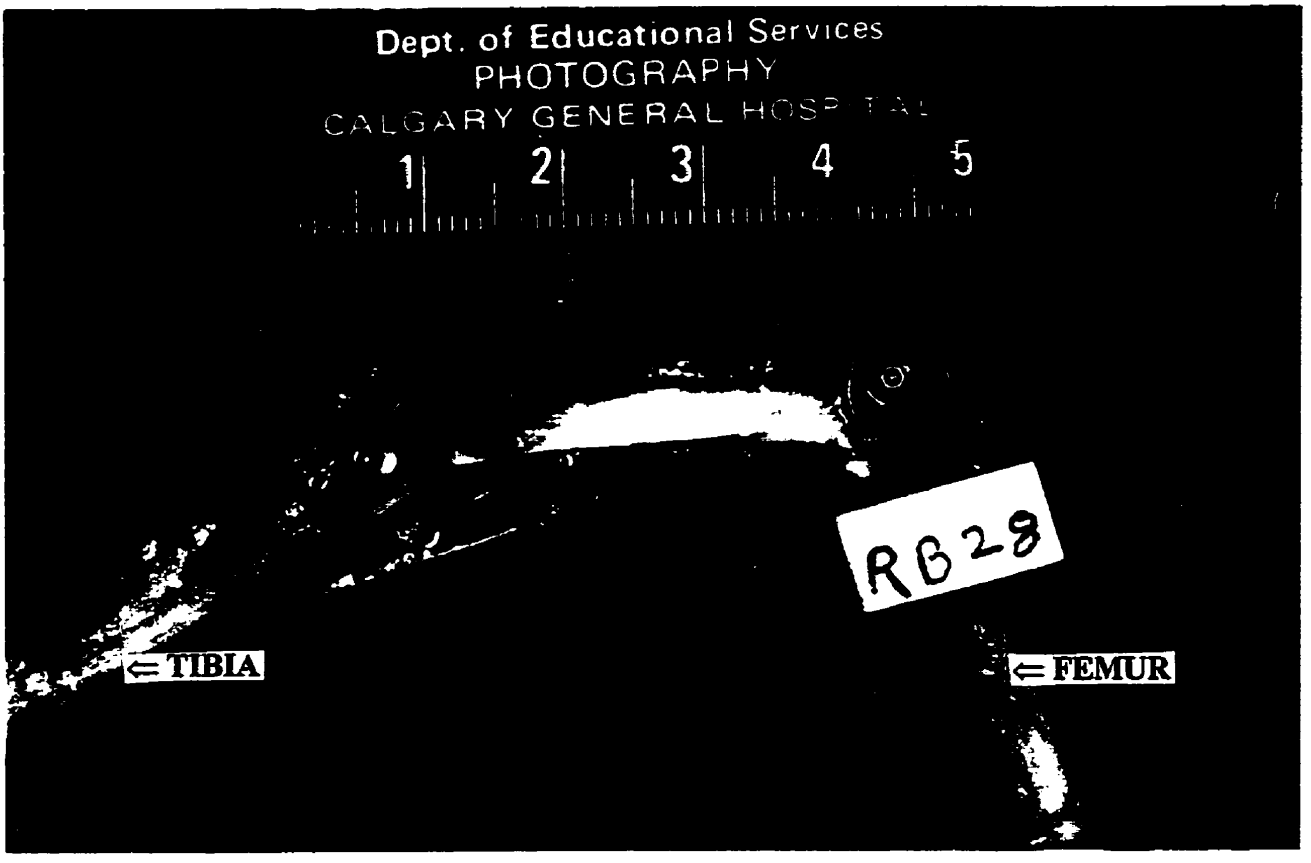

Figure 14. Gross photograph of same 3 week immobilized joint as shown in Figure 13 after dissection of soft tissues and surrounding scar tissue. MCL autograft can be seen between the screw fixation in femur and tibia. All grafts looked grossly larger than normal MCL. Note translucency of graft, especially in femoral and tibial thirds.

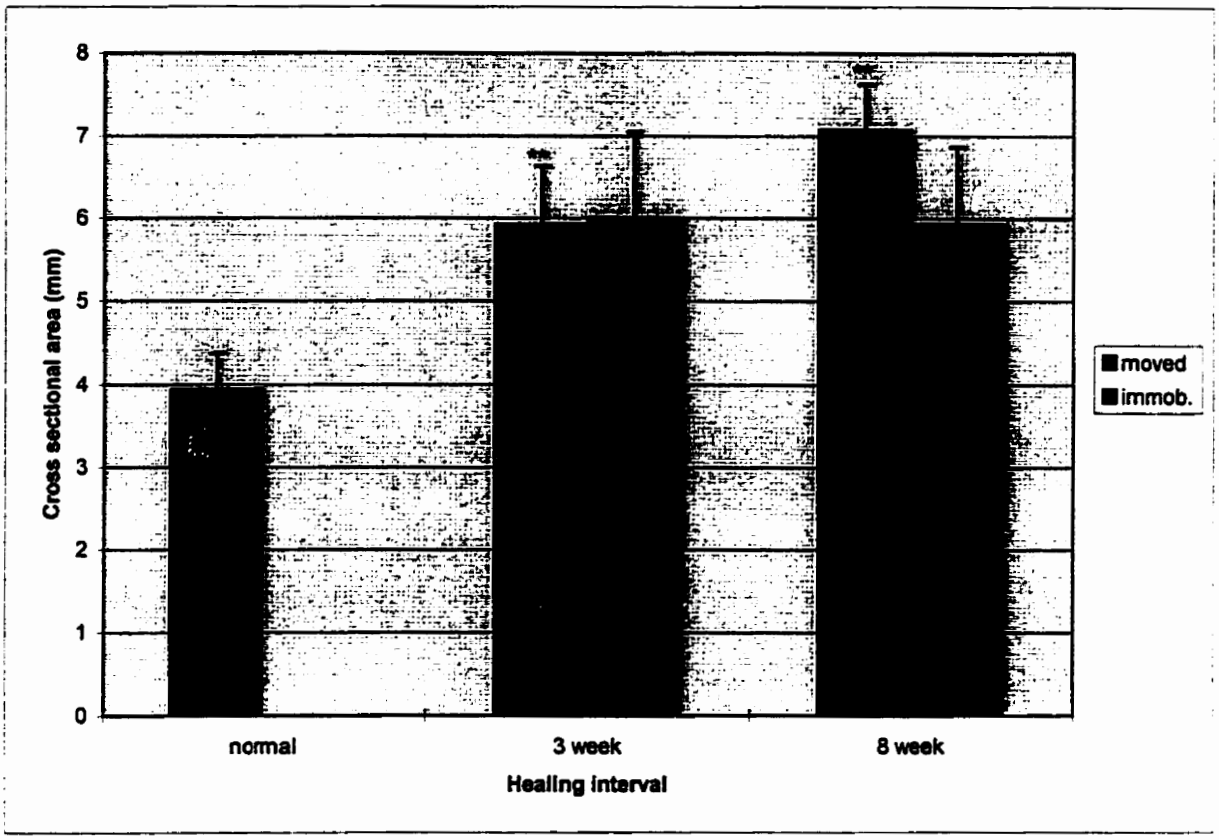

Figure 15: Cross sectional area of grafts. Group means and standard deviations shown. Grafts are larger than normal MCL controls ($p<0.05$). ANOVA reveals increased cross sectional area in non-immmobilized (moved) group with time ($p=0.0002$, **), however similar time trend not seen in immobilized group (immob.) ($p>0.05$). n=8-11 per group

In terms of joint laxity, Figure 16 shows that the immobilized joints were far less lax than the non-immobilized joints, at both 3 and 8 weeks (Tukey's, $p<0.05$). The non-immobilized MCL autografted joints were more lax than normal joints at three weeks (t-test, $p=0.03$), with no subsequent increase in laxity between 3 weeks and 8 weeks (ANOVA showed no time effect, $p>0.05$)(Figure 16).

Graft Creep

Table 2 summarizes all the graft creep data. Figure 17 represents the cyclic creep strain of the grafts. After the 30^{th} cycle all grafts were found to creep significantly more than normal MCLs. By three weeks the non-immobilized grafts crept more than three times as much as normal controls (t-test, $p=0.0004$), while there was little further increase in creep susceptibility between three and eight weeks, and the apparent increase did not reach statistical significance (ANOVA, $p>0.05$). Immobilization significantly increased the susceptibility of the grafts to cyclic creep at the 3 week interval (ANOVA-Tukey's, $p<0.05$), however the apparent immobilization effect on cyclic creep susceptibility at the 8 week healing interval did not reach statistical significance, possibly due to the larger variance seen in the 8 week grafts (Figure 17).

Similar results were seen in the static creep test (Figure 18). All the grafts crept significantly more than normal MCLs (t-tests, $p<0.05$), with the increase in creep vulnerability occurring within the first three weeks, and no subsequent increase between three and eight weeks (Figure 18)(Tukey's comparisons, $p>0.05$). During static creep, the immobilized grafts crept significantly more than the non-immobilized grafts, as the 2 way

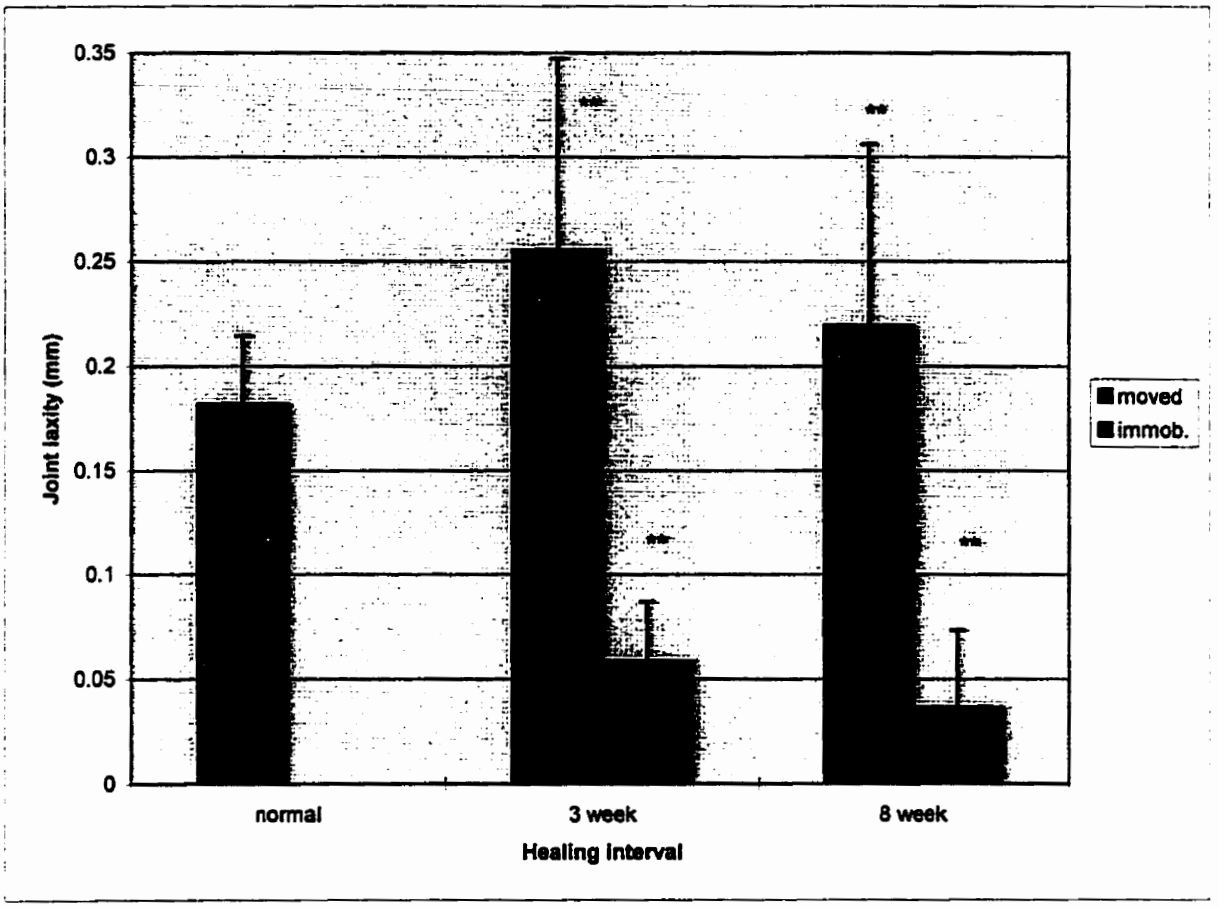

Figure 16:Joint laxity. Group means and standard deviations. Immobilized grafts less lax than moved grafts ($p<0.05$, **). No statistical difference in laxity between 3 weeks and 8 weeks in the moved group. n=8-11 per group.

Table 2. Summary of Creep Data. Group means +/- standard deviations. Immob.= immobilized .grafts; moved= non-immobilized grafts.

GROUP	CYCLIC CREEP STRAIN %	STATIC CREEP STRAIN %	TOTAL CREEP STRAIN %	UN-RECOVERED CREEP STRAIN
Normal MCL n=8	0.17+/-0.17	0.71+/-0.13	0.97+/-0.20	0.74+/-0.21
3 week moved n=10	0.51+/-0.11 (a,b)	1.00+/-0.20 (a,b)	1.63+/-0.32 (a,b)	1.24+/-0.27 (a)
3 week immob. n=8	0.85+/-0.14 (a,b)	1.20+/-0.14 (a,b)	2.25+/-0.29 (a,b)	0.97+/-0.56
8 week moved n=10	0.59+/-0.11 (a)	1.02+/-0.16 (a,b)	1.74+/-0.25 (a,b)	1.21+/-0.23 (a)
8 week immob. n=11	0.77+/-0.22 (a)	1.09+/-0.27 (a,b)	2.13+/-0.45 (a,b)	1.16+/-0.56

"a"= statistically different to normal MCL
"b"= statistical difference between immobilized and moved group
"c"= statistically different to between 3 and 8 weeks

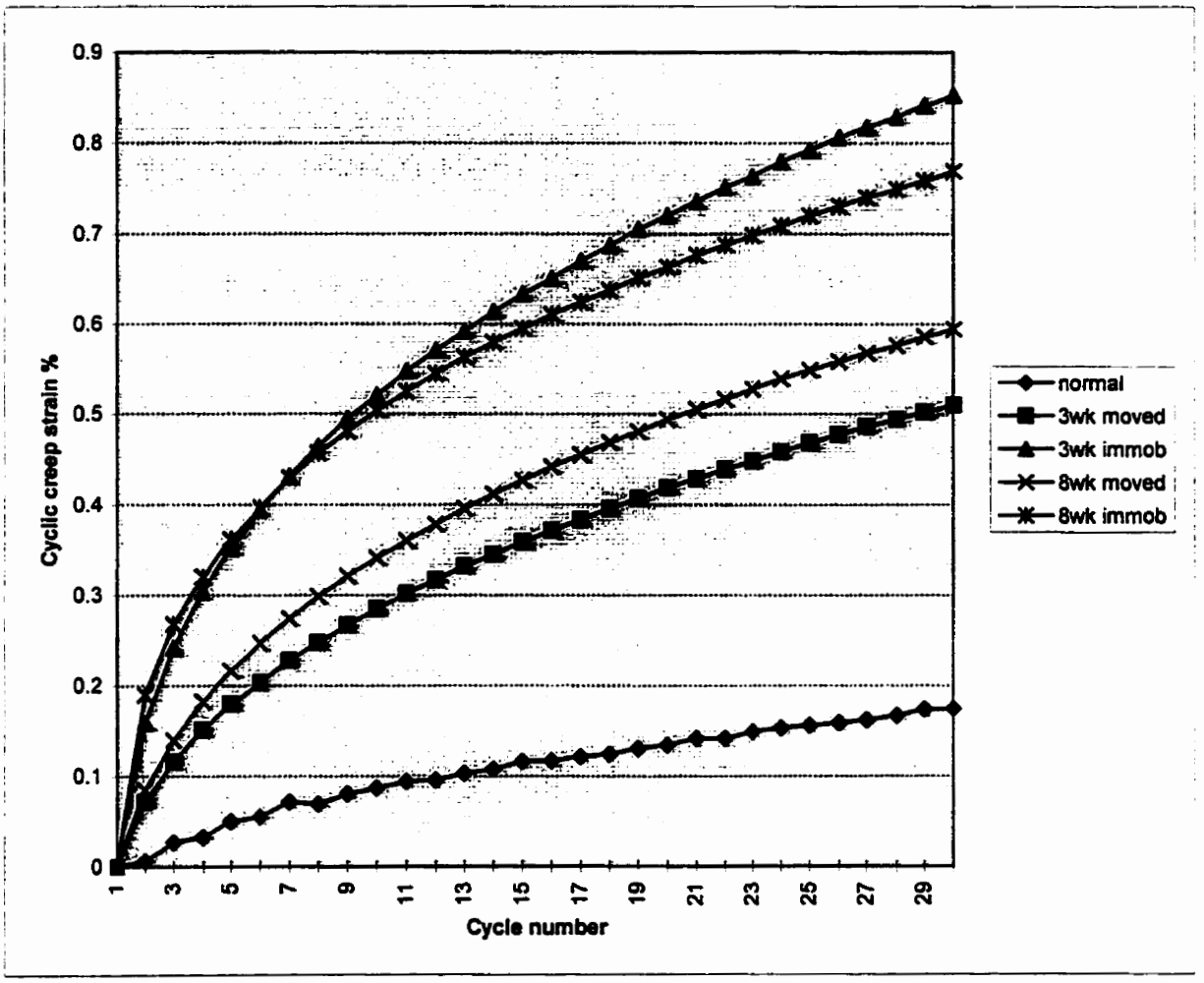

Figure 17: Cyclic creep strain %. Group means for each cycle number. Specimens repetitively stressed to 4 MPa. at 1 Hz. Immobilization significantly increased cyclic creep strain at 3 weeks ($p<0.05$), but no statistical difference reached at 8 weeks. All grafts crept significantly more than normal MCL controls ($p<0.05$). n=8-11 per group.

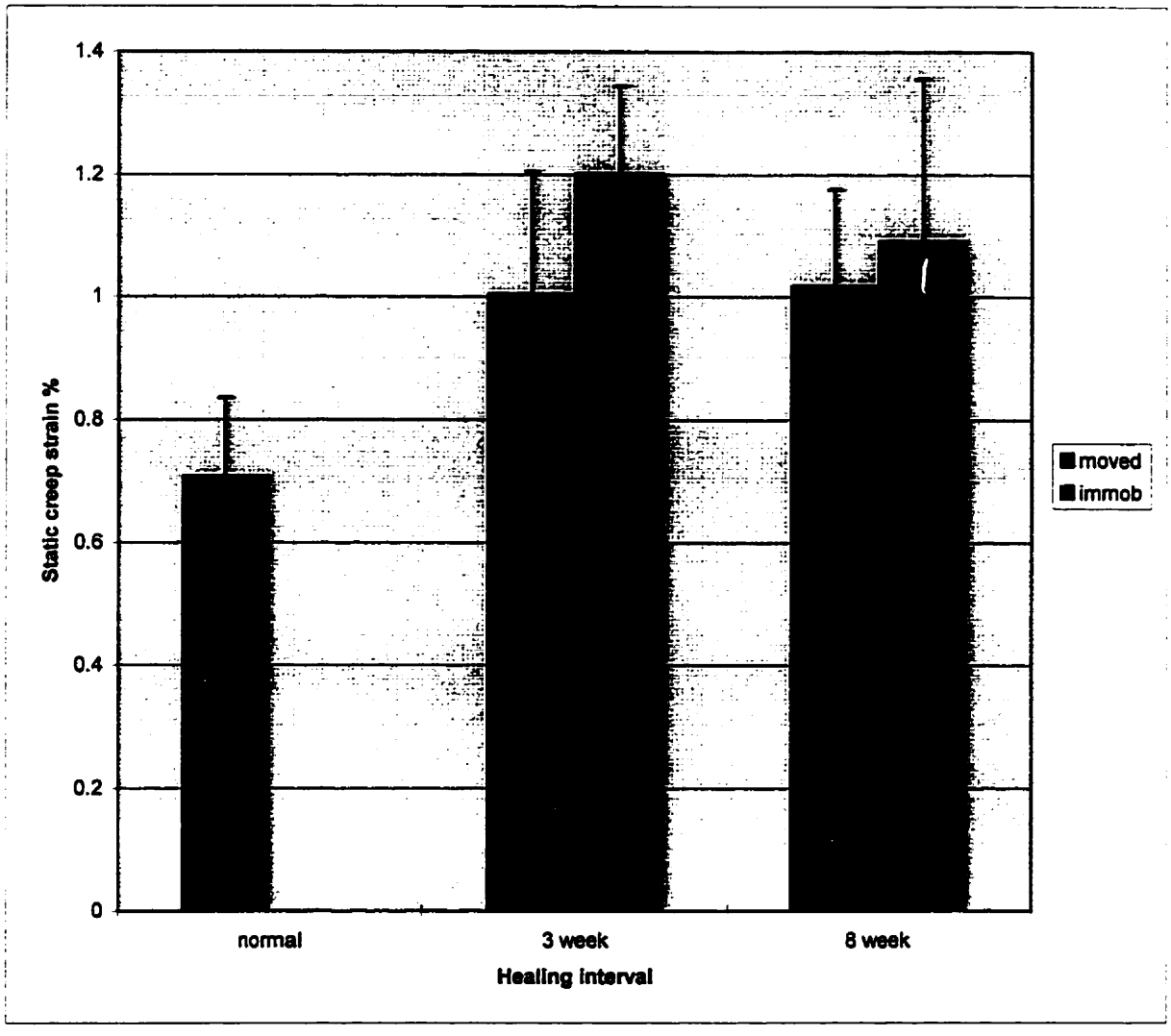

Figure 18: Static creep strain %. Group means with standard deviations. Specimens stressed to 4 MPa for 20 mins. All grafts crept significantly more than normal MCL controls ($p<0.05$). Immobilized groups crept significantly more than moved grafts ($p=0.04$). No statistically significant increases within treatment groups between 3 and 8 weeks. n=8-11 per group.

ANOVA revealed a significant treatment effect, with no time-treatment interaction ($p=0.04$).

If the total creep strain % is considered (creep strain which occurred in both the cyclic and static creep phases of the test protocol), the results reflect those which were seen in the static creep test. Statistically significant differences were seen between all grafts and normal MCLs, and there was a significant effect of immobilization on the total creep (ANOVA, $p=0.0007$) (Figure 19). There was no statistically significant increase within treatment groups between 3 and 8 weeks (Figure 19).

Following the creep protocol, the ligaments were allowed to recover for 20 minutes at zero load. The un-recovered strain following this recovery period is shown in Figure 20. In these *in vitro* test conditions, even normal MCLs were seen to have some un-recovered strain. All the moved grafts however, at both 3 and 8 weeks were seen to have statistically significantly more un-recovered creep than the normal MCLs, under the same test conditions ($p=0.0009$,$p=0.0002$) (Figure 20). The variance within the immobilized grafts however was large, and although they appeared to recover less than normal MCLs, they did not reach statistical significance with direct comparison. However, the 1 way ANOVA and Tukey's comparisons revealed no effect of immobilization on un-recovered creep ($p>0.15$). In fact, no statistically significant differences between any of the grafts could be detected.

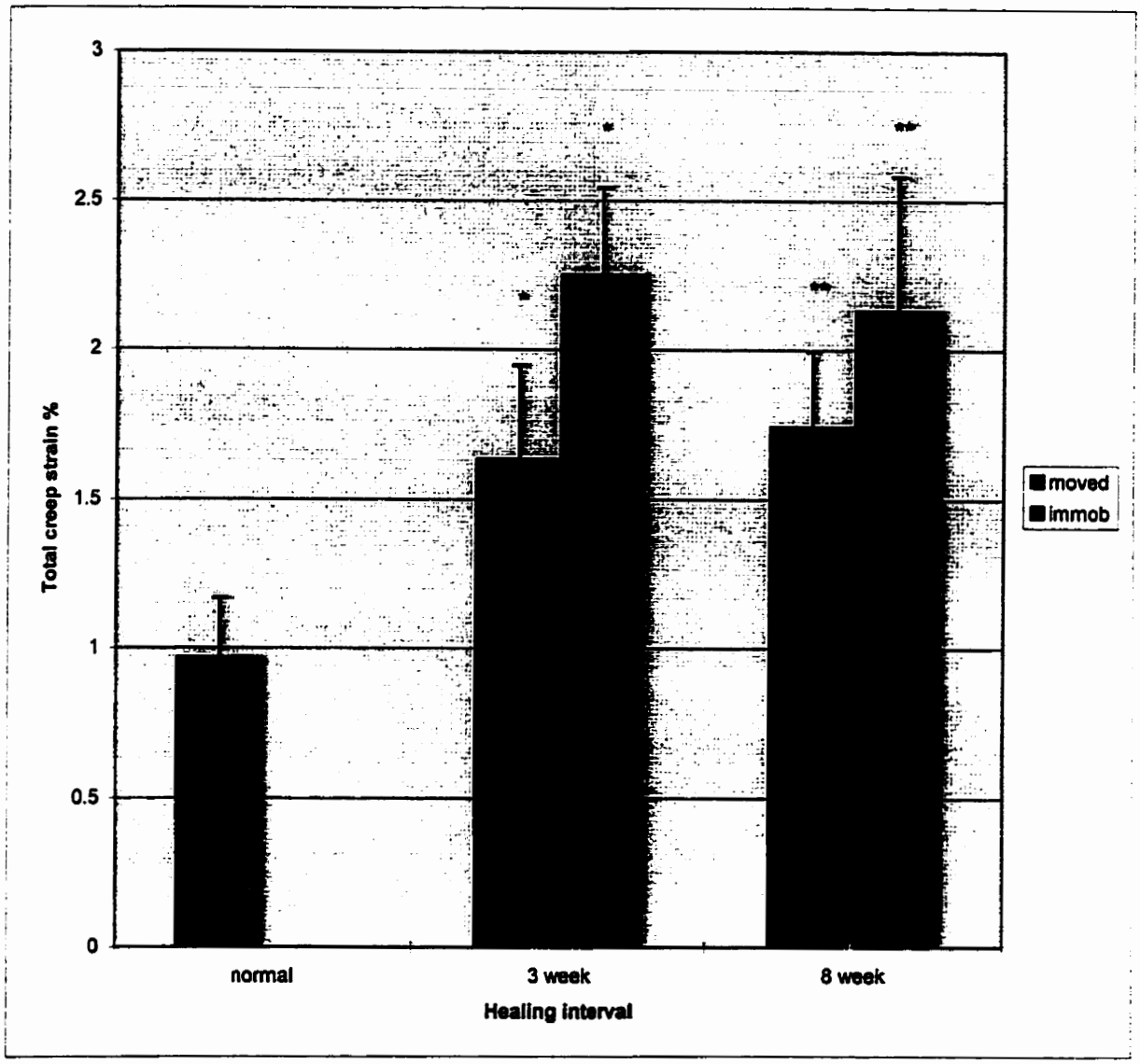

Figure 19: Total creep strain %. Group means and standard deviations. Total creep strain represents creep strain during both the cyclic and static creep strain tests. All grafts crept significantly more than normal MCL controls ($p<0.05$). Immobilization resulted in a statistically significant increase in the total creep strain at both 3 weeks (*)and 8 weeks (**)($p=0.0007$). No statistically significant increase with time within treatment groups. n=8-11 per group.

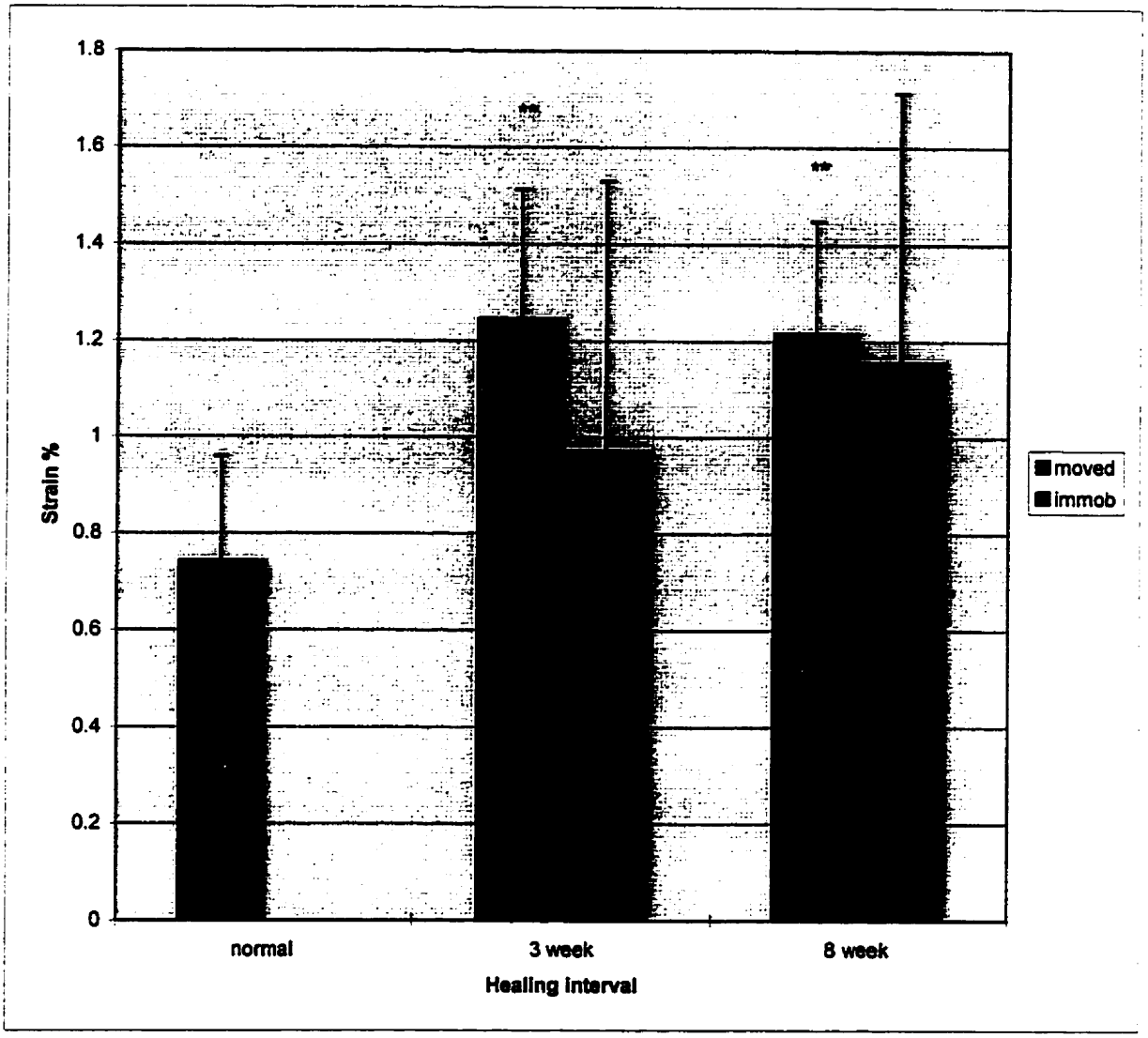

Figure 20: Unrecovered creep strain % . Group means and standard deviations. Strain % after 20 minutes of recovery at zero load. All moved grafts are statistically different to normal MCL controls (**) (3 weeks; p=0.0009, 8 weeks; p=0.0002). No statistical difference between moved and immobilized groups, nor between healing time intervals. n=8-11 per group.

DISCUSSION

These experiments found that rabbit MCL autografts are more susceptible to creep than normal MCL controls. This study further revealed that immobilization of rabbit MCL autografts resulted in a significant increase in the susceptibility of these grafts to creep. These results were seen even with this relatively non-provocative test of short duration, under low physiological loads, and in an extra-articular graft model. Such increased susceptibility of soft tissue grafts to creep following immobilization could result in functionally significant graft elongation when loaded over longer periods of time and at potentially higher stress levels *in vivo*. This progressive elongation could also accumulate because of the apparent inability of the grafts to completely recover from the creep strain (plastic deformation) (Figure 20).

Piper et al 1980 (118), immobilized canine ACL/MCL injuries for four weeks, and subsequently re-mobilized them for another four weeks. They found that the previously immobilized joints had increased valgus laxity over immediately mobilized joints. At biomechanical testing, the MCL scar in the immobilized joints was less strong and less stiff than the scar in the mobilized joints, and it had appeared that the previously immobilized scar had stretched out leading to increased valgus laxity. This study supports our results and hypothesized *in vivo* implications, since it demonstrates the increased susceptibility of immobilized scar to *in vivo* irrecoverable creep.

An important consideration in this study and other studies on ligament laxity and creep is defining a "ligament zero" at which to begin the test. We defined it as the cross-head position at which the ligament just began to take up load (0.1N). In this experiment

the immobilized grafts were fixed in a flexed joint position (about 170°). At the time of testing the knee joints were uniformly tested at about 70° of flexion, and therefore the immobilized joints had to be straightened out for creep testing. This may have put an initial strain on the grafts, and thus put them slightly higher (farther right) on the stress-strain curve at the beginning of the testing (150). In a normal ligament this would correlate with an increased collagen fiber recruitment (146,150), and this may also have recruited more fibers in the immobilized grafts as well, at the start of the creep testing. One would predict that soft tissues which have an initial strain (and more fibers recruited), would creep less than those tissues which are truly starting at zero strain ("ligament zero"). We however found that despite the possibility that the immobilized grafts may have been creep tested at higher initial strain levels on the stress strain curve, that they still crept significantly more than the non-immobilized grafts. This substantiates the finding that immobilization likely resulted in significant graft matrix changes, which appears to make them more vulnerable to creep.

The mechanisms by which immobility increases the susceptibility of ligament grafts to creep are currently unknown. Previous studies have looked at the effect of immobilization on the collagen fiber alignment in scar tissue. In a rabbit midsubstance MCL gap injury, immobilization was found to result in better collagen fiber alignment, in the first six weeks of healing (27). This has also been seen in healing rabbit patella tendon injuries (77). Immobilization has also been shown to decrease the total amount of scar production in ligament gap injuries, thus if less scar material (which creeps more than normal ligament (134)) invades the graft collagen matrix, more of the original graft would be available to resist creep forces. For these reasons we had hypothesized that

immobilization would decrease the creep seen in the MCL autografts. Instead, the results reveal that immobilization caused other matrix changes which have further compromised the grafts' ability to resist creep stresses, since by three weeks the immobilized grafts crept almost one and a half times more than the non-immobilized grafts. The most likely explanation for this dramatic effect of immobilization on graft creep, is that the immobilized grafts had more graft matrix degradation, and scar infiltration than the non-immobilized grafts. It has been shown that immobilized normal ligaments undergo increased turnover of the collagen matrix (111,149). For the first several weeks collagen synthesis is thought to keep pace with increased collagen degradation, however with prolonged immobilization (about 12 weeks), the ligaments begin to atrophy, likely due to an imbalance in the collagen turn-over towards degradation(5). It has also been shown that immobilization of ligaments in tension results in less biomechanical deterioration than immobilization without tension (103). With the knee joint immobilized in flexion the majority of the MCL graft is likely unloaded, with potentially just some of the anterior fibers taking up tension. Thus this unloaded condition may be potentially accentuating the graft degradation.

Ligament grafts have been shown to undergo cellular necrosis, and an initial decrease in cellular metabolism of the resident fibroblasts(83,84,151). Therefore one might hypothesize that the collagen degradation by inflammatory enzymes would outpace synthesis immediately, after transplantation. This may explain the increased susceptibility of both moved and immobilized grafts to creep. Immobilization may enhance this imbalance theoretically, by decreasing new collagen production, by accentuating the inflammatory response and the amount of degradative enzymes present,

or by increasing the intrinsic susceptibility of the collagen itself to degradation. In support of this latter hypothesis, it has been shown *in vitro,* that a loaded acellular patellar tendon is better able to maintain its strength, than unloaded tendons, when subjected to high concentrations of collagenase (99). Also it has been shown that even in the absence of invading cells into a graft, acellular grafts which are loaded, are degraded less than stress shielded grafts, suggesting an intrinsic increase in the susceptibility of unloaded collagen to inflammatory degradative enzymes (151).

Conversely the shift in collagen balance to degradation may also be due to decreased new collagen production. Our study, as well as many other previous studies have shown that moved, or loaded grafts have larger cross-sectional areas, suggesting an enhanced collagen production(43,60,151). Mechanical stimulation of cells can signal an increase in matrix production (28). It is possible that movement is necessary to stimulate cells to produce matrix. Histological analysis to quantify the relative volumes of scar tissue and degraded matrix within the grafts, may help explain these immobilization results.

Another possible explanation for these results may be that the immobilization resulted in changes in the ligament insertion, which increased the susceptibility of the grafts to creep. Normal ligaments which are immobilized, undergo bony resorption around the ligament enthesis, which is thought to result in weakening (decreased insertional failure strength) (111). The immobilized grafts may therefore have been creeping at the ligament insertion sites. One might hypothesize however, that the bony resorption, which is mediated by osteoclasts at normal ligament insertions(149), may be decreased in the grafts because the bone is avascular, and possibly less metabolically

active. Previous studies from our lab (122), have shown that at the three and six week healing intervals, only 10% of MCL autografts failed at the ligament insertion, but this rate was increased to 65% by 24 weeks. This might suggest a delayed ability for the bones to remodel at the insertion site, but it underscores the relative strength of the insertion site as compared to the ligament substance before 24 weeks.

The extra-articular environment of the MCL graft could be considered to be an optimal situation for graft healing (80,122). It might be speculated that an ACL graft would be even more susceptible to creep, since previous biomechanical experiments have found that stress relaxation behaviour and high load properties of these grafts are inferior to those reported for rabbit MCL grafts (80,122). On the other hand the human ACL is much larger than the rabbit MCL graft, and thus one might conversely hypothesize that the relative rate and possibly extent of increased creep susceptibility would be less because more of the native well aligned, creep resistant collagen scaffolding would remain. Future studies need to address whether our current results could be extrapolated to other grafts, particularly ACL grafts.

The increased susceptibility of the grafts to creep may not have any functional significance with respect to increased joint laxity *in vivo*, if they were able to sufficiently recover during periods of rest from the creep strain. We have however shown in this study, that the ligament grafts had more unrecovered creep strain than normal ligaments after 20 minutes at zero load, in these test conditions. We recognize that *in vivo*, the grafts may be able to better recover, due to potential vascular contributions to the recovery process, and potentially longer periods for recovery (as the normal MCLs did not fully recover in these test conditions). However, it has been shown that the majority

(90%) of creep recovery of a patellar tendon occurs in the first 10 minutes at zero load (70). Both the normal ligaments, as well as the grafts were allowed to recover in the same test conditions in this experiment, and the results reveal that some change in the graft matrix must have decreased the grafts' ability to recover from creep strain compared to the normal ligament.

The ultra-structural changes which are responsible for this apparent decrease in the ability of the grafts to recover from creep strain is unknown. It might be speculated however that the smaller and weaker collagen fibers present in the grafts are damaged/fail, leading to permanent stretch. Alternatively, the lack of organization of the collagen fibers within the grafts may lead to increased individual fiber stress with tissue loading, and progressive fiber failures result. Or it may be that the grafts have a decreased propensity to recover the lost water content which occurs in normal tendons and ligaments during creep (61,87,89). Pilot studies from our lab have revealed that normal ligaments are able to recover the majority of the lost water content during 20 minutes of recovery, in our test environment. Future studies will address water movement in grafts during creep and creep recovery.

With time, it has been previously shown that the stress-relaxation properties of MCL autografts returns remarkably close to normal (122). At 48 weeks the stress relaxation of the grafts was found to be within 10% of normal (122). It is still unknown what the long term creep properties of these MCL grafts are, however it appeared in this study that there was a plateau between 3 and 8 weeks. There was also less of a discrepancy in the total creep between the immobilized and non-immobilized groups at 8 weeks. This may be due to a remodeling of the invading scar tissue (151), even in the

immobilized grafts, which may be improving the ability of the scar to resist creep. Future experiments will investigate the long term creep potential of these grafts with and without periods of immobilization.

CONCLUSIONS

Based on these results, it is evident that rabbit MCL autografts become more susceptible to "stretching out" or creeping during the early intervals of healing. Therefore, if too much load was placed on these creep-sensitive soft tissue grafts during the early phases of healing, it could potentially lead to permanent graft stretch, and their subsequent dysfunction. In addition, it is clear from these results that complete immobilization of soft tissue grafts is also potentially detrimental, as this greatly enhances the susceptibility of a graft to creep when loading does occur. Future studies need to further determine how much load can be applied, and when it can be applied in order to optimize graft healing and to minimize graft creep. These results may have significant implications on the timing and aggressiveness of rehabilitation after soft tissue reconstructions.

CHAPTER IV

Overall Discussion of Methods and Results

The Model

The rabbit knee has been a useful model for the study of knee ligaments for many years (14). The MCL autograft model in particular has been shown to be reproducible for the study of autograft healing as its structure is simple, well defined, and consistent in rabbit populations (14,80). From one perspective this autograft model may represent the "best case scenario" for ligament graft healing as that ligament complex is cellular, extra-articular, and can be replaced in an anatomic, low stress environment (14,122). Furthermore, the biomechanical outcome of rabbit MCL grafts is one of the best reported in the literature, with a return to about 90% of normal structural failure load at 48 weeks post transplantation (122). This result occurs despite the fact that there is an initial biomechanical deterioration (65% of control failure load at 24 weeks), similar to other intra-articular graft models. Unpublished data has shown that there is a cellular necrosis even after autograft transplantation, followed by infiltration of scar-like material into these MCL grafts, which may account for this biomechanical deterioration (80,122).. It may be however, that the extra-articular environment which is normally well vascularized, has a more abundant population of cells (of potentially more appropriate phenotype) which are involved in the autograft repair, and thus the grafts have a superior biomechanical outcome to the intra-articular graft (80). Although the results from this autograft model may not be extrapolated directly to the intra-articular graft, it is the

various phases of this "healing" response which we had planned to correlate with graft creep to define the principles of when and why graft creep can occur.

Orthotopic grafts such as this may have more biomechanical success, because the individual collagen fiber bundles may be in a more normal load environment (74,105,122). Based on data which suggests that unloaded collagen fibers are more easily degraded (99), one might hypothesize that if all collagen bundles were returned to their anatomic load environment then they will all be less susceptible to degradation. It has also been shown that the native collagen scaffold may actually direct new collagen formation, with the new matrix being deposited along the surfaces of the collagen scaffold (100). Under these conditions the new collagen fibers would potentially be optimally arranged to resist the forces normally seen by the structure. A widely accepted hypothesis for why poor graft structural and material properties exist when patellar tendons are used in the reconstruction of the ACL is that the graft is placed in an intra-articular environment with abnormal loading demands for a tendon. Studies have shown that the biochemical composition, morphology, and structural properties of the patellar tendon graft, in an intra-articular environment becomes more like ligament with time. This process has been termed "ligamentization". The tendon however never completely transforms into a ligament, and careful analysis of the data reveals that the matrix which replaces the tendon collagen scaffold is in fact scar-like, even after long-term healing (19,114,131). Clearly, one of the largest differences between this MCL autograft model, and the intra-articular tendon grafts used clinically, is this apparent partial transformation of a tendon to a ligament in ACL grafting. The MCL autograft is also physically smaller than most human grafts, and thus potentially more easily enzymatically degraded and

replaced by scar. Therefore it is difficult to extrapolate the absolute time-line of our creep results to human or other larger grafts.

An important consideration with using this model, is that the rabbit MCL is in a low stress environment (150). It could therefore be hypothesized that the MCL grafts would not creep *in vivo* (unlike ACL grafts), but instead will still be susceptible to creep at the time of loading during our biomechanical testing. Indeed it has been shown that the MCL autograft does not become lax *in vivo* (122). This was an important consideration in choosing this model as it was the intention of this study to determine the intrinsic creep susceptibility of the grafts at the time of biomechanical testing. If the grafts had crept *in vivo*, they may have been less susceptible to further creep during our biomechanical testing.

Even in these potentially ideal conditions for graft healing, our results revealed a significant increase in the vulnerability of the grafts to creep under low loads. After only two days post-transplantation the grafts were found to creep more than the time zero grafts and normal MCL controls. Immobilization enhanced this susceptibility, since by three weeks the immobilized grafts crept about one and a half times more than the non-immobilized grafts. Our lab is now investigating whether similar creep vulnerability will be seen in intra-articular patellar tendon ACL grafts. It could be hypothesized that because these grafts typically have very poor biomechanical properties at early healing intervals, they will be even more susceptible to creep than these orthotopic, extra-articular MCL autografts.

Method of Immobilization

We wanted to test the hypothesis that immobilization of the knee would lead to a decrease in the creep potential of the MCL autografts. In order to properly test this hypothesis a complete and rigid immobilization method was required. We used a method of internal fixation developed by Akeson et al. (4,145). We found that even after 8 weeks of immobilization the legs were still rigidly immobilized. Qualitatively the rabbits were seen not to weight bear very much on the immobilized limbs, and previous studies using force plates have confirmed this observation (141). Studies have also attempted to estimate the *in situ* stresses within the rabbit MCL at various joint angles (150). With the knee in a flexed position, it has been estimated that there is less than 1% strain in the posterior region of the MCL with slightly higher strains through the anterior region (150). Therefore, in this experiment with knee immobilized in a flexed position certain areas of the graft may have been under some load, while other areas may have had almost none. It is therefore difficult to make any solid conclusions regarding *in situ* load during healing (although all evidence suggests that it was low), and the resulting creep susceptibility of the tissue. However, we are able to make the conclusion that the lack of knee motion during healing does enhance the MCL grafts' susceptibility to creep.

Specimen freezing

In this study all specimens were treated similarly and frozen to -70^{0}C immediately after harvesting, and stored until such time that they could be biomechanically tested. Studies have shown that that freezing/thawing of soft tissues does not affect their high load biomechanical properties (107,148). However the effects of freezing on the creep

properties of ligaments has not been previously studied. Pilot data from our lab has revealed no statistical difference in the creep of frozen and fresh 2 day grafts (n=4-5 per group). There is however, very little creep data in the literature with which to compare our results, and previously frozen, otherwise normal rabbit MCLs have never been creep tested. If the freezing/thawing procedure altered the water content of the grafts, then this could potentially affect the creep results. In these experiments care was taken to avoid any potential desiccation of the grafts. Future studies will be done to determine what effects freezing has specifically on creep properties of soft tissues, and any potential water content changes.

Test Conditions

The method we used to determine the tensile strain of the grafts during the creep testing, was to measure the deformation of grafts over time, as determined by cross head displacement (linear variable differential transformer), from the "ligament zero" graft length. Firstly, the definition of this "ligament zero" is important. We defined it as the cross-head position at which the ligament began to take up any detectable load (0.1N). In this experiment the immobilized grafts were fixed in a flexed joint position (about 170°). For consistency at the time of testing, all knee joints were uniformly tested at about 70° of flexion, and therefore the immobilized joints had to be straightened out for creep testing. This positioning may have put an initial strain on the grafts, as there was subjectively some mild resistance of the joints to full knee extension. It has been shown previously that rabbit MCL autografts placed in a lax position, tighten up over time and become of similar laxity to an anatomically placed graft (80). Therefore the grafts which were

immobilized in a position of knee hyper flexion may have also contracted somehow to become tight in this knee position. The initial extension of the knee at during mounting on the MTS may therefore have put some initial strain on the grafts, and thus put them slightly higher (farther right) on the stress-strain curve at the beginning of the testing (150). In a normal ligament this would correlate with an increased collagen fiber recruitment (146,150). This may also have recruited more fibers in the immobilized grafts as well, at the start of the creep testing. Based on this assumption, one would predict that soft tissues which have an initial strain (and more fibers recruited), would creep less than those tissues which are truly starting at zero strain ("ligament zero"). We found however that despite the possibility that the immobilized grafts may have been creep tested farther right on the stress strain curve, that they still crept significantly more than the non-immobilized grafts. This supports the concept that our immobilization procedure resulted in significant graft matrix changes which made them more vulnerable to creep.

Previous studies have shown that the measured strain of a bone-ligament-bone complex is greater if one uses the cross-head displacement method (as we have in this experiment) than if the video-dimension analyzer (or similar system) is used (143,150). This is because the cross-head displacement represents potential strain within every structure between the clamps (ie. fixtures, bone-pot interface, bone, ligament insertions, as well as the entire ligament substance). The video dimension analyzer measures strain of some portion the ligament substance, or an insertion area which has been defined/marked. This would have created some concern if we had been testing at high loads or to failure. In our experiment only very low loads were used for creep testing

(typically between 12-30N). Furthermore, we were interested in the structural properties of the entire graft, as *in vivo* any or all components of the graft could be creeping. Also, because the test environment is so crucial to creep testing, we were technically unable to use the video dimension analyzer with the humidity chamber. To ensure that we were not measuring creep at the bone fixation sites (a particular concern at the very early healing intervals) several steps were taken. Firstly, we video taped a creep test of a two day autograft (no bony healing) at high magnification, with markers spanning the bone graft and the adjacent bone. This revealed no movement at the bone graft site. Secondly we creep tested time-zero grafts, and found that they were not statistically different to normal MCL controls. Thirdly, we were able to incorporate the bone grafts into the polymethylmethacrylate, thus re-enforcing the bony interfaces. At three and eight weeks there was considerable bony healing present. From this, we are confident that virtually no creep was measured at the bone graft fixation sites and that creep was occurring in the graft itself.

Our results do however reflect potential strains in both the ligament substance, as well as at the insertions. A pilot study in which we creep tested two 3 week immobilized grafts using the VDA to measure strain revealed that the mid-substance as well as the insertional areas were straining. The relative percent contributions were not however quantified. It appears that both areas are important contributors to the overall creep of the ligament graft.

The test environment in which the ligaments are crept is extremely important (36,39,63,88,146,150). Increases in temperature causes an increase in stress relaxation or creep of tissue (39,88,147), while decreases in water content within the tissue causes a

decrease in the stress relaxation and creep properties (36,63,87). We were able to control the temperature (+/-0.5^{o}C), and provide a physiological humidified test environment throughout our creep test protocol by using a custom designed environment chamber (142). This chamber maintained the temperature of the specimen at 36.5+/-0.5^{o}C and at a relative humidity of 99% (142). The water content of rabbit MCLs has been shown to be maintained at a normal level (65%) for 30 minutes in this test environment (142).

Static versus cyclic creep

Our results revealed that more creep strain was generally seen in all tested tissues during the static creep tests as compared to the cyclic creep tests. However, the cyclic creep test was only for 30 seconds while the static creep test lasted for 1200 seconds. Thus relatively more creep per unit time occurred during the cyclic creep test. In terms of percent strain per second, the static creep was only 8% that of cyclic creep for the time zero grafts, 4.7% for the 3 week grafts, and 4% for the 8 week grafts. Theoretically static creep is a more provocative test than cyclic creep because in cyclic creep testing, the tissue is allowed to recover some/all of the strain between cycles (87).

Water movement out of tissues has been shown to occur during viscoelastic creep tests (89). It has been reported that more water is lost during static creep than during cyclic creep, because during the relaxation phases of cyclic creep testing the water is allowed to move back into the tissue (87). Water is considered important for decreasing inter-fibrilar friction/adhesion, and thus it is thought that it allows for easier sliding between collagen fibers and makes the tissue less stiff (87). If relatively more water is lost during the static creep test than during the cyclic creep test, then this may decrease the amount of creep which can occur. This may be one explanation for our results.

Another possible, and likely more important explanation, is that the cyclic creep test was performed first in this experiment, and their was no recovery time between the cyclic and static creep tests. Therefore, there was likely a significant fiber recruitment which had occurred during the cyclic creep test, which would decrease the creep which would occur during the static creep test which immediately followed. There would also potentially be a decreased water content at the beginning of the static creep test as compared to the cyclic creep test, which would also relatively decrease the amount of static creep. Similar results as we have found here have also been found in stress relaxation experiments where cyclic relaxation preceded static relaxation testing (87). It is likely that if the order of the tests had been reversed, then there would have been relatively more creep during the static creep test.

Creep Recovery

In these experiments we found that the grafts had significantly more un-recovered creep strain than normal MCLs after the 20 minute recovery period. During this recovery period, the testing system was programmed to hold zero load, however it was only able to do so within +/- 1N. Therefore at the end of the 20 minute recovery period the system was manually dialed down to zero Newtons, and the residual strain was recorded. It was this residual strain which represented un-recovered creep. Because we were only able to record this single recovery data point, a mathematical prediction of irrecoverable creep using the quasi-linear viscoelastic theory (56), was not possible. We therefore were unable to know absolutely, how much of the graft creep would never be recovered (ie. amount of plastic deformation). Clearly, the test conditions did not exactly mimic the *in*

vivo situation, since the normal MCLs had some residual strain after the 20 minute recovery period. Several explanations could account for this: 1) Lack of potential vascular contributions to recovery, 2) Insufficient recovery time, 3) Higher than physiologically normal stress levels/ high individual fiber stresses in certain areas of the ligament. It might be hypothesized that the vasculature may be able to help restore lost water from normal ligaments *in vivo*, and that the ligaments may have much longer recovery periods, while the animal is resting. Also contributing to the lack of complete recovery of the normal ligaments is the possibility that the levels/distribution of stress was causing damage to the normal ligaments. Only estimates of normal *in vivo* stresses of rabbit MCLs are known. Furthermore the ligaments were creep tested in longitudinal tension, which is likely not a normal functional loading situation for the MCL. Different collagen bundles of the MCL are loaded at various joint angles (150). The amount of load which was necessary to achieve a stress level of 4 MPa, was based on the assumption that an equal distribution of load would be carried by all fibers within the cross-section of the ligament. This was likely not what occurred in reality, and certain collagen bundles within the normal MCL may have been subjected to very high stresses, resulting in fiber damage, and possibly plastic deformation. It is clear from our results however, that in these test conditions, and after allowing a recovery time equal to the amount of time for the creep test, that the grafts had more un-recovered creep than the normal MCLs or the time-zero graft controls. This increased residual strain represents (partially or completely) a plastic deformation of the tissue, and implies that some matrix changes have occurred in the grafts which make them less able to recover from creep strain. It has been stated that normal uninjured tendons are able to completely recover (ie.

no damage to structural integrity) from strains of less than about 2-3% (39,89). In the current experiment, under the same stress levels as the normal MCLs (which strained less than 3%), we found that the grafts strained from 3.27% (0.84mm) in the two day grafts, to 5.74% (1.34mm) in the 8 week immobilized grafts. Based on these previous estimates of normal tendons to recover from strain, it could be hypothesized that these grafts would not be expected to completely recover from the creep strain. Furthermore, these grafts are no longer normal ultra-structurally, as histological analysis has shown that the original collagen matrix has been somewhat degraded, and there is invading scar tissue (Figure 21), which has small, randomly organized collagen fibers.

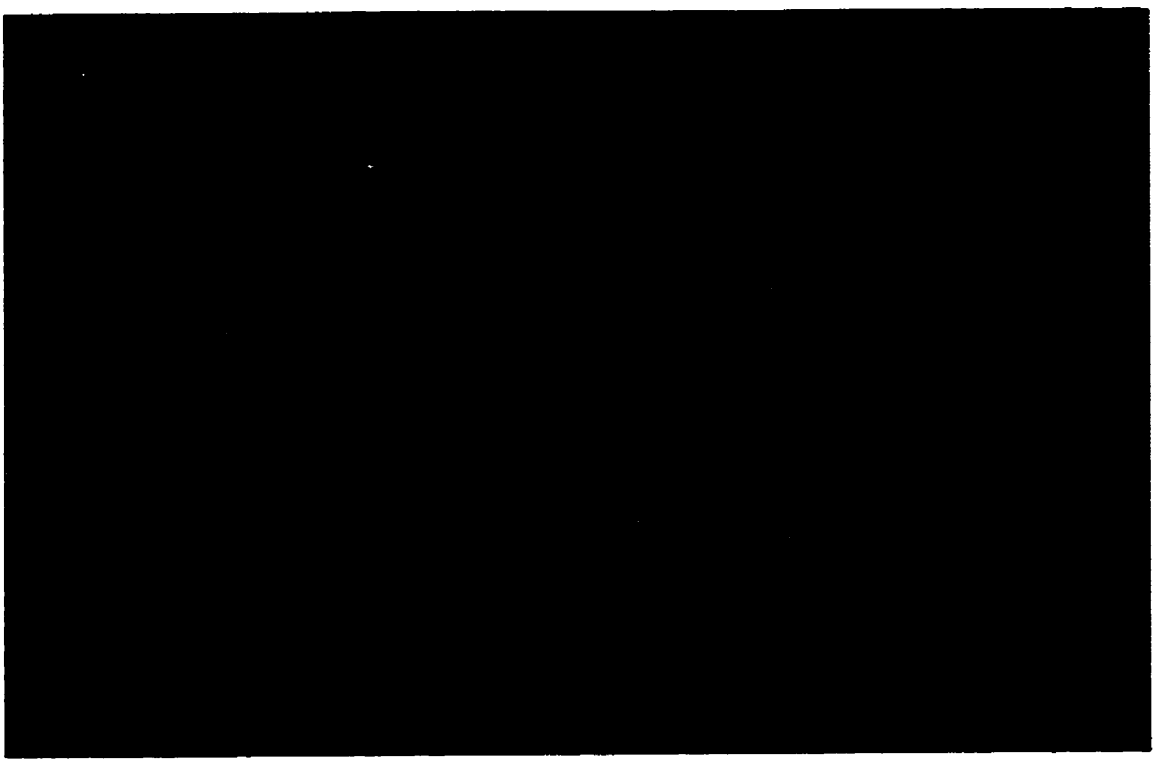

Figure 21. Photomicrograph at 25x magnification of three week MCL autograft. Specimen stained with Sirius red and viewed under polarized light. Regular crimped pattern of normal ligament (A), is being degraded (B). Areas of scar tissue infiltration is also visible (C).

FUTURE DIRECTIONS

The experiments presented in this thesis have revealed several important new findings with regard to the biomechanical creep properties of fresh rabbit MCL autografts. Firstly, these grafts do become more vulnerable to creep within days after transplantation. Secondly, this susceptibility increases for about three weeks post-grafting, and then subsequently plateaus. Thirdly, immobilization further increases the grafts' susceptibility to creep. The rapidity with which this biomechanical deterioration occurs implies that the matrix of the autografts is being degraded by inflammatory enzymes; perhaps more in the immobilized grafts. Future investigations should be done to determine if this is really the case, or whether other mechanisms such as increased graft water content are more responsible for the increased creep (especially at 2 days). It will also be very important to determine whether or not this applies to all grafts including intra-articular grafts (clinically important to ACL surgery). The ultra-structural mechanisms of soft tissue creep must be elucidated, and related to the enhanced susceptibility of the ligament grafts to creep. Similarly, the mechanisms by which immobilization (and more broadly load environment), affects graft creep must be determined. Once the changes in the ultra-structure of the grafts have been documented, and the ultra-structural mechanisms of creep have been elucidated, then potential interventions could be directed at limiting/preventing graft creep. This might include inhibiting early degradation, optimizing the load environment potentially by preventing graft stresses which are "too low" or "too high", preventing the necrosis of the resident

graft fibroblasts, and improving the quality of the invading scar (ie. gene therapeutic manipulation). Ultimately, it must determined if the prevention of creep in soft tissue reconstructions would change the functional outcome of the joint and the clinical success of the these operations.

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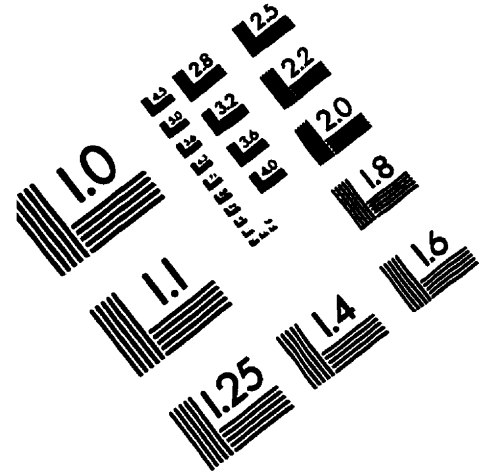
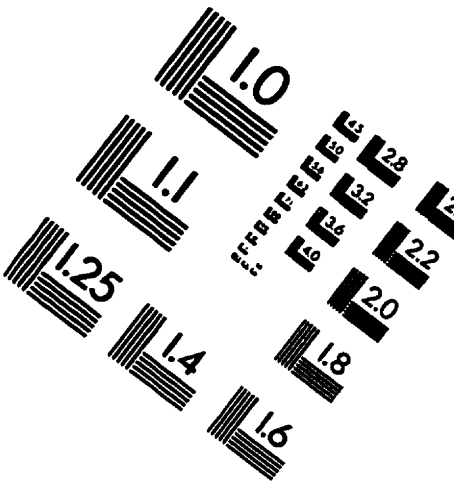
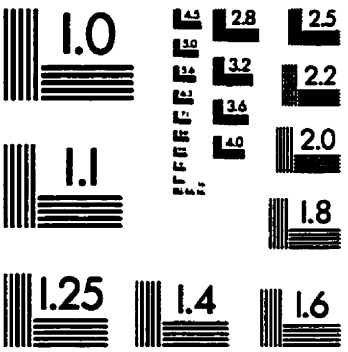
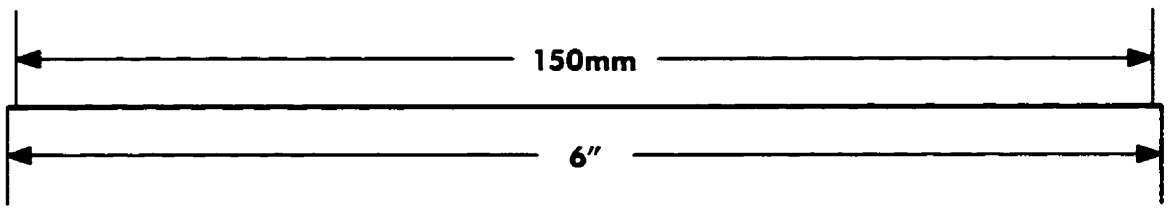
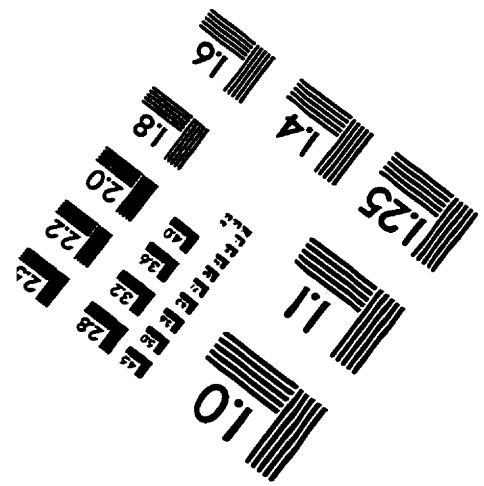
IMAGE EVALUATION TEST TARGET (QA-3)

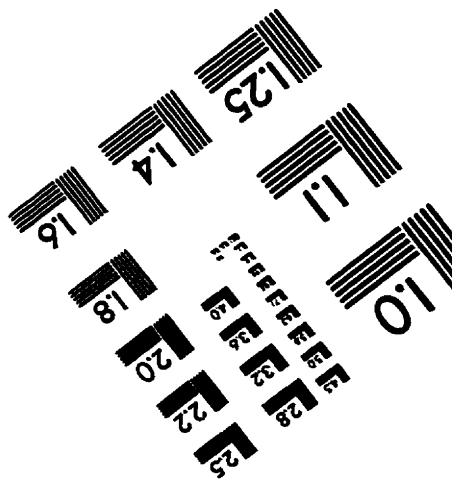












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