Pathophysiology and reversibility of prolonged knee joint immobilization: a comprehensive temporal investigation using an animal model

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Preface

The experimental methods described in the following thesis was approved by the Animal Care Committee at the University of Ottawa (protocol number: ME-2461). Funding for this work was provided by the Canadian Institutes of Health Research Grant MOP 97831 and awarded to Dr. Odette Laneuville and Dr. Guy Trudel. In all original research articles comprising this thesis, I was primary author. My contributions were as follows: execution of experiments, data acquisition, analysis of data, interpretation of results, preparation of figures, drafting of manuscripts, editing and revision of manuscripts, and approval of final version of articles. Regarding the review articles included in the appendix, I contributed as a co-author with sections of text, figures, and was involved in the revision of the final version; with exception to one article in which I was sole author. Specific contributions are listed in each respective article.

In consultation with the various journals, I retain the right as an author to include published articles as part of this thesis and post to thesis repositories, provided it is not published commercially. Copyright permissions have been included in Appendix V, and articles have been properly referenced with the respective journals as the original source. All published articles have since passed their respective 12-month embargo period.

Abstract

The knee joint is a diarthrodial joint that rotates in the flexion-extension axis to provide individuals mobility. A limitation in the passive range of motion (ROM) is detrimental for function and this limitation is termed a joint contracture. A commonly shared characteristic between conditions that lead to contracture formation is prolonged periods of immobilization. However, the etiology of immobility-induced joint contractures is not well described and requires quantitative data on anatomical structures limiting knee mobility to design new interventions aimed at restoring function. In turn, our research group has developed an experimental animal model to study the temporal pathophysiology of knee immobilization and reversibility through unassisted remobilization. With durations of immobilization ranging from 1 to 32 weeks and remobilization up to 48 weeks, our experimental design provides a comprehensive temporal overview on the various stages of contracture formation: initiation, progression, and severity. A combination of muscles and articular structures are involved in the pathophysiology of knee flexion contractures, but the posterior joint capsule is of particular interest. Through histomorphological analysis, we provided quantitative data on the contribution of the reduced posterior capsule length in the limitation of knee extension and increased joint stiffness. Moreover, elucidation of synoviocyte profiles within the synovium of the capsule provided insights to potential mechanisms of capsule shortening. Our novel measurable outcome of mechanical joint stiffness revealed distinct temporal differences with ROM measurements after joint immobilization and remobilization, suggesting that alterations in the biomechanical properties of articular tissue structures are also contributing to the limitation in function. Malleability of the dynamic reciprocal relationship between trabecular bone loss and accumulation of marrow adipose tissue (predominately through adipocyte hyperplasia) after knee immobilization underscores the

sensitivity of the bone marrow microenvironment in response to mechanical stimuli and lack thereof. Remobilization of the knee joint is limited in its capacity to reverse detriments induced by extended periods of joint immobilization. Findings from this work point to the temporal changes detected in different musculoskeletal tissues during knee immobilization and emphasizes the contribution of the joint capsule in limiting joint mobility.

Résumé

Le genou est une articulation diarthrodiale qui tourne principalement dans l'axe flexion-extension et contribue à la mobilité. Une limitation de l'amplitude du mouvement passif (ROM) limite la fonction principale d'une articulation et réfère à une contracture articulaire. Des périodes prolongées d'immobilisation constituent une caractéristique communément partagée entre les différentes conditions conduisant à la formation de contractures. Cependant, l'étiologie des contractures articulaires induites par l'immobilité n'est pas bien connue et nécessite des données quantitatives sur les structures anatomiques limitant la mobilité du genou pour concevoir de nouvelles interventions visant à restaurer la fonction. Notre groupe de recherche a développé un modèle animal expérimental pour étudier la physiopathologie temporelle de l'immobilisation du genou et sa réversibilité non assistée. En utilisant des durées d'immobilisation allant de 1 à 32 semaines et de remobilisation jusqu'à 48 semaines, notre conception expérimentale fournit un spectre temporel des différentes étapes caractérisant la contracture: initiation, progression et sévérité. Une combinaison de structures musculaires et articulaires est impliquée dans la physiopathologie des contractures de flexion du genou et la capsule articulaire postérieure présente un intérêt particulier. Suite à une analyse histomorphologique, nous avons fourni des données quantitatives sur la contribution d'une réduction de la longueur de la capsule postérieure dans la limitation de l'extension du genou et l'augmentation de la raideur articulaire. De plus, l'élucidation des profils de synoviocytes dans la synovie de la capsule a fourni des informations sur les mécanismes potentiels de raccourcissement de la capsule. Notre résultat mesurable de la rigidité mécanique des articulations a révélé des différences temporelles distinctes des mesures de ROM après l'immobilisation et la remobilisation des articulations, suggérant que les altérations des propriétés biomécaniques des différents tissus de l'articulation contribuent également à la

limitation de la fonction. La malléabilité de la relation réciproque et dynamique entre la perte d'os trabéculaire et l'accumulation de tissu adipeux médullaire (résultant principalement d'une d'hyperplasie des adipocytes) après l'immobilisation des articulations souligne la sensibilité du microenvironnement de la moelle osseuse en réponse aux stimuli mécaniques et à leur absence. La remobilisation de l'articulation du genou est limitée dans sa capacité à inverser les changements induits par de longues périodes d'immobilisation. Les résultats de l'ensemble de ces travaux décrivent les changements structuraux et dynamiques qui sont associées aux modifications de différents tissus musculosquelettiques du genou lors de l'immobilisation en itérant la contribution de la capsule articulaire dans la limitation de la mobilité des articulations.

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Lastly, the pursuit of this academic endeavour would have not been possible without the unconditional support and encouragement from my family and friends. As this journey comes to an end, may we celebrate the fruits of this accomplishment together.

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Abbreviations

ACL	Anterior cruciate ligament
ADH	Acquired deforming hypertonia
ADL	Activities of daily life
AMC	Arthrogryposis multiplex congenita
ANOVA	Analysis of variance
BMP	Bone morphogenic protein
BPSD	Behavioral and psychological symptoms of dementia
CI	Confidence interval
CSA	Cross-sectional area
CT	Connective tissue
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FOV	Field of view
GDF5	Growth differentiation factor 5
HA	Hyaluronic acid
H&E	Hematoxylin and Eosin
IHH	Indian hedgehog
MAT	Marrow adipose tissue
mRNA	Messenger ribonucleic acid
RA	Rheumatoid arthritis
ROM	Range of motion
SAT	Subcutaneous adipose tissue

SCJ	Synovio-cartilage junction
SDC	Supplementary digital content
SFRP2	Secreted frizzled-related protein 2
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
TKA	Total knee arthroplasty
UDPGD	Uridine diphosphoglucose dehydrogenase
VAT	Visceral adipose tissue
Wnt	Wingless-related integration site

Introduction

For the knee joint, full range of motion (ROM) is critical for maintaining function. Knee flexion and extension are essential for movement and mobility. A limitation in the passive ROM of an affected joint is described as a joint contracture and can cause impairments to gait and reduce overall mobility [1]. In addition, joint contractures can bring numerous detriments to cartilage, bone, muscle, and soft tissues surrounding the joint [2,3]. Passive ROM focuses on joint tissues limiting ROM and eliminates any potential contribution of the central nervous system and/or muscle activation observed in active ROM. The development of joint contractures can vary due to the heterogeneous nature of conditions leading to contracture formation (Appendix I) [4]. However, prolonged durations of joint immobilization have been suggested to be a common cause [5]. Conditions such as arthrogryposis multiplex congenita highlight the importance of movement during stages as early as embryonic/fetal development to mitigate contracture formation prior to birth (Appendix II) [6]. Lack of movement presents many functional complications for individuals that are subject to prolonged durations of immobilization in order to alleviate pain or protect healing tissues where both the injury and period of immobilization can contribute to contracture formation [7,8]. A long post-operative rehabilitation period is a common course of action following medical/surgical management of acute injuries such as anterior cruciate ligament tear, patellar dislocation, or traumatic knee injuries [7–9]. The lack of clinical trials and heterogenous nature of conditions that contribute to contractures have limited the use of human samples to study the effects of joint immobilization [1,10].

The etiology of immobility-induced joint contractures is not well described and requires quantitative data on anatomical structures limiting knee mobility to design new interventions aimed at restoring function. In turn, animal models have been developed to investigate the

temporal effects of prolonged knee immobilization. Rat and rabbit animal models are commonly used to study knee flexion contractures that are secondary to immobilization [3,11,12] and those accompanied by intraarticular trauma [13–15], as they exhibit similar anatomy and function to the human knee. Moreover, joint dysfunction in a mouse model of osteogenesis imperfecta shows the breadth of conditions that can contribute to contracture formation [16]. Our research group has developed an experimental rat animal model to study the temporal pathophysiology and reversibility of immobility-induced knee flexion joint contractures (Figure 1) [17–19]. The large enough size of the rat knee permits mechanical testing for measurable outcomes such as passive ROM, but also small enough that the knee can fit onto a microscope slide for histological analysis to investigate morphological changes of articular structures. Furthermore, the method of immobilization using an extra-articular rigid plastic plate as a fixator does not invade the joint space and therefore preserves the integrity of the joint tissue structures, allowing to isolate the contribution of immobility (from other factors such as injury and trauma) to joint contracture formation. The extra-articular position of the fixator also allows for its removal to study the remobilization of knees with flexion contractures.

Figure 1. Rat model of immobility-induced knee flexion contractures. Screws are inserted into the proximal femur and distal tibia with a rigid plastic plate that extra-articularly fixates the knee at a 45-degree angle in flexion. Illustration by the Bone and Joint Research Laboratory.

Range of motion and posterior capsule length

The knee joint is a diarthrodial joint which rotates primarily in the flexion-extension axis, and this movement is facilitated by structural features such as articular cartilage, ligaments, and a fibrous joint capsule [20]. Passive ROM is the main measurable outcome for assessing contracture severity and an increase in the duration of joint immobilization in flexion results in a progressive decrease in ROM of knee extension [3]. Our rat model has further elucidated the contribution of different groups of joint tissue structures responsible for the limitation in ROM [17–19]. For knee flexion contractures, the limitation in ROM is a deficit in knee extension, and the structures limiting ROM can be categorized as with or without muscle contribution: myogenic and arthrogenic restrictions. Myogenic structures are primarily comprised of muscle, tendon, and fascia. Arthrogenic restrictions include bone, cartilage, ligaments, and capsule [17]. In addition to mechanical factors surrounding the knee joint, our research group has shown that intrinsic genetic factors can also contribute to the severity of contracture formation [21]. To determine the contribution of each group to the restriction in ROM, biomechanical testing of the knee in extension is conducted twice on the same knee. The first ROM is measured with all tissue structures intact (except for skin), followed by a second measurement after a myotomy is performed on all posterior trans-articular muscles and tendons [19]. The myogenic restriction can be attributed to the gain in ROM post-myotomy. The arthrogenic restriction can be attributed to the remaining deficit in ROM when compared to a contralateral control. Previous work in our animal model studying joint immobilization up to 32 weeks indicated that muscles largely contribute during the early stages of a knee joint contracture. As duration of immobility increased, the arthrogenic contribution to the contracture gradually increased in magnitude [18].

More recently, our research group conducted a comprehensive temporal study aimed at investigating the reversibility of contractures of knee joints immobilized in flexion for 1 to 32 weeks through unassisted remobilization (removing the fixator and allowing the rats voluntary movement). The results showed that short durations of immobilization (up to 2 weeks) resulted in a limitation of ROM caused primarily by myogenic restrictions and recovered full range of extension after remobilization, while longer durations of immobilization (beyond 2 weeks) were caused by arthrogenic restrictions and had no significant recovery with remobilization [11]. This suggests that prolonged durations of knee immobilization in flexion are causing changes to the articular structures surrounding the joint and ultimately affecting its function negatively. The work presented in this thesis further investigates the reversibility and dynamics of tissue structure changes through joint remobilization.

An articular structure commonly investigated following joint immobilization is the joint capsule, more specifically the posterior joint capsule in the context of flexion contractures. The joint capsule is a soft connective tissue responsible for enclosing synovial fluid within the joint space and providing passive stability to joint kinematics [22]. Two distinct layers are present in the joint capsule: an external fibrous layer that provides structural support, and an inner synovial layer responsible for the secretion of synovial fluid into the joint cavity [23]. The superficial layer (intima) of the synovial lining is 1 to 3 cells thick and is composed of specialized cells called synoviocytes with two primary types that have been identified: macrophage-like cells (type A) and fibroblast-like cells (type B) [23,24]. The underlying tissue (subintima) is composed of blood and lymphatic vessels with fibroblasts in a collagenous extracellular matrix [25]. Synovial tissue can be categorized based on the structure and content of the subintimal layer such as adipose, areolar, or fibrous [25]. The capsule inserts into bone at the synovial-cartilage junction on both femoral

and tibial sides and the type of insertion has been described to be fibrocartilaginous [22]. The joint capsule is a deformable structure that allows movement of adjacent, relatively non-deformable tissue, and maintains a non-adherent tissue surface to facilitate this action [25]. Furthermore, the synovium contributes to the maintenance of cartilage lubrication, synovial fluid volume, and nutrition of chondrocytes within joints [25].

The joint capsule is a dynamic tissue that can change in response to forces acting on it; mechanical stimulation during joint movement appears crucial for maintaining capsule elasticity [26]. In circumstances of trauma and immobilization of a limb, thickening and shortening of the capsule can have negative consequences on joint mobility [22]. Such mechanisms can occur in arthrofibrosis where aberrations in the synthesis and orientation of collagen fibrils contribute to the thickening of the capsule and contracture formation [27]. Although the posterior capsule is thought to have a passive role in knee extension, it instead plays an active role in limiting knee extension after joint immobilization and has been documented in our rat model [28]. The role of the posterior capsule in limiting knee extension is further supported by the fact that posterior capsulotomy is able to restore most of the deficit in knee extension [29,30]. In contrast, the anterior capsule is constantly under tension during flexion and is not antagonistic to knee extension. However, this is a technically demanding operation that risks damaging neurovasculature in the posterior regions of the knee and is not routinely selected as a course of treatment for patients with knee flexion contractures.

In the experimental model, a predominant pathophysiological change that occurs with knee immobilization in flexion is a reduction in length of the posterior capsule [12,28,31]. Similarly with ROM, there is a progressive decrease in capsule length as duration of immobilization increases [28]. When the knee is immobilized in flexion, the attachments of the posterior capsule

to the femur and tibia are positioned closer, depleting the capsule of tension in comparison to full extension and allowing for capsular folds to form. A sustained point of contact between the inner layer of these folds during immobilization have been suggested to result in the adhesion of the synovium (inner layer) and ultimately shorten the length of the capsule [28]. This anatomical change and structural reorganization of the posterior capsule contributes to the deficit in knee extension [26]. Adhesion of the synovium may be mediated by cadherin adhesion molecules that have been shown to have a role in the homophilic adhesion of synovial fibroblasts [32,33]. More specifically, cadherin-11 mediates the formation of intercellular junctions between fibroblast-like synoviocytes [33]. That being said, mechanical strain in the capsule likely modulates the biology of synoviocytes [34]. The mechanosensitive nature of synoviocytes and implications in hyaluronan secretion during joint use have been shown in vitro [35]. Additionally, our experimental model has shown a decrease in proliferation of synoviocytes following immobilization [36]. A morphological characteristic of interest in type B synoviocytes is the development of cytoplasmic processes that extend radially to form a course network of processes that cover the intimal surface and have antenna-like microvilli that project into the joint cavity [23]. These cellular morphologies in conjunction with the role of synoviocytes in synovial tissue organization may be pertinent in the ability of capsule folds to adhere together and result in a shortened capsule length (distance from femur to tibial capsule insertions). Furthermore, given the distinct roles that type A and type B synoviocytes have, an imbalance in proportion of synoviocytes may indicate losses in homeostatic roles as seen in joint pathologies such as arthritis [37]. With potential to contribute and be involved in the mechanism of capsule shortening, this provides rationale for further investigation on the temporal response of type A and B synoviocytes during knee immobilization and remobilization. The joint capsule plays an integral part in knee kinematics following joint immobilization, but

much remains unknown regarding the mechanisms of capsule shortening and whether the shortening of the capsule observed after joint immobilization is reversible.

Knee joint stiffness following immobilization and remobilization

The involvement of the joint capsule in limiting knee extension and articular stiffness may not be limited to its length as the only factor contributing to the impairment of knee function. Fibrosis in the capsule represents a possible mechanism of increased joint stiffness during prolonged knee immobilization [38]. The lack of mechanical stress to the capsule tissue during prolonged knee immobilization can disorganize collagen fibers in the posterior joint capsule and has been shown to be associated with increased joint stiffness in a rat model [39]. Joint stiffness after immobilization has also been shown to be associated with an increase in collagen turnover and increased collagen cross-linking in the joint capsule [40]. Changes in tissue composition of the capsule and other articular structures may be affecting other biomechanical properties of the knee but lack quantitative data on mechanical joint stiffness. Although passive ROM remains a primary quantitative measure of contracture severity, joint stiffness is an additional measurable outcome that requires consideration. Joint stiffness provides insight to the change in resistance during displacement of the knee in extension, while ROM provides an angle of extension for a given torque. A customized arthrometer developed for our experimental model is able to provide ROM in extension of the rat knee joint at multiple torques following a precise and user-independent method [19]. The investigation of joint stiffness in animal models serves as an appropriate and novel measurable outcome for function after knee immobilization. The relevance in testing the biomechanics of the knee is highlighted when patients report a difficulty in ranging their joints with a slower active angular velocity [41]. Furthermore, stiffness is relevant in the

assessment of end-feel in clinic and limitations to passive ROM [42–44]. Determining the amount and type of resistance the joint provides when arriving at the end-feel can provide insight to how well the joint will respond to treatment, such as stretching. Articular stiffness with a soft end-feel may have better success in recovery with progressive stretching, whereas an abrupt hard end-feel may have limited chance for improvement [45]. The characterization of end-feel in contractures by clinicians is qualitative in nature and quantitative data on mechanical joint stiffness is lacking. Identifying the biomechanical behaviour of the knee joint in addition to changes in anatomical structures after immobilization may provide insight to what is contributing the most to articular stiffness and identify which treatment modalities would have better potential for success.

Effects of joint immobilization and remobilization on marrow adipose tissue and trabecular bone

The effects of joint immobilization are not limited to a loss of function in ROM and changes to structures surrounding the knee joint. The reduced mechanical stimuli transmitted throughout the knee joint during immobilization can result in changes to underlying components such as the bone marrow. The bone marrow is a unique microenvironment found in the central cavities of axial and long bones [46]. It is one of the largest organs in the body and the main site of hematopoiesis [47,48]. The bone marrow is comprised of hematopoietic stem cells and mesenchymal stem cells that differentiate to form the primary components of the marrow space: hematopoietic tissue, trabecular bone, and adipose tissue [46–48]. Knowledge regarding the dynamics and temporal nature of these tissues during joint disuse are limited, partly owing to the relative inaccessibility of the marrow space. It has been described that a competitive balance exists between osteoblastogenic and adipogenic pathways, where the differentiation of mesenchymal stem cells is influenced by mechanical signals transduced through ground-reaction forces and/or contractile

forces generated by muscles [49,50]. Without mechanical stimuli, the three main tenants of the marrow space are subject to changes in composition. Homeostasis of bone marrow tissue can be dysregulated by a reduction in mechanical stimuli, which enables adipose generation and bone resorption pathways [51,52]. In contrast, exercise and physical interventions that introduce mechanical stimuli have been shown to increase bone mass and reduce adipose tissue [53–57]. The multifactorial regulation of bone marrow tissue is largely mediated by two main transcription factors, PPARγ and RUNX2 [58,59]. However, the regulation of this competitive differentiation is far more complex and can include cofactors that participate in the active suppression of opposing pathways while promoting either adipogenesis or osteoblastogenesis [49,60,61].

Models of spinal cord injury, bed rest, and limb suspension all exhibit a reduction in mechanical stimulation, mainly lack of loading, and have reported trabecular bone loss to be most prominent in the epiphysis of the proximal tibia [62–65]. As a weight-bearing joint, the tibial epiphysis is particularly sensitive to reduced mechanical stimuli and is subject to higher strains when compared to the metaphysis [66,67]. The association of higher marrow adipose tissue (MAT) levels and low bone density have been observed during skeletal disuse [54,68,69]. Additionally, the accumulation of MAT occurs concomitantly with aging but can be accelerated by skeletal disuse; this process is characterized by a combination of marrow adipocyte hyperplasia and/or hypertrophy. Whether joint immobilization promotes MAT accumulation through changes in cell morphology is unclear [54]. Although common in sports medicine and general clinical practices, knowledge regarding the malleability of marrow adiposity and trabecular bone following prolonged joint immobilization and remobilization remains limited. The trabecular bone loss experienced during periods of immobility can introduce bone fragility and the reciprocal increase in MAT can negatively impact bone properties and hematopoiesis [70]. The reversibility of these

deleterious effects after prolonged joint immobilization have not been thoroughly investigated. Therefore, in our rat model of immobility-induced knee flexion contracture, the epiphysis of the proximal tibia serves as a suitable region to understand how the composition of the bone marrow adapts to changes in mechanical environments, mainly joint immobility.

Purpose and Objectives

The detriments of joint immobilization are vast and unavoidable in some clinical contexts. Using our rat model of immobility-induced knee flexion contractures, we can investigate the dynamics of tissue changes, pathophysiology, and reversibility of detriments caused by joint immobilization through unassisted remobilization. Our temporal study design provides us a comprehensive overview of joint immobilization of up to 32 weeks and subsequent remobilization of up to 4 times the duration of immobilization. Through these chapters, this thesis aims to address 3 main research questions: 1) Is there an association between posterior capsule length and knee ROM in extension following remobilization and what potential mechanisms contribute to capsule shortening? 2) As a measurable outcome of function, does mechanical joint stiffness differ in its temporal response to joint immobilization and remobilization when compared to passive ROM? 3) What are the effects of joint disuse on trabecular bone and MAT within the epiphysis of the proximal tibia and are these effects malleable through remobilization?

This thesis is divided into 5 different chapters of published works and can be grouped into 2 sections: 1) joint contracture pathophysiology and 2) effects of disuse on bone marrow composition. Section 1 is comprised of Chapters 1-3. Chapter 1 investigates the pathophysiology and reversibility of immobility-induced knee joint contractures with a focus on providing quantitative data on the posterior joint capsule and its contribution to the limitation of function. In

Chapter 2, the mechanical adaptation and temporal response of synoviocytes in the joint capsule are evaluated for their involvement in the pathophysiology of capsule shortening. Lastly, Chapter 3 investigates mechanical joint stiffness as a novel measurable outcome for knee function following joint immobilization. Section 2 consist of Chapters 4 and 5. Chapter 4 examines the effects of joint immobilization towards marrow composition, with a focus on the relationship between trabecular bone and MAT. Furthermore, the cellular morphology of MAT accumulation through adipocyte hyperplasia and/or hypertrophy are investigated in Chapter 5. In all chapters, the malleability and reversibility of the detriments induced by prolonged joint immobilization are evaluated by unassisted remobilization of the knee joint.

Chapter 1

Range of extension correlates with posterior capsule length after knee remobilization

Medicine & Science in Sports & Exercise

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Keywords: Posterior joint capsule; knee; range of motion; flexion contracture; rehabilitation.

Abstract

Introduction: Knee injuries are common in sports, and post-injury immobilization is often required to protect healing tissues and alleviate pain, but both the injury and the immobilization can lead to a knee contracture. Knee flexion contractures limit performance. Previous research has identified posterior knee capsule fibrosis as a contributor to immobility-induced knee flexion contractures. This study aims to measure posterior knee capsule length at various durations of remobilization after knee immobilization and to correlate with the recovery of knee range of motion. **Methods:** 259 male Sprague-Dawley rats had one knee extra-articularly immobilized in flexion with a Delrin® plate at a 45° angle for one of six durations: 1, 2, 4, 8, 16, or 32 weeks, followed by spontaneous remobilization after plate removal, which lasted zero, one, two, and four times the duration of immobilization. The contralateral knees served as controls. The posterior knee capsule length was measured by histomorphometry. These measures were correlated with previously published range of motion data from the same cohort of specimens. **Results:** Knees immobilized for 1 and 2 weeks partially recovered posterior capsule length ($P>0.05$). Knees immobilized beyond 2 weeks failed to recover posterior capsule length irrespective of the duration of remobilization ($P<0.05$). The residual posterior capsule shortening correlated with the lack of knee extension ($P<0.003$). **Conclusion:** For knee injuries requiring more than 2 weeks of immobilization, unassisted remobilization will not restore posterior knee capsule shortening and the reduction in knee extension. These results support the role of the posterior capsule in knee joint contracture and the need to minimize the duration of immobility and to assist the recovery of the range of knee extension after a sport injury.

Key Terms: Posterior joint capsule; knee; range of motion; flexion contracture; rehabilitation.

Introduction

The knee is commonly injured with multiple sports, and joint immobilization often must be implemented in the initial management to protect damaged tissues and alleviate pain (1,2). The acute unstable knee, ACL tear, patellar dislocation, and other acute traumatic knee injuries initially require complete immobilization (1,2). After medical and/or surgical management of the acute injury, the athletes face a long post-operative rehabilitation time that includes joint remobilization (3). However, prolonged use of a cast, or a knee orthosis restricting motion after knee injury or surgery risks many complications (4). Prolonged immobilization has detrimental effects on cartilage, bone, and soft tissues and can result in a loss of range of motion (ROM) (5). Flexion contractures are common complications following knee replacement and ACL reconstruction (6,7). The loss of knee extension increases joint contact pressures, quadriceps muscle activity, fatigue, and impairs gait, limiting performance for active athletes (5,8–12). Quantitative data on anatomical structures limiting knee mobility are limited but necessary to design and test new interventions to restore knee joint mobility post-injury. The limited number of clinical studies has precluded the use of human samples (13). In turn, the rat model exhibits similar anatomy and physiology to the human knee and has shown ROM limitations in response to immobilization (13,14).

Experimental models of knee joint contractures showed that long durations of immobilization caused contractures (14). The tissues responsible for the limitation in ROM have been grouped into myogenic and arthrogenic categories (11,15,16). Myogenic restrictions are caused by muscle, tendon and fascia, whereas, arthrogenic restrictions are caused by bone, cartilage, synovium, capsule and ligaments (15,16). After division of the skin and muscles, the remaining restriction in knee extension can be attributed to arthrogenic restrictions (15,17).

Previous experimental studies have documented decreased ROM with increasing durations of immobility (14,16), and as well as decreased posterior synovial length beyond 2-4 weeks of immobilization (8,18). The importance of the posterior capsule in limiting knee extension during joint contractures has been documented in a rat model (8,16,17); with posterior capsulotomy restoring some knee extension deficits (19–21). The anatomical changes of the posterior capsule with immobilization support investigating its reversibility during remobilization after injury (22,23).

A previous exhaustive temporal study quantified the ROM of knees during unassisted recovery, after immobilization (17). The results indicated that knees immobilized for 1 and 2 weeks recovered full range of extension, however, knees immobilized for more than 2 weeks, had no significant recovery with remobilization.

The present study investigated anatomical changes in the posterior capsule of rat knee joints with contractures during remobilization and correlated those with previously published ROM data from the same specimen (17). Our objectives were to 1) measure the posterior knee capsule length after 1-32 weeks of immobilization and 1-48 weeks of remobilization, and 2) correlate the posterior capsule length measurements with previously published ROM data. Our hypotheses were that 1) the posterior capsule shortening following long durations of immobilization will not reverse after any duration of unassisted remobilization, and 2) shorter posterior capsules correlate positively with reduced knee ROM in the remobilization phase following immobilization.

Materials and Methods

Experimental methods

The experimental method was previously detailed by Trudel et al. (17) and approved by the Animal Care Committee (#ME-2461). In summary, 259 male Sprague-Dawley rats (350g) had one knee extra-articularly fixed with a Delrin® plate spanning from proximal femur to distal tibia at a 45° angle for one of six durations: 1, 2, 4, 8, 16, or 32 weeks (Fig. 1). The side of surgery was alternated. The contralateral knees served as the control group. Immobilization was lifted by removing the plate and each period of immobilization was followed by four different durations of spontaneous remobilization. Rats were allowed free activity in their cages for zero, one, two, or four times the duration of immobilization, with exception to the longest durations of immobilization (Fig. 1). At the end of the remobilization period, the rats were killed by carbon dioxide inhalation and the knees were mechanically tested for angle of knee extension using a fully automated arthrometer with a force of 12.5 N-cm (24) and immediately harvested. ROM measurements of the knee in extension after division of skin and muscles were used in this study to attribute the remaining knee extension deficit to arthrogenic restrictions. Groups are defined as week-week, where the first number is the duration of immobilization and the second is the duration of remobilization (e.g. group 2-4 was immobilized for 2 weeks and remobilized for 4 weeks). We measured the posterior capsule length in the same rat knees that had ROM measured (17). It was previously reported that 250 rats had been used for ROM in the study, however, due to the lengthy experimental design, additional rats were included to account for potential loss and replacement. As a result, 9 additional rats were available for histological analysis and used in the current study, despite not being tested for ROM.

Tissue Preparation and Staining

The knee joints and surrounding tissues were removed *en bloc*, fixed in Bouin's solution (in its natural repositioning after remobilization) for 24 hours, decalcified in 10% Tris-EDTA solution for 2 months, and embedded in low melting point paraffin (18). Standardized serial sections at the medial mid-condylar level were made in the sagittal plane. The 7μm sections were stained with 1% Alcian Blue for 5 minutes and 0.5% Direct Red for 5 minutes. Alcian Blue was selected for staining to create an optimal differentiation between the intimal and subintimal layers of the capsule; Direct Red acted as a counterstain (8).

Histomorphometric Analyses: Measurement of Posterior Capsule Length

The mounted sections were examined at a low magnification (3.3-6.6X) on a light microscope (Olympus BH-2, Tokyo) and histologically analyzed using imaging software ImageJ (NIH, Bethesda, USA). This study focuses on the posterior capsule since we are studying knee flexion contractures. The synovial intima length of the posterior capsule was measured. The femoral and tibial sections of the posterior synovial length were measured separately, with the medial meniscus as the anatomical landmark used for the division. The posterior femoral synovial length was measured from the posterior-superior horn of the medial meniscus to the synovio-cartilage junction on the posterior femur and the posterior tibial synovial length was measured from the posterior-inferior horn of the meniscus to the posterior tibia synovio-cartilage junction (see Supplemental Figure 1, SDC 1, illustrating the histological measurement). Anatomically the tibial posterior capsule is shorter than the femoral section (see Supplementary Figures 1 and 2, SDC 1 and SDC 2, illustrating this difference). Results from both sections are combined to equal

the posterior capsule length. Capsule length was measured by the same person blinded to the experimental group and slides were randomized prior to analysis.

Statistical Analysis

All capsule length data were expressed as mean ± standard deviation. Statistical analysis was conducted using SPSS version 24.0 (IBM Corp., Armonk, NY). Differences between experimental and contralateral knees were compared at each time point by paired t-test. The temporal effects of recovery among groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. In the ANOVA, the dependent variable was posterior capsule length and the independent variable was time of immobilization/remobilization. Values of ($P<0.05$) were considered statistically significant.

Pearson correlation coefficient analysis was conducted to determine the strength of the linear relationship between posterior capsule length and ROM data previously published from the same samples (17) after immobilization and remobilization. All immobilization groups were pooled together for a fixed duration of remobilization (0, 8, or 16 weeks) and posterior capsule length was paired with individual ROM measurements for each rat knee. The total, femoral, and tibial posterior capsule lengths were correlated separately with ROM. Rats that did not have both ROM and capsule length measurements were excluded from analysis. Multiple correlations were accounted for using a single step post-hoc Bonferroni correction. Values of ($P<0.003$) were considered statistically significant.

Results

Of 518 knees, 74 (32 experimental and 42 contralateral knees; 7 rats had both knees excluded = 14 knees or 7 rats) were damaged by prior mechanical testing or histological processing, leaving n=444 knee specimens from 252 rats for histomorphological analysis. The distributions per group are illustrated in supplementary Table 1 (see Supplementary Table 1, SDC 3, which shows sample sizes).

Synovial Folds

The contralateral groups, after immobilization and remobilization, showed a folded posterior joint capsule on both the femoral and tibial sites (Fig. 2); synovial folds were in close proximity. Immobilized groups showed that synovial folds had adhered together, leading to decreased posterior capsule length.

Posterior Capsule Length After Fixed Durations of Immobilization with Increasing Durations of Remobilization

Quantitative measures of the posterior capsule length after various durations of knee immobilization and remobilization are illustrated in Figure 3. Posterior capsule length of knees immobilized for 1 week followed by 1 to 4 weeks of remobilization were comparable to contralateral knees (all $P>0.05$; Fig. 3A). In knees immobilized for 2 weeks and remobilized for 2 and 8 weeks, the posterior capsules were shorter than the corresponding contralateral knees ($P<0.05$; Fig. 3B). Knees immobilized for 2 weeks also had a longer posterior capsule after 4 weeks of remobilization when compared with no remobilization (group 2-0) ($P<0.05$; Fig. 3B).

The shorter posterior capsule of knees immobilized for 4 weeks failed to recover its length after any duration of remobilization (4, 8 and 16 weeks), when compared with no remobilization (4-0) (all 3 $P>0.05$; Fig. 3C). The posterior capsules of knees immobilized for 4 weeks and remobilized for 0, 8 and 16 weeks were also significantly shorter than that of contralateral knees (all 3 $P<0.05$; Fig. 3C). For knees that were immobilized for 8, 16 or 32 weeks, the posterior capsule failed to significantly increase in length after any duration of remobilization up to 48 weeks, when compared with no remobilization (8 comparisons $P>0.05$; Fig. 3D-F), with the exception of group 16-32 ($P<0.05$). The posterior capsule of knees immobilized for 8, 16, or 32 weeks and remobilized for any duration up to 48 weeks were also significantly shorter than corresponding contralateral knees (all 12 comparisons $P<0.05$; Fig. 3D-F). Immobilization for 1, 2, 4, 8, 16 and 32 weeks and remobilization up to 48 weeks did not affect the posterior capsule length of contralateral knees when compared with no remobilization (all $P>0.05$).

Posterior Capsule Length After Increasing Durations of Immobilization with Fixed Duration of Remobilization

Despite 8 or 16 weeks of remobilization, the posterior capsule length of knees immobilized for 2, 4, 8, 16 or 32 weeks remained shorter than the contralateral knee (all 8 comparisons $P<0.05$). Also, the posterior capsule of immobilized knees shortened as the duration of immobilization was increased. For knees remobilized for 8 weeks, the posterior capsule length was significantly shorter after 16 weeks of immobilization when compared with the earliest duration of immobilization (2-8) ($P<0.05$ Fig. 4A). Knees remobilized for 16 weeks had significantly shorter posterior capsule lengths after 8, 16, and 32 weeks of immobilization, when compared with the earliest duration of immobilization (4-16) (all 3 $P<0.05$; Fig. 4B). Posterior capsule length of knees contralateral to

the knees immobilized for 2, 4, 8, 16 and 32 weeks and remobilized for 8 or 16 weeks were unchanged (all $P>0.05$).

Femoral and Tibial Posterior Capsule Length

Changes in posterior capsule length were mainly attributable to changes to the longer postero-superior (femoral) section of the posterior capsule and less to the postero-inferior (tibial) section (see Supplementary Figures 2 and 3, SDC 2 and SDC 4, which shows differences in length).

Correlation Between Posterior Capsule Length and Range of Motion

Posterior capsule length positively correlated with range of knee extension in all immobilization groups combined ($r=0.542$; $P<0.001$) (Table 1, Fig. 5). In knees immobilized for 2, 4, 8, and 16 weeks followed by 8 weeks of remobilization, the reduced posterior knee capsule length positively correlated with the range of knee extension but failed to reach statistical significance after correcting for multiple comparisons ($r=0.456$; $P=0.007$). For knees immobilized 4, 8, 16, and 32 weeks and remobilized for 16 weeks, the reduced posterior knee capsule length correlated with decreased knee extension ($r=0.541$; $P<0.001$). In the contralateral knees of the 16-week remobilization group, no significant correlation was measured between posterior knee capsule length and the range of knee extension ($r=0.342$; $P=0.031$).

Discussion

We report reversibility of posterior capsule length shortening following knee contractures induced by immobilization of 2 weeks or less. However, unassisted remobilization of any duration, even when 4 times longer than the period of immobilization, failed to reverse the posterior capsule

shortening caused by immobilization longer than 2 weeks, confirming our first hypothesis. This finding shows a biological difference between short and long durations of immobilization in response to remobilization. Secondly, posterior capsule shortening correlated with loss of knee range of extension, both in knees that were and those that were not remobilized, confirming our second hypothesis. The current comprehensive study adds to the literature multiple durations of immobilization, up to 32 weeks, and of remobilization up to 48 weeks. The range of immobilization and remobilization durations are compatible with the clinical presentation of patients treated for weeks and months after their knee injury.

Posterior Capsule Length Shortening

Our data adds quantitative data to the literature supporting that the posterior knee capsule changes are irreversible, at least for unassisted recovery. Two experimental studies have reported irreversibility of capsule shortening after knee immobilization and remobilization. Kaneguchi et al. used transarticular Kirschner wires to immobilize rat knees for 3 weeks and remobilized up to 2 weeks (25). They found that the posterior capsule length decreased after immobilization and further decreased following remobilization. Furthermore, they showed that the arthrogenic contracture had continued to develop during the short period of remobilization. Ando et al., adopted our method for internal fixation, processing, and histological assessment to show similar results using 16 weeks as the single duration of remobilization with 1 to 16 weeks of immobilization (26). These and other experiments, as well as clinical reports, have established the posterior capsule as a major contributor to the lack of knee extension in immobility-induced knee contractures (18–21,27).

Our study establishes the synovial layer as a valid and responsive surrogate marker of underlying posterior capsule contribution to an arthrogenic limitation in joint contractures secondary to immobility. Previous studies have explained the shortening of the posterior capsule as a result of adhesions of synovial villi to neighboring synovial villi, or to articular cartilage after immobilization (14,18). Since the knee is immobilized in flexion, the posterior part of the joint is not under tension, allowing for the loose synovial layer to fold and adhere to one another (14,18). Moreover, the lack of tension permits capsular cellular elements such as fibroblasts, synoviocytes, and adipocytes to proliferate, which impacts changes in proteoglycans, collagen proteins, and crosslinks between collagen fibers in the extracellular matrix (14,16,23). Additionally, the rabbit model of post-traumatic joint contracture has shown increased number of myofibroblasts in the posterior capsule after immobilization (28,29). These changes all contribute to the fibrosis and stiffness of the posterior capsule, which further increases the resistance to joint motion (14,18,23,28). The importance of capsular adhesions can be appreciated since the shortened capsule length persisted after remobilization following immobilization beyond 2 weeks. In this study, shortening of posterior capsule length occurred predominately in the femoral part of the capsule (see Supplementary Figures 2 and 3, SDC 2 and SDC 4, showing this change). The femoral posterior capsule is anatomically longer than the tibial capsule, permitting more synovial folds to coalesce, more synovial-synovial contact, and opportunities for adhesions to form between folds.

Posterior Capsule Length and Knee Range of Motion in Extension

ROM is the main functional outcome measure when evaluating the severity of joint contractures (22), and the only one available to clinicians treating athletes with after knee injury. Pairing the anatomical posterior capsule length data with the range of knee motion allowed

correlating the mechanical limitation to an articular structure. Correlating posterior capsule length with ROM data in the same knee joints greatly reduces data variability. Previously published data showed decreased ROM in knees immobilized for 1 to 2 weeks (17). However, remobilization led to recovery in the range of knee extension, reaching levels comparable to the contralateral. The range of motion tested before myotomy allowed distinguishing the restrictions mainly of myogenic origin, that led to reduced ROM after short durations of immobilization, with little contribution from arthrogenic structures (14–17). The current study confirms that intraarticular synovial length changes after short periods of immobilization were reversible and support that myogenic changes might account for the temporary decrease in knee range of extension (17). However, as arthrogenic contractures develop, the loss of knee ROM becomes irreversible (17). In this study, posterior capsule length positively correlated with knee range of extension after myotomy as durations of immobilization increased, regardless of whether the knees were remobilized or not. This reinforces the contribution of the posterior capsule to arthrogenic contractures. The posterior capsule length of contralateral knees also showed a weaker correlation with knee extension. Possible reasons include ageing, causing both reduced ROM and synovial length, or gait adaptation, by flexing the knee opposite to the immobilized knee to compensate for discrepancy in leg length (12). The stronger correlation in immobilized legs confirms that the correlations we report are not due to age only. In our study, we applied a Bonferroni correction, a very conservative method, to account for the multiple correlation analyses performed. Positive correlations between posterior capsule length and ROM were measured in all remobilization groups but failed to reach significance in the 8-week remobilization group. The positive correlation between the capsule structure and ROM after

immobilization and remobilization highlights the functional importance of the reduction in length of the postero-femoral capsule.

Clinical Relevance

Knee injuries are most common in sports medicine and many are treated with immobilization during conservative management or after surgery. The development of a knee joint contracture poses a significant challenge to athletes and the treating team, which may constitute a career-ending complication for runners, jumpers, and cyclists (5). Current rehabilitation treatments for established knee joint contractures include sustained stretching and exercises to increase ROM, while severe contractures may require surgical intervention (11,22). We provided quantitative data pointing to an anatomical deficit, the posterior knee capsule shortening, which correlated with the lack of knee extension after immobilization. Progressive capsule shortening with incremental durations of immobilization was an irreversible process that correlated with irreversible arthrogenic contractures. Unassisted remobilization was insufficient to restore posterior capsule length and range of knee extension when immobilized beyond 2 weeks. The data indicated a short window of opportunity for intervention where anatomical reversibility of the capsule adhesions was possible. This study provides experimental evidence for minimizing the duration of knee immobilization after a knee injury. The data also support that should immobilization extend past 2 weeks, as it is the case for many acute traumatic knee injuries (30), unassisted remobilization may be insufficient to reverse the anatomical and range of motion changes. Assisting the passive and active knee mobilization of the athlete is recommended. Considering the use of an animal model, this study is limited by the use of a quadruped rat model, whose knee's habitual position is in flexion, which may cause the knee to be more resistant to flexion contractures (17). However,

the similar rat knee anatomy and function in comparison to human provides valuable knowledge about the lack of reversibility after the development of a flexion contracture caused by prolonged immobilization.

Conclusion

Immobilized knees developed flexion contractures characterized anatomically by shortening of the posterior capsule that correlated with the mechanical lack of knee extension. This exhaustive study provided quantitative and temporal evidence that both joint alterations were irreversible by unassisted remobilization when the duration of knee immobilization exceeded 2 weeks. Interventions aimed at restoring knee extension must be implemented as unassisted remobilization will not reverse knee flexion contractures.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s). The authors declare that the results of the present study are presented clearly, honestly, without fabrication, falsification or inappropriate data manipulation, and does not constitute an endorsement by ACSM.

Author Contributions: GT, OL, and HKU conception and design of research; HZ performed experiments; HZ analyzed data; HZ, GT, and OL interpreted results of experiments; HZ prepared figures and drafted manuscript; HZ, GT, HKU, and OL edited and revised manuscript; HZ, GT, HKU, and OL approved final version of manuscript.

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Figures and Tables

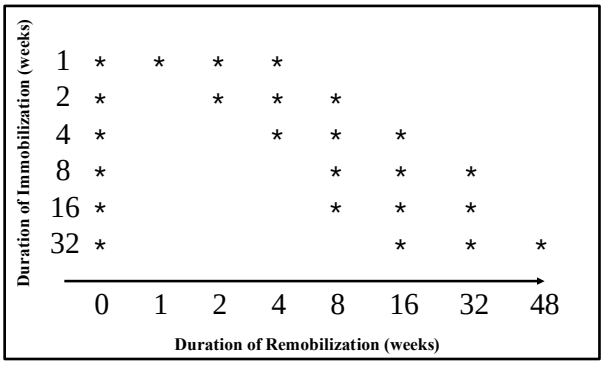

Figure 1. Study design. Sprague Dawley rats were divided into 6 durations of immobilization. Each duration of immobilization had four durations of remobilization, indicated by an asterisk (*). At the end of the period of remobilization, the rat knees were measured for range of motion and harvested for histomorphological analysis. The contralateral knee constituted the control group.

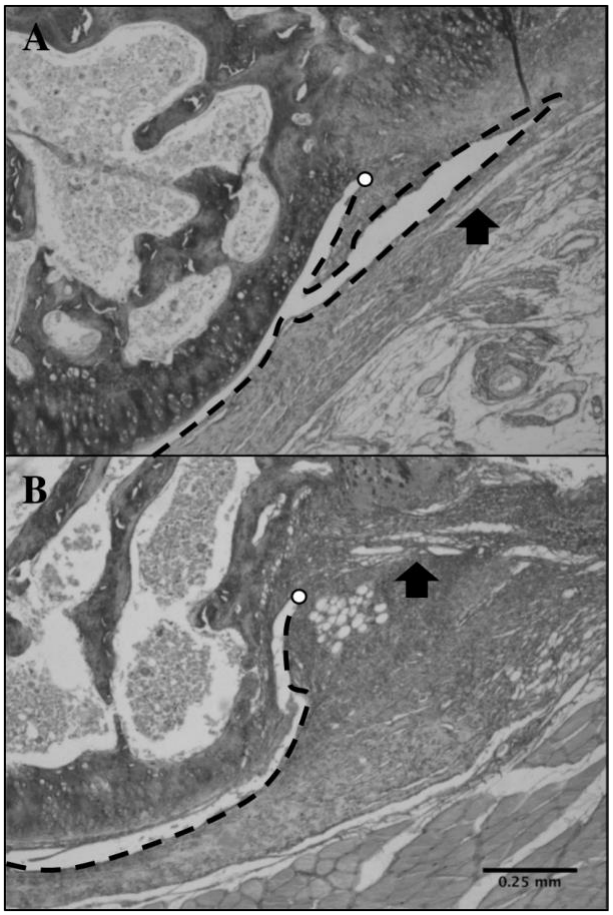

Figure 2. Shortening of the posterior joint capsule after immobilization. Microphotograph of a sagittal section of the postero-femoral knee capsule. A) Contralateral knee immobilized for 2 weeks with no remobilization B) Immobilized knee. The posterior capsule length was shortened after immobilization; shortening caused by adhesions of synovial folds and obliteration of joint recess. Segmented line delineates the posterior capsule length and open circles indicate the synovio-cartilage junction. The arrows indicate the area in which a synovial adhesion may have occurred, thereby obliterating the joint recess and reducing the posterior capsule length. Magnification of 13.2X.

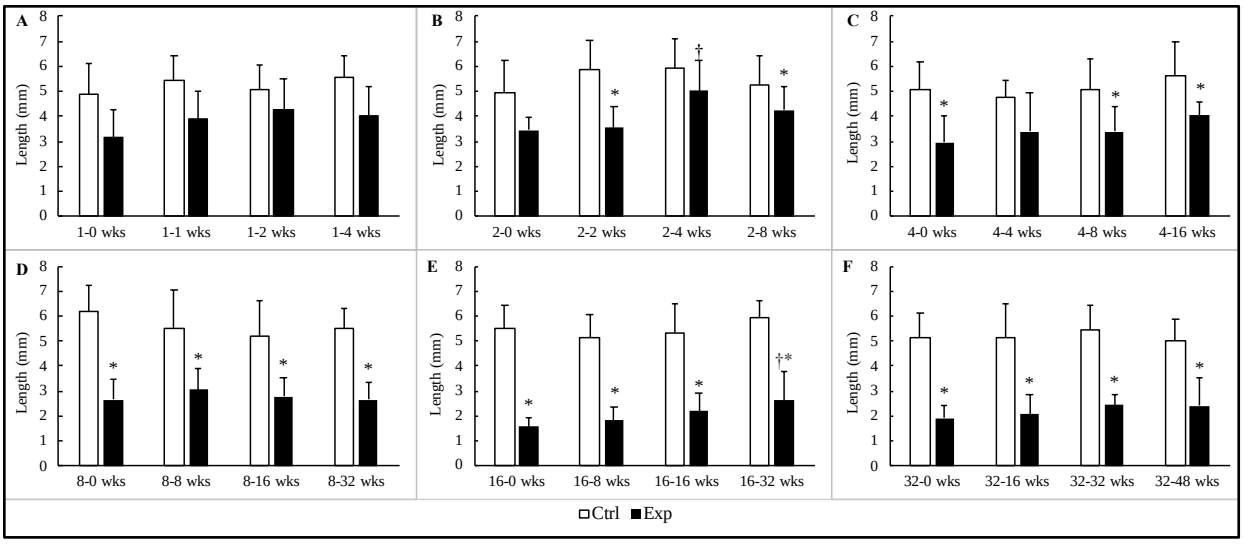

Figure 3. Posterior capsule length (mm) of rat knee joints following a fixed duration of immobilization and increasing durations of remobilization. A-D) Immobilization of 1, 2, 4, or 8 weeks, followed by remobilization that were one, two, and four times the immobilization durations. E-F) Immobilization of 16 and 32 weeks, followed by remobilization that were one-half, one, and two times the immobilization duration, with the exception of group 32-48. *: significant difference compared to contralateral knee ($P<0.05$). †: significant difference compared to no remobilization ($P<0.05$). Corresponding statistical and significance values are listed in supplementary Tables 1 and 2 (see Supplementary Tables 1 and 2, SDC 3 and SDC 5, which shows summary of statistics).

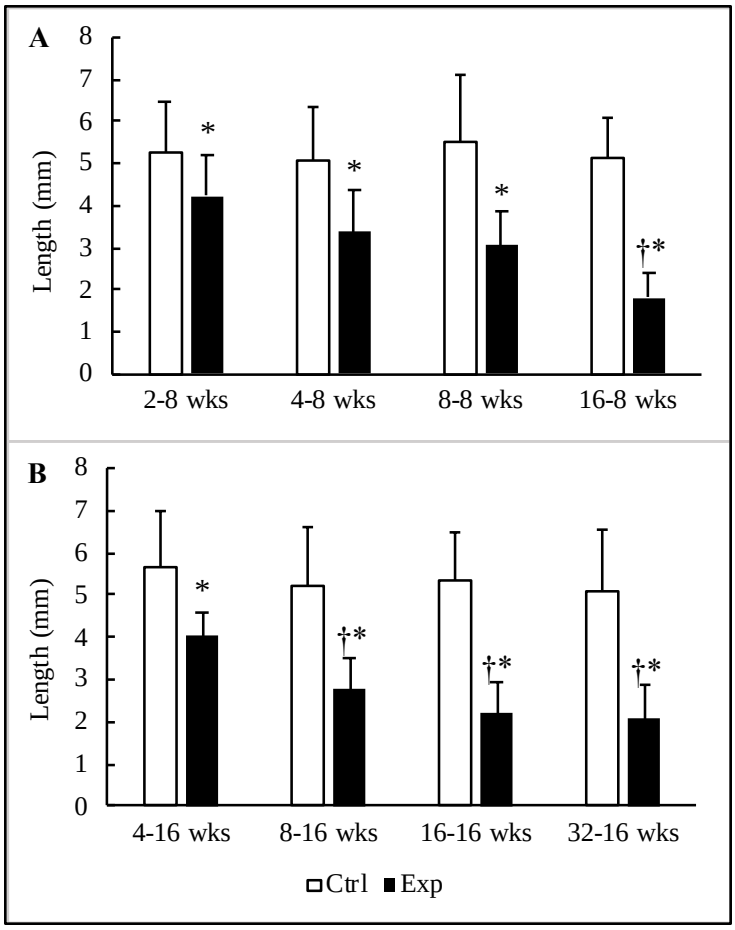

Figure 4. Posterior capsule length (mm) of rat knee joints following increasing durations of immobilization with a fixed duration of remobilization. A-B) Remobilization of 8 and 16 weeks, each with durations of immobilization that were one-quarter, one-half, one, and two times the duration of remobilization. *: significant difference compared to contralateral knee (P<0.05). †: significant difference compared to earliest time point of immobilization (P<0.05). Corresponding statistical and significance values are listed in supplementary Tables 2 and 3 (see Supplementary Tables 2 and 3, SDC 5 and SDC 6, which shows summary of statistics).

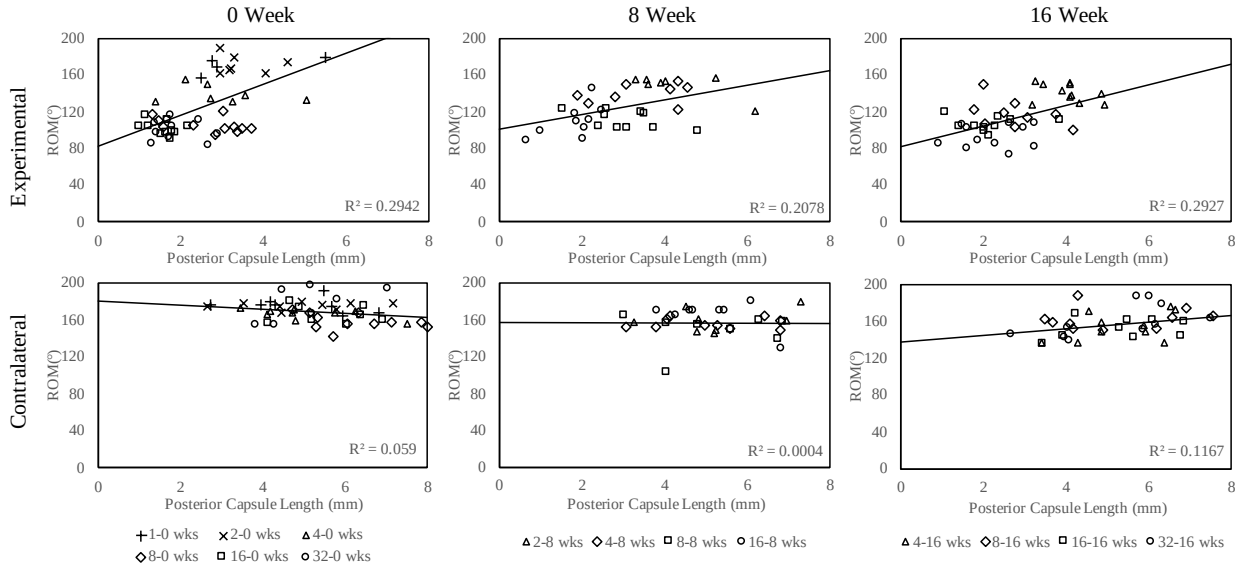

Figure 5. Correlation between posterior capsule length and corresponding range of knee extension. For fixed durations of remobilization (0, 8, or 16 weeks), all samples were pooled from the incremental durations of immobilization. 0 weeks: groups immobilized for 1 to 32 weeks with no remobilization. 8 weeks: groups immobilized for 2 to 16 weeks with 8 weeks of remobilization. 16 weeks: groups immobilized for 4 to 32 weeks with 16 weeks of remobilization. Corresponding correlation coefficient and significance values are listed in Table 1.

Table 1. Pearson correlation coefficients and respective P-values. Comparison between posterior capsule length and ROM in the same knees. For a fixed duration of remobilization, all samples were pooled from the incremental durations of immobilization. Comparison of femoral and tibial sections of the posterior capsule length with ROM were also conducted. Alpha value was adjusted using the Bonferroni correction. *: (P<0.003).

Comparison	n	Time Remobilized (wks)	Correlation Coefficient (r)			*P*-value		
			Total	Femoral	Tibial	Total	Femoral	Tibial
Contralateral	52	0	-0.243	-0.249	-0.063	0.083	0.076	0.660
	35	8	-0.019	-0.174	0.474	0.915	0.318	0.004
	40	16	0.342	0.395	-0.03	0.031	0.012	0.853
Experimental	50	0	0.542	0.550	0.222	<0.001*	<0.001*	0.122
	34	8	0.456	0.452	0.299	0.007	0.007	0.086
	41	16	0.541	0.506	0.348	<0.001*	<0.001*	0.026

Supplemental Digital Content

- Supplementary Figure 1.
- Supplementary Figure 2.
- Supplementary Figure 3.
- Supplementary Table 1.
- Supplementary Table 2.
- Supplementary Table 3.

Chapter 2

Mechanical adaptation of synoviocytes A and B to immobilization and remobilization: a study in the rat knee flexion model

Journal of Molecular Histology

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Keywords: Synoviocyte; posterior joint capsule; knee; immobilization; gene expression; immunohistochemistry.

Abstract

The objective of this study was to quantify the *in vivo* response of synoviocytes type A and B in the posterior joint capsule to knee immobilization and remobilization. Also, to correlate the immunohistochemical data with selected mRNA expression in the posterior joint capsule. Forty-two adult male Sprague-Dawley rats had one knee joint immobilized in flexion for durations of 1 to 4 weeks. Fifteen were harvested after immobilization and 15 were remobilized for 4 weeks. They were analyzed immunohistochemically with CD68 and CD55 antibodies as markers for synoviocytes type A and type B, respectively. Controls were 15 age-matched rats. The remaining 12 rats had their posterior capsule harvested and synoviocyte-specific CD68, CD55, and uridine diphosphoglucose dehydrogenase (UDPGD) mRNA expression was measured. Controls were 12 sham-operated knees. Knee immobilization for 2 weeks significantly increased synoviocytes A:B staining ratio compared to controls (3.88 ± 1.39 vs. 1.83 ± 0.76; $p<0.05$). Remobilization for 4 weeks abolished the increase. Remobilization of knees that were immobilized for 1 week also significantly lowered the synoviocytes A:B staining ratios compared to immobilized-only knees (0.66 ± 0.23 vs. 2.19 ± 0.54; $p<0.05$) and to controls (0.66 ± 0.23 vs. 1.32 ± 0.29; $p<0.05$). Consistent with the immunohistochemistry, mRNA expression of synoviocyte type B-specific CD55 and UDPGD genes were significantly lower in the capsules immobilized for 2 weeks (both $p < 0.05$). Knee immobilization and remobilization significantly modulated synoviocytes *in vivo*, stressing their mechanosensitive nature and possible contribution to immobility-induced changes of the joint capsule.

Keywords: Synoviocyte; posterior joint capsule; knee; immobilization; gene expression; immunohistochemistry.

Introduction

The synovial membrane lines the articular side of the joint capsule and is responsible for secretion of the synovial fluid and for phagocytosis (Smith 2011). The synovium is a few cell layers thick populated with specialized cells termed synoviocytes (Iwanaga et al. 2000). Two main types of synoviocytes have been identified: type B synoviocytes, which possess secretory functions of lubricating molecules such as hyaluronic acid (HA), and type A synoviocytes, which have been associated with a phagocytic function (Iwanaga et al. 2000; Smith 2011; El-gabalawy 2013; Shanaj and Donlin 2019). Synoviocytes have been shown to be mechanosensitive to joint movement and mechanical deformation *in vitro* (Momberger et al. 2005; Ingram et al. 2008). Conversely, prolonged knee immobilization has resulted in the decreased proliferation of synoviocytes and as well as decreased synovial fluid and HA content (Pitsillides et al. 1999; Trudel et al. 2003). Mechanical strain in the capsule, or lack thereof, likely modulates the biology of synoviocytes (McCarty et al. 2011), but the temporal response and quantification of type A and B synoviocytes within the synovium *in vivo* during knee immobilization and subsequent remobilization have yet to be examined.

Determining the cell type ratios in the synovium was proposed as an important measure to identify predominating cellular interactions during various joint pathologies (Shanaj and Donlin 2019). In chronic inflammatory diseases such as rheumatoid arthritis (RA), the role of synoviocytes are well established: type B synoviocytes are associated with the promotion of synovitis, pannus growth, and ultimately cartilage degeneration, whereas type A synoviocytes are associated with production of proinflammatory mediators (Falconer et al. 2018). However, in the context of immobility-induced joint contractures, Trudel et al., have shown that extraarticular immobilization of the knee joint in flexion did not induce intraarticular pannus proliferation

(Trudel et al. 2000). Thus, contrary to RA, changes in synoviocyte type A to B ratio in joint contractures are not associated to an inflammatory/proliferative response, but rather as an adaptation to its mechanical environment. The main objective of this study was to investigate how synoviocyte type A and type B immunostaining in the posterior synovium changes with knee immobilization and remobilization and to correlate these changes with synoviocyte type A and type B-specific mRNA expression.

Using the open source plugin IHC profiler (Varghese et al. 2014), we quantified CD68 and CD55 staining in the posterior synovium of rat knees that have been immobilized in flexion for 1, 2, or 4 weeks and remobilized for 4 weeks. Additionally, we extracted the temporal mRNA expression data of CD68, CD55, and uridine diphosphoglucose dehydrogenase (UDPGD) genes in the posterior joint capsule of rat knees immobilized for the same durations from genome-wide expression data (Wong et al. 2015). Considering the lack of HA production and non-inflammatory response during joint immobilization, we hypothesized that immobilization would increase the synoviocyte A:B staining ratio (due to a decrease in type B staining), and remobilization would restore type B synoviocytes and the ratio between synoviocyte types.

Materials and Methods

Experimental Methods

The study was approved by the University of Ottawa Animal Care Committee, which is certified by the Canadian Council on Animal Care (#ME-2461). Thirty adult male Sprague-Dawley rats had one knee extraarticularly fixed with a Delrin® plate at a 45° angle for durations of 1, 2, or 4 weeks as per Trudel et al (Trudel et al. 2014). The side of surgery was alternated. Rats

were then divided into immobilization-only and remobilization groups. During unassisted knee remobilization, the fixator was removed, and the rats were allowed unrestricted movement within their cages for 4 weeks. Controls were 15 unoperated age-matched rats. Each experimental group contained n=5 knees for analysis compared with n=5 controls. The nomenclature for the groups consists of the immobilization-remobilization durations (e.g. group 2-4 was immobilized for 2 weeks and remobilized for 4 weeks).

Tissue preparation and immunohistochemistry

At the end of the immobilization-remobilization period, the knee joints were removed *en bloc*, fixed in Bouin's solution for 24 hours, decalcified in 10% Tris-EDTA solution for 2 months, and embedded in low melting point paraffin (Trudel et al. 2000). Standardized serial sections (7µm) were cut at the mid-medial condylar level in the sagittal plane.

In order to detect the synoviocytes, we used CD68 antibodies as a marker for type A and CD55 antibodies as a marker for type B synoviocytes (Smith 2011; El-gabalawy 2013; Shanaj and Donlin 2019). Deparaffinized knee sections underwent a heat-induced epitope retrieval in a 10mM sodium citrate buffer (pH 6) at 60ºC for 80 mins. Endogenous peroxidase was removed with Peroxidased 1 for 5 min (Biocare Medical PX968) and nonspecific background staining was reduced with a universal blocking reagent for 20 min (Biocare Medical BS966). Serial sections were incubated with polyclonal rabbit anti-rat CD68 (Abcam, ab125212, 1:50) and CD55 (Abcam, ab231061, 1:50) antisera for 1 hr at room temperature and subsequently incubated overnight at 4ºC. The secondary antibody, a rabbit-on-rodent horseradish peroxidase polymer (Biocare Medical, RMR622G), was incubated for 30 min at room temperature. The chromogenic detection step was conducted using 3, 3'-diaminobenzidine (DAB) (Biocare Medical, BDB2004L) incubated on the

sections for 2 min. Counterstaining was conducted in diluted hematoxylin. A negative isotype control consisted of using a polymer negative control serum (mouse & rabbit) (Biocare Medical, NC499) as the primary antibody. Positive tissue controls consisted of rat spleen for CD68 and rat intestine for CD55.

Microscopy and analysis with IHC Profiler

The stained slides were visualized by light microscopy with an Olympus BH-2 (Tokyo, Japan) microscope at 33X magnification and captured using a Marlin F080C digital camera (Allied Vision Technologies) with AVT Smartview 1.5.1 software. Distribution of synoviocytes A and B staining were investigated in a field of view in the postero-superior knee synovium. This region was selected as previous results have shown that the postero-superior knee capsule undergoes the largest shortening compared to the postero-inferior side in this model of knee immobilization (Zhou et al. 2018). Images were processed using ImageJ (NIH, Bethesda, MD) with the IHC Profiler plugin; a software developed for clinical histopathological sample analysis (Varghese et al. 2014). IHC Profiler provides an automated and unbiased method to quantitatively assess antibody staining intensity and percentage in DAB and hematoxylin stained tissue sections using a spectral deconvolution method of the DAB/hematoxylin colour spectra and computerized pixel-by-pixel analysis of the entire field of view (Varghese et al. 2014). IHC Profiler determines the percentage of pixels that return a positive outcome from DAB staining, rather than the number of positively stained cells, and provides a four-tier scoring system (high positive, positive, low positive, negative) of its pixel-by-pixel analysis. Positive scores were combined to provide a binary outcome (i.e. positive or negative score). The type A:B synoviocyte staining ratios were calculated

for each knee between serial sections. We compared the mean ratio of all 5 knees at each time point.

Gene expression data

The temporal mRNA expression of CD68, CD55, and uridine diphosphoglucose dehydrogenase (UDPGD), were extracted from previously generated genome-wide expression data of the posterior joint capsule in rat knees that followed the same experimental methods of joint immobilization (Wong et al. 2015). We analyzed data from 12 rats immobilized for durations between 1 and 4 weeks, similar to the immunohistochemical data, and 12 controls. This cohort differed from the specimens used for immunohistochemistry in that each group consists of n=4 rats (instead of n=5) and the control group was sham-operated age-matched (instead of only age-matched). In short, posterior joint capsules from operated and control knees were harvested at 1, 2, or 4 weeks after immobilization or after sham-surgery and total RNA was isolated for gene expression analysis on microarray. The Affymetrix GeneChips microarray and protocols was used with the Rat Genome 230 2.0 Array and the identity of probe sets were confirmed with the National Center for Biotechnology Information GenBank database. Expression of mRNA for CD68, CD55, and UDPGD for each capsule were calculated from the median of 5 replicates per probe and the mean of 4 capsules per group are presented for each of the durations of immobilization.

Statistical Analysis

Statistical analyses were conducted using SPSS version 24 (IBM Corp., Armonk, NY). Synoviocyte A:B staining ratios were reported as the mean ± standard deviation at each time point. To test the effect of immobilization duration, we conducted non-parametric Kruskal-Wallis

between time points. Differences between experimental and age-matched controls at each time point were compared using non-parametric Mann-Whitney U tests. Differences in gene expression between experimental and sham-operated controls were also compared using Mann-Whitney U tests. P values < 0.05 were considered statistically significant.

Results

Immunohistochemical staining of synoviocytes type A and B

Representative images of type A and type B-specific staining in the synovium for immobilized and control knees are presented in Fig. 1. For the knees immobilized for 1 week, remobilization resulted in significantly lower mean synoviocyte A:B staining ratio compared to immobilized-only knees and compared to controls ($p<0.05$; Fig. 2a). Knee immobilization for 2 weeks significantly increased the synoviocyte A:B staining ratio compared to controls ($p<0.05$; Fig. 2b), and remobilization abolished this difference ($p>0.05$; Fig. 2b). Immobilization and remobilization had no significant effect on knees immobilized for 4 weeks ($p>0.05$; Fig. 2c). Lastly, age had no effect on the synoviocyte A:B staining ratio in the unoperated controls ($p>0.05$; Fig. 2).

mRNA expression of synoviocyte-specific markers

The temporal gene expression of CD68, CD55, and UDPGD during immobilization are presented in Fig. 3. CD68 mRNA in the posterior capsules from immobilized knees showed no significant differences in expression when compared to controls at all immobilization durations ($p>0.05$; Fig. 3a). However, CD55 mRNA expression was significantly lower in the posterior capsules from knees immobilized for 1 and 2 weeks compared to controls (both $p<0.05$; Fig. 3b).

Lastly, UDPGD mRNA expression was significantly lower in the posterior capsule from knees immobilized for 2 weeks compared to controls ($p<0.05$; Fig. 3c).

Discussion

Consequences of joint immobilization include losses in range of motion and reduced HA content in synovial fluid, causing functional deficits (Pitsillides et al. 1999; Trudel et al. 2014). Knee immobilization in flexion significantly shortened the posterior capsule, a change correlated with reduced range of motion. Both the lack of extension and the shortened posterior capsule were irreversible after prolonged immobility despite long durations of remobilization in an animal model (Zhou et al. 2018). The mechanisms for joint capsule shortening may involve mechanosensitive changes of synoviocytes type A and B residing in the synovium. Here we show that eliminating knee motion increased the synoviocyte A:B staining ratio and that it was restored by remobilization. Gene expression data of CD68, CD55, and UDPGD corroborated the changes seen at the protein level and suggested that the increased synoviocyte A:B staining ratio was attributable to a decrease in synoviocyte B and not an increase in synoviocyte A staining. These findings highlighted the sensitivity of synoviocytes to joint movement and supported both of our hypotheses.

The temporal response of synoviocyte staining in the posterior capsule during knee immobilization and remobilization provide insight into the cellular interactions involved in synovial and knee pathologies accompanied by reduced joint motion. The increased synoviocyte A:B staining ratio after joint immobilization was caused by decreased type B synoviocyte staining (Fig. 1). Since type B synoviocytes are involved in HA production (Smith 2011; El-gabalawy 2013; Shanaj and Donlin 2019), these findings are biologically relevant to the well-described decrease

in HA concentration in the synovial fluid of immobilized joints (Pitsillides et al. 1999). The primary role of HA in diarthrodial joints is to contribute to the high viscosity and lubricating properties of the synovial fluid, and in turn protects the joint from the deleterious effects of repetitive use (Ingram et al. 2008). Thus, a decrease in type B synoviocytes during joint immobilization may partially contribute to limited joint function by reducing HA secretion. These data are further supported by the decreased expression of CD55 during immobilization and the decreased expression of UDGPD, an enzyme essential for HA production and also a marker for type B synoviocytes (Pitsillides et al. 1999). Immunostaining of type A synoviocytes did not change with immobilization, as reflected by the expression of CD68. These characteristic changes in synoviocyte subpopulations with joint immobilization may also be involved in capsular shortening and both types of synoviocytes have previously been observed in the adhesion regions of the synovial membrane (Ando et al. 2010). The synovial membrane must maintain a non-adherent tissue surface to allow movement and type B synoviocytes may play an important role in the inhibition of adhesion (Smith 2011). A decrease in type B synoviocytes with a relaxed and folded posterior capsule during knee immobilization in flexion may facilitate adhesion of facing capsular folds that eventually result in a shortened capsule length (Trudel et al. 2003; Zhou et al. 2018).

The response of synoviocyte type A:B staining ratio after knee immobilization and remobilization highlights the mechanosensitive nature of synoviocytes (Momberger et al. 2005). It has been suggested that the synoviocyte subpopulations are modulated by mechanical strain in the posterior capsule during joint motion (McCarty et al. 2011). Moreover, increased HA production has been observed with cyclic joint motion in a rabbit model (Ingram et al. 2008). In this model of immobility-induced knee flexion contracture, the loss of knee extension was shown

to be reversible only if the duration of immobilization was 2 weeks or less (Trudel et al. 2014). These results predicted that an elevated synoviocyte A:B staining ratio would be reversible after short durations of immobilization, which was also the case for the recovery in posterior capsule length (Zhou et al. 2018). However, reversibility of the synoviocyte ratio after durations of immobilization longer than 4 weeks is unknown. Our results suggest that the synoviocytes were not responsive after 4 weeks of immobilization.

Determining synoviocyte subpopulations has become increasingly important in understanding their role in various joint pathologies and is essential for the development of targeted therapeutic strategies (Croft et al. 2019; Shanaj and Donlin 2019). This is pertinent to immune-mediated inflammatory diseases such as RA, as synoviocytes exhibit different morphologies and functional roles (Iwanaga et al. 2000). Different subtypes drive the disease process through inflammation/tissue damage. Similarly, the profile of synoviocyte subtype can provide insight on the optimal choice of therapies to modulate the disease (Croft et al. 2019; Shanaj and Donlin 2019). In our study, we quantified the modulation of synoviocytes type A and B secondary to immobilization, which is largely devoid of an immune-mediated component. These data provide insight on the cellular changes in the synovium occurring primarily as an adaptation to its mechanical environment. The ratio of type A to B synoviocytes is dependent on conditions invoked by various pathologies, as determined by the distinct roles of the synoviocyte types; type A synoviocytes are involved in immune-mediated and inflammatory responses, while type B synoviocytes are involved in secretory functions with greater sensitivity to movement. An imbalance between synoviocyte type A and B proportions may indicate losses in homeostatic roles and implications in joint pathologies such as arthritis (Shanaj and Donlin 2019). The most common symptoms reported by patients with arthritis are pain and increased joint stiffness. These symptoms

inhibit joint movement through their full range of motion and can lead to contractures. Our results suggest that the synoviocyte phenotype in arthritis is not only reflective of immune-mediated changes, but also of the accompanying mechanical effects.

The use of IHC profiler provided an objective quantification, eliminating the subjective nature of traditional manual scoring methods (Varghese et al. 2014). Staining ratios between synoviocytes type A and B on serial sections eliminated the experimental variability in sample preparation, which could skew the staining percentage, and isolated the differences in synoviocyte-specific staining. The limitations in this study include that immunohistochemical and gene expression measures were carried out on different groups of rats with their respective controls. However, they were all the same species, sex, and underwent the same extraarticular internal knee fixation at the same age. Secondly, the field of view for analysis was limited to one region of the capsule; however, the postero-superior capsule undergoes the largest capsule shortening following knee immobilization. Third, IHC profiler does not quantify the number of cells that are positively stained, but rather the percentage and intensity of positively stained pixels from a histological sample.

Conclusion

Synoviocytes in the posterior capsule responded specifically to knee immobilization in flexion. Synoviocyte type A:B staining ratio increased during immobilization, corresponding to a decreased expression of genes specific to synoviocyte type B. Knee remobilization restored the staining ratio. These findings match mechanical data and support a mechanosensitive modulation of the synovium during joint movement. Synoviocyte type B may be mechanistically involved in

the posterior synovial shortening of immobilized knees and other forms of arthritis that limit joint range of motion.

Conflicts of interest

The authors declare no conflicts of interest.

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Author Contributions: Conception and design of research: OL, GT. Acquisition of data: HZ. Data analysis: OL, HZ. Interpreted results of experiments: OL, GT, HZ. Prepared the figures and drafted the article: OL, HZ. Edited and revised the article: OL, GT, HZ. Approved final version of the article: OL, GT, HZ.

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Figures

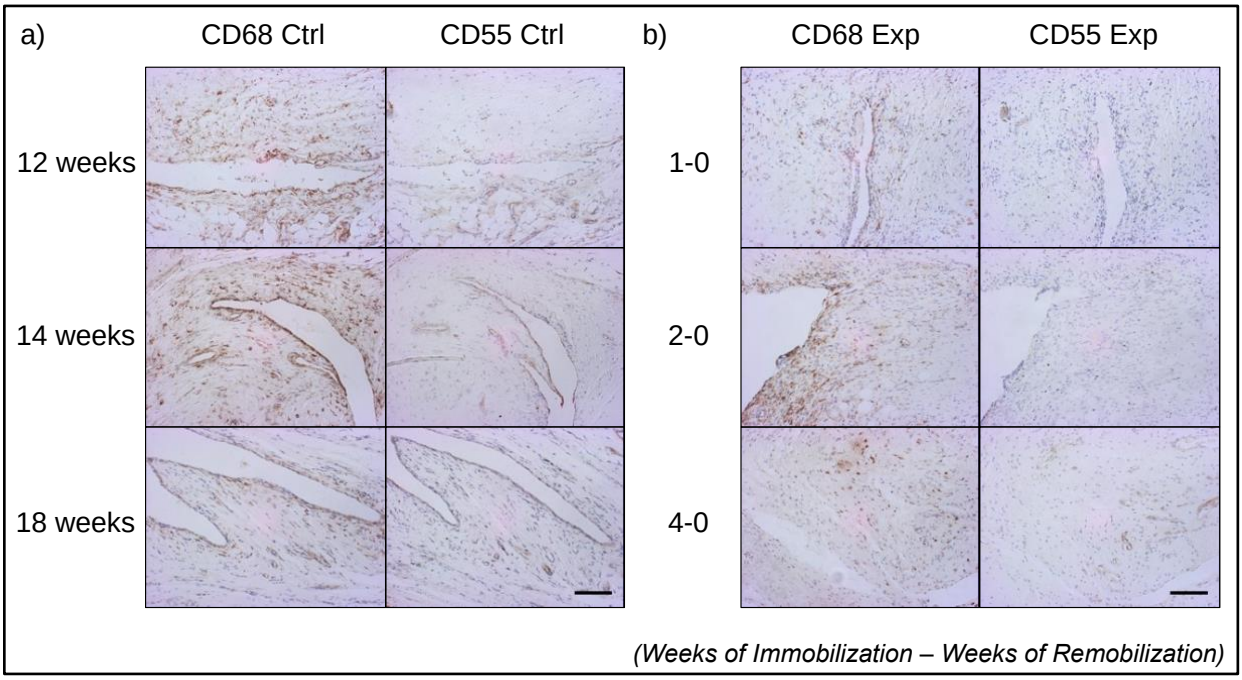

Fig. 1 Effect of knee immobilization on synoviocyte type A and B immunostaining in the posterior synovium using CD68 and CD55. Fields of view were selected on serial sections of the postero-superior synovium. a Age-matched controls (Ctrl). b Immobilized knees (Exp). Standardized serial sections (7 µm) were made in the medial mid-condylar plane. Brown staining indicates positive synoviocyte type A-specific CD68 or type B-specific CD55 staining in the respective images. Scale bar represents 0.1mm

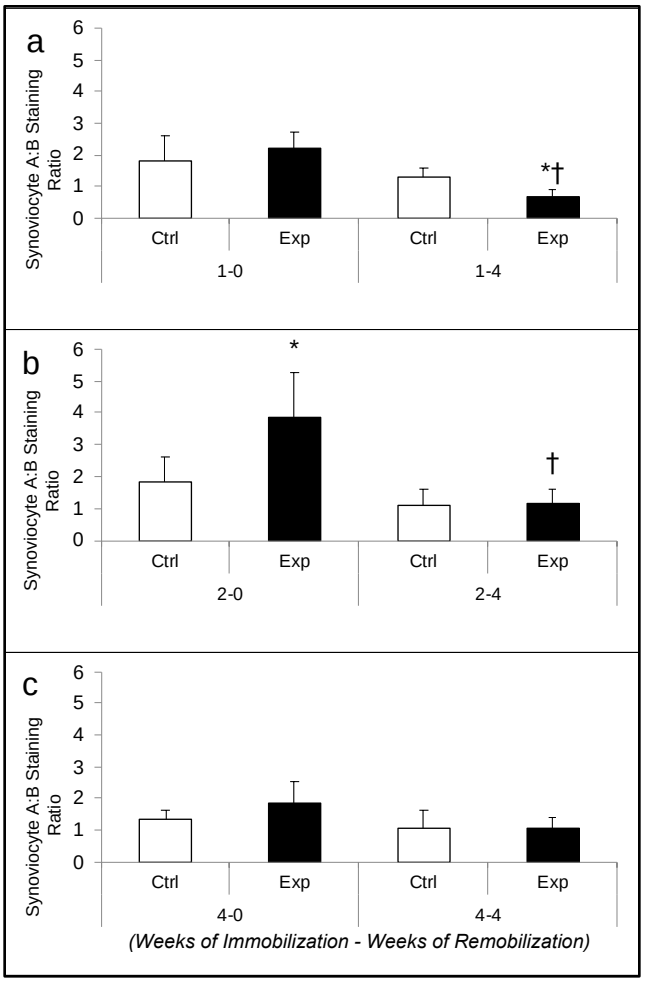

Fig. 2 Effect of knee immobilization and remobilization on synoviocyte A:B staining ratio in the postero-superior synovium. a One week of immobilization and 4 weeks of remobilization lowered the synoviocyte A:B staining ratio compared to immobilized and control knees. **b** Two weeks of immobilization increased the synoviocyte A:B staining ratio compared to controls and 4 weeks of remobilization abolished this difference. **c** Four weeks of immobilization had no statistically significant effect. Values are presented as mean±SD; n=5 knees per group. *; $p<0.05$ in comparison to control knee within a time point. †; $p<0.05$ in comparison to immobilized-only knee. Exp: immobilized knee. Ctrl: age-matched control. Group nomenclature; Weeks of immobilization – Weeks of remobilization

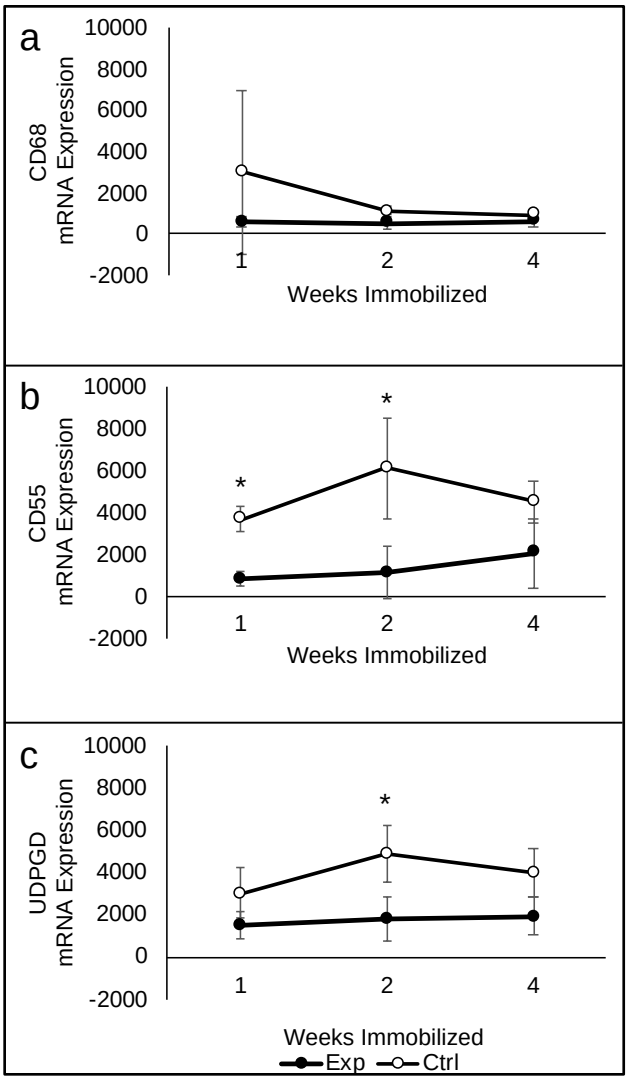

Fig. 3 Effect of knee immobilization on mRNA expression in the posterior joint capsule. Differences in mRNA expression between immobilized and sham-operated knees of synoviocytes type A and B-specific genes. **a** Synoviocyte type A-specific CD68 mRNA expression was unchanged after knee immobilization **b** Synoviocyte type B-specific CD55 mRNA expression was consistently lower in the posterior capsule of knees immobilized in flexion for 1 and 2 weeks. **c** Synoviocyte type B-specific UDPGD mRNA expression was lower in the posterior capsule of knees immobilized in flexion for 2 weeks. Values are presented as mean±SD; n=4 knees per group. *: $p<0.05$ between immobilized control knees within each time point

Chapter 3

Knee joint stiffness following immobilization and remobilization: a study in the rat model

Journal of Biomechanics

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Keywords: biomechanics; knee stiffness; extension deficit; range of motion; animal model

Abstract

Deficits in extension can limit the function and performance of the knee joint. The range of motion (ROM) deficit in knee extension is often measured and reported at a single torque value applied in the flexion-extension axis. This static measurement of ROM omits key details about the biomechanical properties of the knee, such as its mechanical stiffness. Our objectives were 1) to quantify knee extension stiffness after various periods of immobilization and remobilization, and 2) to evaluate how stiffness correlated with the length of the posterior knee capsule. Two hundred fifty-six male Sprague Dawley rats had one knee immobilized at a 45° angle in flexion using a Delrin® plate for 6 different durations ranging from 1-32 weeks. Remobilization was initiated by removing the plate and lasted for 0-48 weeks. The contralateral knee and unoperated age-matched rats were used as controls. An automated arthrometer extended the knee at four pre-determined torques and these data were used to calculate mechanical stiffness. The stiffness of knees immobilized for 8 or more weeks was significantly greater than controls and persisted despite remobilization ($p < 0.05$). Remobilization after 16 and 32 weeks of immobilization resulted in a progressive increase in mechanical stiffness ($p < 0.05$). The length of the posterior capsule significantly correlated with knee stiffness in extension ($p < 0.05$). Deficit in knee extension was characterized by increased stiffness, which was irreversible upon unassisted remobilization.

Keywords: biomechanics; knee stiffness; extension deficit; range of motion; animal model

Introduction

Complete knee extension is required for normal gait and to perform daily activities such as ascending and descending stairs or standing from a seated position (Dietz et al., 2017). Restricted range of movement of the knee, either in flexion or in extension, may contribute to a functional deficit and to an increased risk of osteoarthritis (Shelbourne et al., 2012). The terms stiffness and contracture are also used to describe the limited range of motion (ROM) of the knee. Stiffness is reported by patients as the sensation that the motion of a joint, active and/or passive, is difficult or limited and disrupts activities of daily living. The term joint contracture refers to the limitation in the passive ROM of a joint (Born et al., 2017; Laneuville, 2016). The inability to fully extend the knee is called a knee flexion contracture. Achieving full knee extension is a key factor in the clinical assessment of the knee and in determining the outcome of surgical interventions such as total knee arthroplasty (TKA) (Flierl et al., 2019).

Knee extension deficits are common and were reported to arise from soft-tissue fibrosis, capsular adhesions, poor rehabilitation compliance, technical errors during surgery, or unknown (Núñez et al., 2009). Prolonged immobilization of otherwise normal knee joints is a common cause (Laneuville, 2016). Functional deficits in knee extension can be improved with physiotherapy or surgical intervention, but treatment failure rate is high. Knee stiffness is the second most common complaint of patients with arthritis, such as osteoarthritis or rheumatoid arthritis. Specifically, patients identified the lack of joint mobility as a desired area of improvement, only secondary to pain (Heiberg and Kvien, 2002).

Stiffness and ROM are key indicators for the clinical evaluation of knee function. While both are related to the function of the knee joint, they measure different biomechanical properties. Knee ROM is an angle measurement based on the application of a single torque value in the

flexion-extension axis, whereas stiffness describes the increased resistance to forces; commonly expressed as the change in torque divided by angular displacement produced at the knee joint ($\Delta T/\Delta\theta$) (Markolf et al., 2016; Oatis et al., 2006). Knee stiffness can be calculated by taking the slope of the relationship between torque applied and angular displacement produced (Hufschmidt and Mauritz, 1985; Markolf et al., 2016; Sung et al., 2010; van der Steen et al., 2018).

Animal models have been used to study the mechanical properties of joints and understand the pathophysiology of knee extension deficits, including their reversibility. Most animal studies of knee flexion contractures reported ROM using only a single torque (Abdel et al., 2012; Hazlewood et al., 2018; Kaneguchi et al., 2016; Trudel et al., 2014b). These models do not inform on the contractile and stiffness properties of the knee joint (Reina et al., 2018). In 2014, a comprehensive study investigated the reversibility of immobility-induced knee flexion contractures with unassisted recovery in a rat model and reported the ROM using a single torque of 12.5N-cm (Trudel et al., 2014b). In the current study, we used the angle of extension measured at 4 incremental torques, ranging from 2.5 to 17.5 N-cm, to compute the stiffness of structures limiting complete extension of the knees. The objectives of this study were to 1) calculate stiffness in extension of rat knees immobilized for 1 to 32 weeks and remobilized for up to 48 weeks, and 2) correlate knee stiffness with a key articular structure limiting knee extension; the posterior knee capsule. We hypothesized that 1) knee stiffness will increase as duration of immobilization increased, 2) knee stiffness will improve following remobilization, and 3) knee stiffness will be negatively correlated with posterior capsule length.

Materials and Methods

Experimental methods

Details of the knee immobilization surgery and remobilization were previously described (Trudel et al., 2014b) as approved by the Institutional Animal Care Committee (ME-2461). In summary, 256 male Sprague Dawley rats (10-week-old, 350g) had one knee immobilized at a 45° angle in flexion using a Delrin® plate and screws, which spanned the proximal femur and distal tibia. The side of surgery was alternated. Rats were divided into six different durations of immobilization: 1, 2, 4, 8, 16, or 32 weeks. Immobilization was terminated by surgically removing the plate and each period of immobilization was followed by one of four different durations of unassisted remobilization; meaning remobilization without intervention. Rats were allowed free activity in their cages for zero, one, two, or four times the duration of immobilization, with exception to the longest durations of immobilization. Groups are designated as week-week, where the first number is the duration of immobilization and the second is the duration of remobilization (e.g. group 4-16 was immobilized for 4 weeks and remobilized for 16 weeks). The results of the immobilized knee, "experimental," were compared with the knees contralateral to the immobilized knees, "contralateral," and knees from unoperated age-matched rats, "control" (Supplementary Table 1 and 2). At the end of the remobilization period, rats were killed by carbon dioxide inhalation and the knees were mechanically tested.

Mechanical testing

The arthrometer used to measure angle of knee extension for a rat animal model has previously been described (Campbell et al., 2018). In brief, the animal being tested is positioned on its side with the leg being measured facing upwards. The lower extremity was first degloved to

remove skin from underlying fascia. The femur was secured in a grooved metal clamp and the lateral femoral condyle was positioned over the center of rotation of the arthrometer. The movable arm was positioned behind the leg (superior to the calcaneus) to push the knee into passive extension at four pre-determined torques: 2.5, 7.5, 12.5, and 17.5 N-cm, hereon referred to as T1, T2, T3, and T4, respectively. The range of torques was determined based on mechanical testing on normal rat knee joints; T1 was determined as the point of resistance just above measurable amounts, and T4 was determined to be the amount of force that led to angular extension beyond full extension (180°) and capsular failure after myotomy (Campbell et al., 2018; Trudel et al., 2000, 1999; Trudel and Uhthoff, 2000). The movable arm pushed the leg into extension at a speed of 0.69 rad/s until the first pre-determined torque was reached. Upon reaching the torque, the arthrometer stopped for 2.1s while a digital camera positioned above the arthrometer automatically took a picture of the leg. This process was repeated until all four torques were applied. Only data measured before myotomy are reported in this study.

Knee range of motion measurement

Anatomical landmarks were used to determine angle of knee extension. The images obtained during mechanical testing were analyzed using ImageJ (NIH, Bethesda, MD). The 'angle tool' was used to trace the femorotibial angle. The femoral line was drawn from the middle of the femur clamp to the lateral femoral condyle and the tibial line was drawn from the lateral femoral condyle to the lateral malleolus. The angle was then calculated using the 'measure' function.

Knee stiffness calculation

The angle of extension reached at each torque minus the initial angle of immobilization (45°) represented the knee displacement in extension for each torque. The displacement data at each torque from the same knee were used to calculate knee joint stiffness. For this, torque (N-cm) angular displacement (°) graphs were generated (Figure 1) and the slope of the torque-displacement regression line was used to derive the stiffness (*k*) using this equation:

$$k = \frac{\sum(x - \bar{x})\,(y - \bar{y})}{\sum(x - \bar{x})^2}$$

The rationale for graphing the torque (independent variable) on the y-axis and displacement (dependent variable) on the x-axis is justified by the rotational stiffness equation; where k is stiffness, T is applied torque, and θ is the produced angular displacement:

$$k = \frac{\mathrm{T}}{\theta}$$

Thus, in order to calculate stiffness from the slope (Δy/Δx), torque was plotted on the y-axis and angular displacement on the x-axis.

Posterior knee capsule length

The rat knees were mechanically tested and had the length of their posterior capsule measured histologically. The histological methods are described in a previous publication (Zhou et al., 2018). The data from the posterior capsule length was used to correlate with the mechanical stiffness of the knees.

Statistical analysis

Descriptive statistics were used to summarize the data. Slope values obtained from individual knees for a group were presented as mean values ± standard deviation. The temporal effects of remobilization among groups were compared using a one-way ANOVA followed by Tukey's *post hoc* test. In the ANOVA, the dependent variable was knee stiffness in extension and the independent variable was time of immobilization and remobilization. The differences between stiffness of immobilized and contralateral knees at each time point were compared using paired *t*-tests, while differences between immobilized and controls were compared using unpaired *t*-tests. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was conducted using SPSS version 24.0 (IBM Corp., Armonk, NY).

The strength of the linear relationship between overall knee stiffness and previously published posterior capsule length data from the same rat knees (Zhou et al., 2018) was determined by a Pearson correlation coefficient analysis. All immobilization groups were pooled together for a fixed duration of remobilization (0, 8, or 16 weeks) and the posterior capsule length was paired with individual knee stiffness measurements for each rat knee. Rats that did not have both posterior capsule length and knee stiffness measurements were excluded from analysis. Values of $p < 0.05$ were considered statistically significant.

Results

Of the 512 knees measured, 25 were damaged during mechanical testing or did not provide measurements at all 4 torques (16 experimental and 9 contralateral). As a result, 487 knees from 253 rats were available for analysis (Supplementary Table 1). In the controls, 14 knees were

excluded for similar reasons, leaving 74 knees available for analysis. The final sample size for each group and corresponding experimental groups are listed in Supplementary Table 2.

Difference in profiles of torque-displacement curves

Figure 2 shows the relationship between median values of knee displacement in extension measured at increasing torques for the immobilized and for the contralateral knees without remobilization. Increased durations of immobilization reduced ROM and shifted the torque-displacement curves to the left when compared to corresponding contralateral knees (Figure 2A-B). Fixed periods of 8 or 16 weeks of unassisted remobilization did not restore the torque-displacement curves to the contralateral positions (Figure 3A-B) and the torque-displacement curves remained left-shifted compared to contralateral knees (Figure 3C-D).

Knee stiffness: fixed duration of immobilization

The stiffness of knees immobilized for 1, 2, and 4 weeks were comparable to the contralateral knees except for groups 1-2, 2-0, and 4-8 ($p < 0.05$; Figure 4A-C). Knee stiffness was significantly greater than controls for some groups ($p < 0.05$; groups 1-1, 1-4, 2-4, 4-4, and 4-16). In the knees immobilized for 1, 2, 4, and 8 weeks, there were no significant differences in knee stiffness in all remobilized groups when compared to immobilization only ($p > 0.05$; Figure 4A-D). Beyond 4 weeks of immobilization, knee stiffness was significantly greater than contralateral knees and controls at all time points and persisted after remobilization (all 12 comparisons $p < 0.05$; Figure 4D-F). Moreover, in knees immobilized for 16 and 32 weeks, knee stiffness was significantly greater after 16 and 32 weeks of remobilization when compared to no remobilization ($p < 0.05$; Figure 4E-F).

Remobilization up to 48 weeks had no effect on knee stiffness in contralateral knees immobilized from 1 to 32 weeks when compared to immobilization only and increased age did not have a significant effect on knee stiffness (all $p > 0.05$; Figure 4).

Knee stiffness: fixed duration of remobilization

With immobilization only, knee stiffness was significantly greater than contralateral and controls beyond 4 weeks of immobilization ($p < 0.05$; Figure 5A). Furthermore, stiffness was significantly greater in knees immobilized beyond 4 weeks when compared to the shortest duration of immobilization (1 week) ($p < 0.05$; groups 8-0, 16-0, and 32-0) (Figure 5A). Despite a fixed remobilization period of 8 or 16 weeks, knee stiffness was still greater than both controls beyond 4 weeks of immobilization ($p < 0.05$; Figure 5B-C). There was a significant increase in knee stiffness as duration of immobilization increased, shown in groups 8-8, 16-8, 16-16, and 32-16, when compared to their respective shortest duration of immobilization (2-8 and 4-16) ($p < 0.05$; Figure 5B-C).

There were no changes in knee stiffness in the contralateral or control knees when duration of immobilization or age increased (all $p > 0.05$; Figure 5A-C).

Correlation between knee stiffness and posterior capsule length

Knee stiffness negatively correlated with posterior capsule length when all immobilization groups were combined ($r = -0.452$; $p < 0.001$). For knees that were immobilized for 2, 4, 8, and 16 weeks, followed by 8 weeks of remobilization, increased knee stiffness negatively correlated with posterior capsule length ($r = -0.395$; $p < 0.05$). Similarly, in knees immobilized for 4, 8, 16, and 32 weeks, followed by 16 weeks of remobilization, the negative correlation between knee stiffness

and posterior capsule length remained significant (r = -0.375; $p < 0.05$). There were no significant correlations between knee stiffness and posterior capsule length in the contralateral knees ($p > 0.05$; Table 1).

Discussion

We report the effects of immobilization and remobilization on knee stiffness in the rat model and the correlation with posterior knee capsule length. This comprehensive study provides novel data on the biomechanical properties of posterior knee tissues with relevance for knee extension deficits. Our results showed that knees immobilized for 1, 2, or 4 weeks were comparable to the controls, but knees immobilized beyond 4 weeks had significantly greater stiffness. These results confirmed our first hypothesis. Despite remobilization, knee stiffness persisted and even progressively increased when durations of immobilization exceeded 4 weeks, contradicting our second hypothesis. As expected, knee stiffness negatively correlated with posterior capsule length; confirming our third hypothesis.

Knee stiffness versus range of motion

The measure of stiffness provides insight on the mechanical properties of biological tissues and their impact on knee function. The duration of immobilization and remobilization had distinct effects on knee stiffness and further contributes to previously published ROM restrictions (Trudel et al., 2014b). Knees immobilized for 1 to 4 weeks showed no statistically significant increase in stiffness despite significantly reduced range of extension when compared to controls (Trudel et al., 2014b). Statistically significant increases in stiffness were detected after 8 weeks of immobilization, much later than the significant ROM loss which was reached after 1 week of

immobilization. The significant increase in knee stiffness after 8 weeks of immobilization corresponded to the moment the loss of ROM in extension plateaued (Trudel et al., 1999, 2014b). The temporal difference of ROM restriction and increase in stiffness may be explained by muscular restrictions associated with short-term immobilization and articular restrictions during long-term immobilization (Trudel et al., 2014a). Muscles are more pliable to stretching than articular connective tissues such as capsule and ligaments (De Deyne, 2001). As a consequence, muscular restrictions during short-term immobilization would restrict ROM but with unaltered stiffness. Whereas changes in articular structures become more prominent during long-term immobilization and contribute to the increased stiffness at the angle of restricted ROM.

The effect of various durations of remobilization on stiffness were compared with the ROM. Knees immobilized for 1 week showed no increase in stiffness and they returned to control ROM after remobilization (Trudel et al., 2014b). Immobilization beyond 4 weeks led to significant increases in stiffness that did not remit despite extensive durations of remobilization. This corresponded to a partial return to normal ROM in knees immobilized for 2, 4, 8, and 16 weeks despite long periods of remobilization (Trudel et al., 2014b). Interestingly, knees immobilized for 16 and 32 weeks continued to accrue stiffness during the remobilization period. After long-term immobilization, loss of ROM and increased stiffness is most severe, potentially limiting rat activity during remobilization. A low level of activity would extend the duration of immobilization, further aggravating knee extension deficits and increasing stiffness. Fibrosis of the capsule represents a possible mechanism of increased stiffness during prolonged knee immobilization (Sasabe et al., 2017). Knee stiffness provides additional and valuable measures regarding the onset and progression of knee extension deficits.

Biomechanical versus clinical stiffness

In the evaluation of knee stiffness, one must differentiate between biomechanical stiffness and clinical stiffness reported by patients. Biomechanical stiffness is physically described as an increased resistance to displacement for a given amount of force applied; it is calculated as the relationship between the change in force applied and the change in displacement produced ($\Delta T/\Delta\theta$). Knees with a deficit in extension will experience stiffness due to a pathological limitation in ROM. Clinically, patients report stiffness as the difficulty in ranging their joints, and a slower active angular velocity in accessing their available joint ROM due to tissue resistance to movement (Halls et al., 2014). Clinical stiffness is spontaneously reported by patients with arthritic joints after immobility, such as the morning stiffness after a night in bed or getting up after prolonged sitting (Hider et al., 2019). It is also a common complaint of patients mobilizing their joints after a long period of immobilization in a cast or a brace (Gravlee and Van Durme, 2007). Clinical stiffness improves with the use of the affected joint. Despite being the second highest complaint of patients with various forms of inflammatory arthritis, we are unaware of a study that attempted to correlate the clinical stiffness reported by patients and the biomechanical stiffness of their joints. Our study establishes the proof of concept that immobilization increases mechanical stiffness in normal joints and therefore may contribute to the symptoms of clinical stiffness reported by patients with immobilized joints or with arthritis. Our results also support the clinical recommendation to limit the duration of joint immobilization, and when this is not possible, to assist joint remobilization after long periods of immobilization.

Pathophysiology of knee stiffness and contribution of the posterior capsule

Mechanical knee stiffness may depend on arthrogenic, myogenic, and cutaneous tissues that contribute to the resistance during knee extension. The arthrogenic structures (bone, cartilage, ligaments, and capsule) provide stiffness without muscular contribution (Roy et al., 2011). The myogenic structures (muscle, fascia, and tendons) can provide both passive and active resistance (Roy et al., 2011). A change in mechanical knee stiffness can indicate alterations or adaptations of the elastic properties of arthrogenic and myogenic structures. During joint immobilization, the soft tissues surrounding the knee joint structurally adapt to meet mechanical demands, which in turn impacts their material properties (Hayashi, 1996). The role of ligaments, tendons, and muscles have commonly been investigated in knee restriction deficits (Amankwah et al., 2006; Hayashi, 1996; Stanev and Moustakas, 2019), but less so the posterior capsule. Historically, Wright and Johns suggested that the stiffness experienced by patients with connective tissue diseases was due to changes in and around the joint capsule (Wright and Johns, 1961).

Research on the effect of joint immobilization identified the posterior knee capsule as a key structure limiting knee ROM in extension (Zhou et al., 2018). Reduction of the posterior knee capsule length was positively correlated with decreased range of extension. Similarly, in the current study, a shortened capsule length was highly correlated with increased knee stiffness. To our knowledge, only one group has investigated the material properties of the posterior knee capsule in human cadavers; Rachmat et al., highlighted the heterogenous regional distribution of material properties in the posterior capsule (Rachmat et al., 2015). The significant inverse correlation we report indicates that the shortened posterior capsule may play an important role in increasing knee stiffness and as well as limiting knee extension after immobilization (Zhou et al., 2018). Elucidating the material properties of the posterior capsule in normal knees and knees with

extension deficits would strengthen our understanding of biomechanical changes that occur in knees with increased stiffness; in cases such as osteoarthritis, TKA, or prolonged durations of joint immobilization.

Limitations

We were unable to quantify the contribution of various individual articular structures to the increased knee stiffness. Given the progressive torque applied, various structures could have sequentially contributed to the stiffness; i.e. initial testing may have disrupted the elastic properties of some structures while continued testing other stressed structures. Successive testing cycles increased angular displacement of the knee in a rat model (Markolf et al., 2016). Future studies could perform mechanical testing after myotomy to isolate stiffness strictly attributed to arthrogenic structures. Our mechanical testing did not consider the contribution of active muscle contraction.

Conclusion

Eight or more weeks of immobilization in flexion significantly increased knee stiffness in extension. Unassisted remobilization failed to reverse the increased stiffness. Moreover, remobilization progressively worsened knee stiffness when the immobilization period was longer than 8 weeks. The increased stiffness in extension correlated with a reduction in length of the posterior knee capsule. Future investigation on the biological changes of the posterior knee capsule and their biomechanical properties is warranted to understand its contribution in limiting knee function and to optimize remobilization. Clinical applications include the remobilization of joints

after injury or fracture and the correlation between clinical and biomechanical stiffness in patients with arthritis.

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Conflict of interest statement

The authors declare no conflicts of interest, financial or otherwise.

Author Contributions

Conception and design of research: LG, OL, and GT. Data analysis: OL and HZ. Interpreted results of experiments: OL, GT, and HZ. Prepared the figures and drafted the article: OL and HZ. Edited and revised the article: LG, OL, GT, and HZ. Approved final version of the article: LG, OL, GT, and HZ.

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Figures and Tables

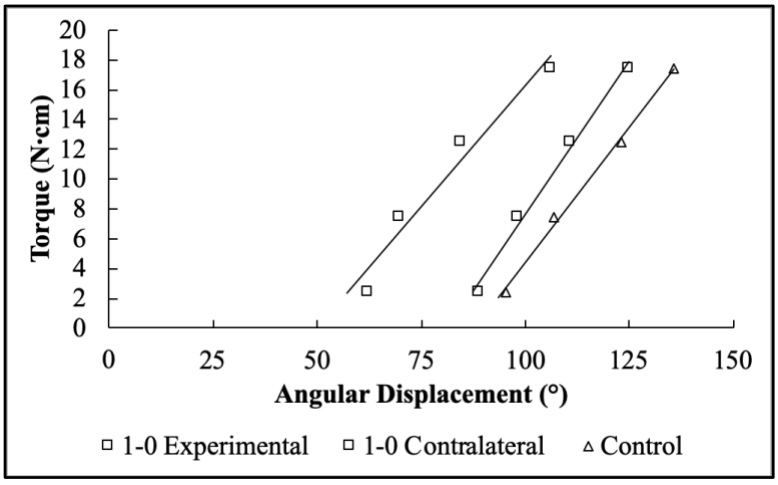

Figure 1. Knee angular displacement in extension at four pre-determined torques. Knee stiffness represented as the median angular displacements of rat knees immobilized for one week at torques 2.5, 7.5, 12.5, and 17.5 N-cm. These are compared to the contralateral and control knees. The slope of the regression line represented the stiffness of the knee in extension. Knees were tested after skin was removed.

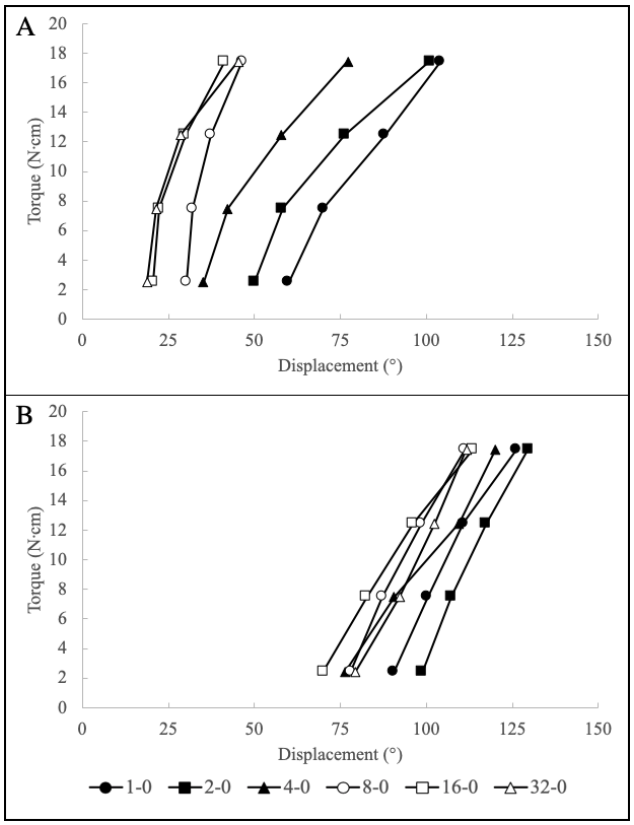

Figure 2. Torque-displacement curves of knee extension after various durations of immobilization. A) The median angular displacements of knees immobilized for 1, 2, 4, 8, 16, and 32 weeks at torques 2.5, 7.5, 12.5, and 17.5 N-cm. B) Contralateral knees.

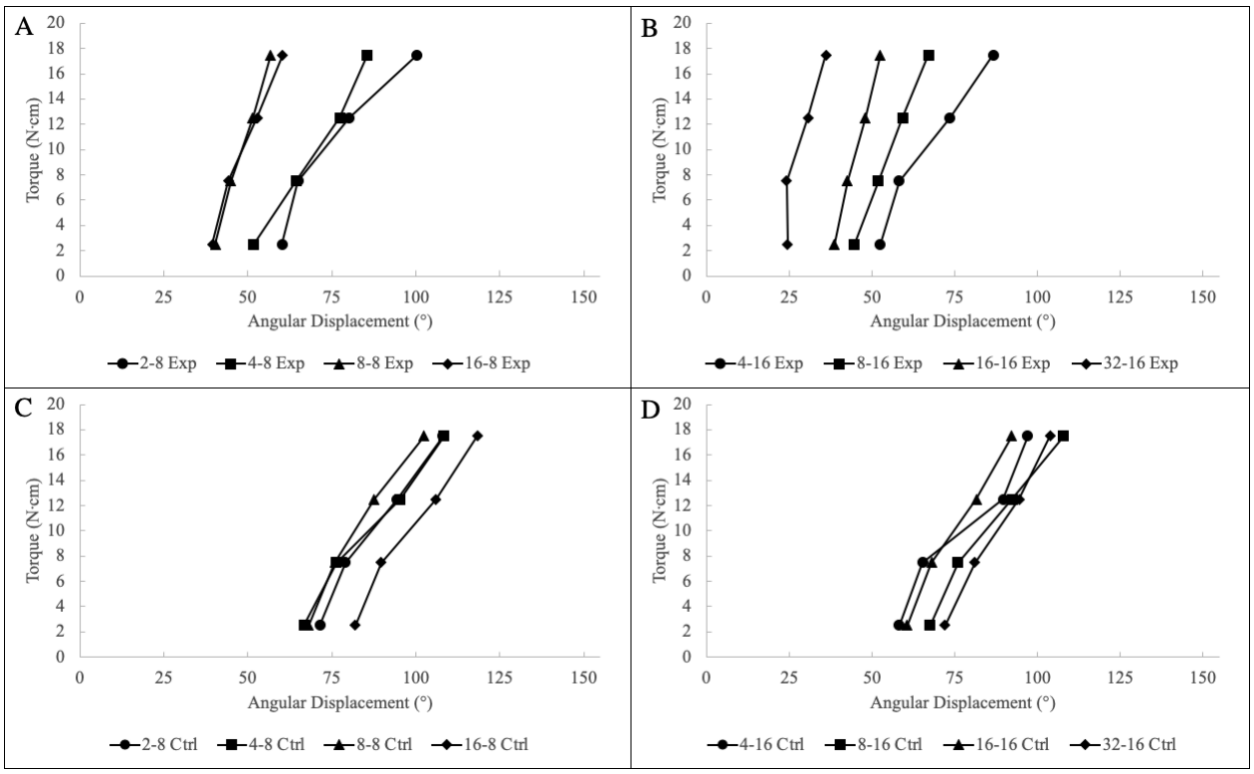

Figure 3. Torque-displacement curves of knee extension after a fixed duration of remobilization for various durations of immobilization. The median angular displacements of knees remobilized for 8 (A and C) and 16 (B and D) weeks at torques 2.5, 7.5, 12.5, and 17.5 N-cm. The durations of immobilization were one-quarter, one-half, one, and two times the duration of immobilization. A-B) Immobilized knees. C-D) Contralateral knees.

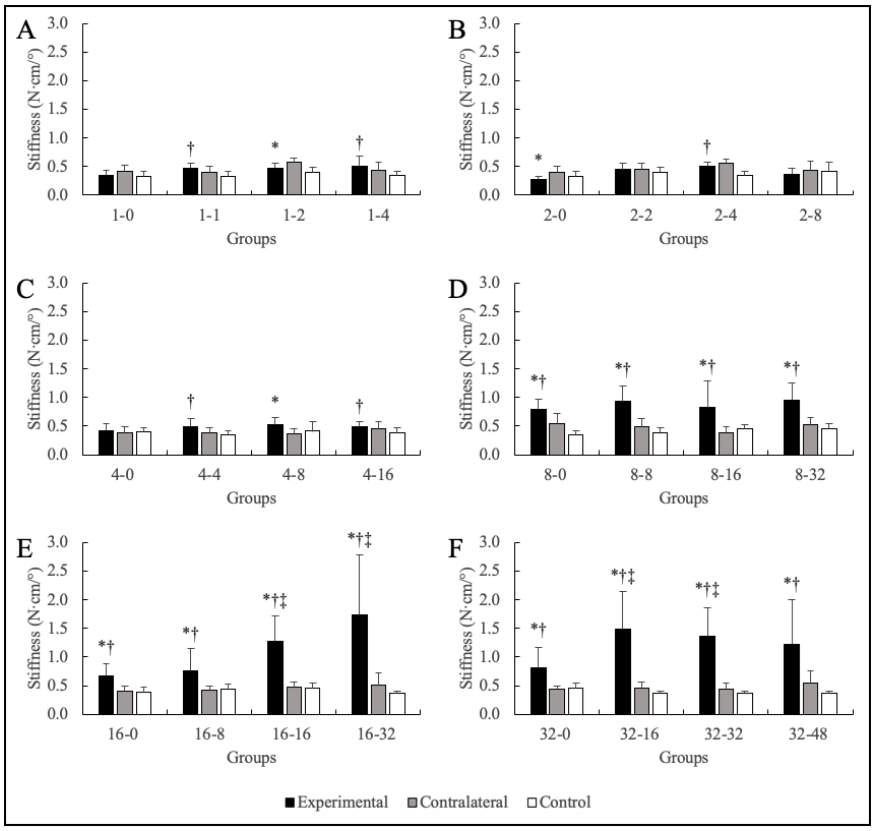

Figure 4. Stiffness in extension of rat knee joints after a fixed duration of immobilization and increasing durations of remobilization. A-D) 1, 2, 4, and 8 of immobilization which were followed by remobilization of zero, one, two, and four times the duration of immobilization. E-F) 16 and 32 weeks of immobilization that was followed by remobilization that was zero, one-half, one and two times the duration of immobilization, with exception to group 32-48 (1.5 times the duration of immobilization). *: Significant difference compared to contralateral knee ($p < 0.05$). †: Significant difference compared to controls ($p < 0.05$). ‡: Significant difference compared to no remobilization ($p < 0.05$).

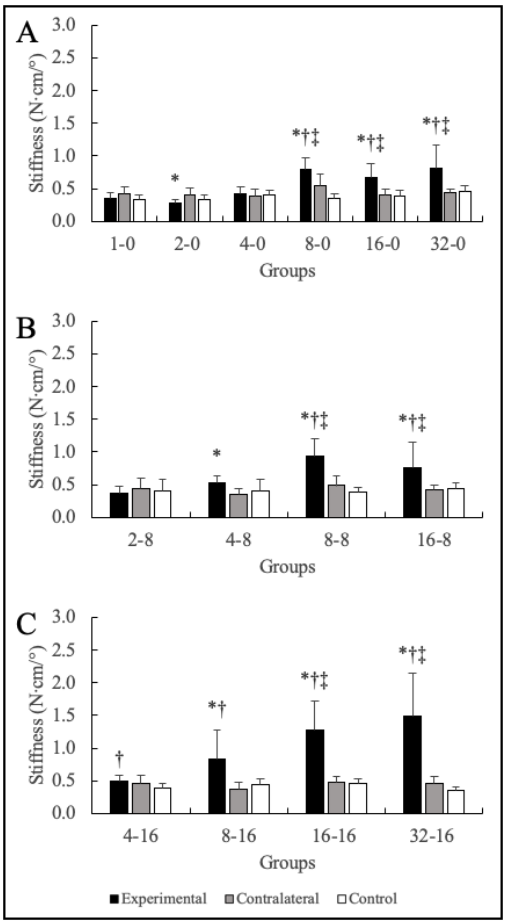

Figure 5. Stiffness in extension of rat knee joint after increasing durations of immobilization and a fixed duration of remobilization. A) No remobilization with immobilization from 1 to 32 weeks. B-C) 8 and 16 weeks of remobilization with immobilization durations that were one-quarter, one-half, one, and two times the duration of remobilization. *: Significant difference compared to contralateral knees ($p < 0.05$). †: Significant difference compared to controls ($p < 0.05$). ‡: Significant difference compared to no remobilization ($p < 0.05$).

Table 1. Pearson correlation analysis between overall knee stiffness measures and total posterior capsule length in the same rat knees. All immobilization groups were pooled for a fixed duration of remobilization. *: ($p < 0.05$).

Comparison	n	Time Remobilized (wks)	Correlation Coefficient (r)	*p* - Value
Contralateral	49	0	0.251	0.081
	35	8	-0.054	0.758
	35	16	-0.151	0.373
Experimental	50	0	-0.452	<0.001*
	32	8	-0.395	0.025*
	36	16	-0.375	0.024*

Chapter 4

Reversibility of marrow adipose accumulation and reduction of trabecular bone in the epiphysis of the proximal tibia

Acta Histochemica

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Keywords: Bone marrow; trabecular bone; marrow adipose tissue; knee immobilization; histomorphometry.

Abstract

Mechanical stimuli play an important role in the homeostasis of trabecular bone and marrow adipose tissue, particularly for the weight-bearing skeleton. Prolonged immobilization and disuse have been shown to reduce trabecular bone content and increase marrow adipose tissue in the bones of lower limb joints such as the knee. However, details on the temporal response of this relationship to prolonged immobilization and its reversibility is limited. Forty rats had one knee immobilized at 45° of flexion for 2, 4, 8, or 16 weeks and subsequently remobilized for 0 or 8 weeks. The contralateral knees were used as controls. Histomorphometric measures of trabecular bone and marrow adipose tissue (MAT) areas were conducted in the epiphysis of the proximal tibia. Knee immobilization for 4, 8, and 16 weeks significantly reduced trabecular bone area by -0.125, -0.139, and -0.161 mm^2/mm^2, respectively, with corresponding 95% CIs of [-0.012, -0.239], [-0.006, -0.273], and [-0.101, -0.221]. MAT area significantly increased at 2 and 16 weeks by +0.008 and +0.027 mm^2/mm^2, respectively, with 95% CIs of [0.014, 0.002] and [0.039, 0.016]. Remobilization for 8 weeks restored trabecular bone area compared to the contralateral knee and the magnitude of change was significantly greater for 8 and 16 weeks of immobilization with effect sizes of 1.69 and 1.86, respectively. The difference in MAT area between immobilized and contralateral knees were eliminated with remobilization. These results characterize the temporal response of trabecular bone and MAT in the epiphysis of the proximal tibia to joint immobilization and remobilization.

Keywords: Bone marrow; trabecular bone; marrow adipose tissue; knee immobilization; histomorphometry.

Introduction

The distal femur and proximal tibia are composed of dense outer cortical bone and porous inner trabecular bone (Crockett et al., 2011). Femur and tibia have the ability to remodel by removing or adding bone in response to changes in body mass, weight-bearing habits, and muscle forces (Burr et al., 2002; Oftadeh et al., 2015). Changes are larger in trabecular bone structure, possibly attributed to its higher turnover and four times greater surface area-to-volume ratio than cortical bone (Oftadeh et al., 2015; Squire et al., 2008). In models of reduced mechanical stimulation, such as bed rest, limb suspension, and spinal cord injury, the absolute trabecular bone losses in the tibial epiphyses predominated (Rittweger et al., 2009, 2006a, 2006b). Adaptation of bone in the epiphyseal compartment to changes in mechanical environment have also been linked to knee extensor forces following spinal cord injury (Rittweger et al., 2006a). Trabecular bone in the tibial epiphysis is particularly sensitive to reduced mechanical stimulation associated with bedrest and joint immobilization as it is typically subject to higher strains during weight-bearing compared to the metaphysis (Rickard et al., 2008; Westerlind et al., 1997).

Trabecular bone encloses hematopoietic tissue and marrow adipose tissue (MAT). As a consequence, a change in trabecular bone content may impact these neighbouring marrow tissues. Strong clinical interest was sparked by associations between higher MAT levels and low bone density during skeletal unloading circumstances (Alexandre and Vico, 2011; Pagnotti and Styner, 2016; Verma et al., 2002). Reduced mechanical stimulation can dysregulate bone marrow tissue homeostasis by enabling adipose generation and bone resorptive pathways (Bikle et al., 2003; Wallace and Cumming, 2000). In contrast, physical interventions and exercises designed to increase bone mass have been shown to reduce MAT (Crockett et al., 2011; Pagnotti and Styner, 2016; Rubin et al., 2002b, 2002a; Srinivasan et al., 2003). *In vitro*, a lack of mechanical stimuli

inhibited the osteoblastic differentiation of mesenchymal stem cells and instead favoured adipogenesis; increased mechanical stimuli had the reciprocal effect (David et al., 2007; Kostenuik et al., 1997; Yen et al., 2009; Zayzafoon et al., 2004). MAT and trabecular bone may have a similar inverse relationship *in vivo,* but this has not been thoroughly investigated during prolonged joint immobilization (Keune et al., 2017; Verma et al., 2002).

Rodent models of skeletal disuse, immobilization, or unloading have produced decreased bone mass (Jian Li et al., 1990; Sessions et al., 1989; Turner and Bell, 1986; Yeh et al., 1993). However, potential reversibility of the concomitant bone losses and trabecular changes are not well documented. The objectives of this study were 1) to investigate the temporal effects of incremental durations of knee immobilization on trabecular bone and MAT areas in the epiphysis of the proximal tibia in rats and 2) to determine whether the changes in trabecular bone and MAT areas were reversible with unassisted remobilization. We hypothesized that in the epiphysis of the proximal tibia 1) the trabecular bone will decrease while adipose tissue will increase in immobilized knees and 2) unassisted remobilization will restore both trabecular bone and MAT areas.

Materials and Methods

Experimental Methods

A description of the knee immobilization surgery and remobilization were previously detailed (Trudel et al., 2014) and approved by the Institutional Animal Care Committee (ME-2461). Range of motion (ROM) data has previously been reported for these knees (Trudel et al., 2014) and this paper reports the changes in trabecular bone and MAT. Forty male Sprague Dawley rats (10-week old, 334g: Charles River Laboratories, St-Constant, Quebec, Canada) had one knee extra-

articularly immobilized at a 45° angle in flexion using a Delrin® plate that spanned the proximal femur and distal tibia (see Supplementary Fig. 1, illustrating the model of immobilization). The side of surgery was alternated. Rats were immobilized for 2, 4, 8, or 16 weeks. Knee immobilization ended upon surgical removal of the plate and screws and was followed by 0 or 8 weeks of unassisted remobilization. Rats had unrestricted activity within their individual cages and a standard rat chow diet was available *ad libitum*. The contralateral knee was used as a control, providing each group with 5 paired knees for analysis. At the end of the remobilization period, the rats were killed by carbon dioxide inhalation and knees were harvested for histological analysis. Groups are defined as week-week, where the first number is the duration of immobilization and the second is the duration of remobilization (e.g., group 2-8 was immobilized for 2 weeks and remobilized for 8 weeks).

Knee Processing and Staining

The knee joints and surrounding tissues were removed *en bloc* and fixed in Bouin's solution for 24 hours. The knees were subsequently decalcified in 10% Tris-EDTA solution for 2 months and embedded in low melting point paraffin. Standardized sections at the medial mid-condylar level were made in the sagittal plane and one section was analyzed per knee. The 7μm sections were histochemically stained with hematoxylin and eosin (H&E) by the Louise Pelletier Histology Core Facility using an automated system (Leica CV 5030 Automatic Stainer) at our institution.

Histomorphometric Analysis

The measurement of trabecular bone area and MAT area was conducted in the epiphysis of the proximal tibia. The mounted sections were examined at a magnification ranging from 3.3X to 33X

on an upright bright field microscope (Olympus BH-2, Tokyo) and histologically analyzed using imaging software ImageJ (NIH, Bethesda, USA). An adjustable grid was superimposed on all images to select the fields of view (FOVs) at 3.3X magnification and placed at equal distances in the anteroposterior axis proximal to the epiphyseal growth plate (Fig. 1A). Trabecular and MAT area were characterized in 6 FOVs that were of equal size (0.30mm^2) at 33X magnification (Fig. 1B).

FOVs were inspected and areas from non-adipocyte cells and acellular interstitial tissue were manually removed using a paintbrush tool. Trabecular bone was manually selected using a freehand selection tool based on pink staining from eosin to isolate bone from the marrow. Trabecular bone area was measured using the 'measure' function (Fig. 1C). To measure MAT area, images were converted to 16-bit images and the 'threshold' function was used to produce a binary image. Residual outliers and noise were removed using the 'despeckle' and 'remove outliers' function. The 'watershed' function was then used to segment individual adipocytes. MAT area was measured using the 'particle analyzer' function in ImageJ to calculate the sum of the adipose tissue area in each FOV (Fig. 1D). The parameters of this function were set to the following: circularity: 0.30-1.00; size (mm^2): 0.00001-infinity.

Calculations

Trabecular bone and MAT area from the 6 FOVs were summated for each knee. The difference in area between the immobilized and contralateral knee were reported and averaged for 5 rats in each group. This area was normalized to provide a value per mm^2.

Statistical Analysis

Statistical analysis was conducted using SPSS version 24.0 (IBM Corp., Armonk, NY). Descriptive statistics for the distribution of trabecular bone and MAT areas are displayed using boxplots. The difference between the immobilized and contralateral knees are reported as means per 5 knees with 95% confidence intervals (CIs) for each group. The effects of immobilization, with and without remobilization, were determined by 95% CIs of the mean difference within groups; a statistically significant difference could be concluded with 95% confidence if the mean difference ± margin of error did not include zero (the null value of the mean difference). The formula for t-statistics was used with degrees of freedom of n – 1 to account for small sample size. To quantify the magnitude of the effect remobilization had on the mean difference in trabecular bone and MAT area, the effect size between immobilized and remobilized groups were calculated using Hedges' *g* method of effect size and reported with 95% CIs.

A Pearson correlation analysis was conducted to determine the strength of the linear relationship between the changes in trabecular bone and MAT area. The changes in area were calculated from pairwise differences between immobilized and contralateral knees. All durations of immobilization were pooled for a fixed duration of remobilization (0 or 8 weeks) and analyzed separately. Values of $P<0.05$ were considered statistically significant.

A one-way ANOVA was conducted to compare the temporal effect of immobilization for each group, followed by a Tukey's *post-hoc* test; the independent variable was duration of immobilization, and the dependent variable was the mean difference of trabecular bone or MAT area. Values of $P<0.05$ were considered statistically significant.

Results

Effect of immobilization on trabecular bone and MAT area.

Descriptive statistics of the trabecular bone and MAT area after immobilization for 2, 4, 8, and 16 weeks are displayed by boxplots in Fig. 2A. The trabecular bone area between immobilized and contralateral knees had mean differences of -0.062, -0.125, -0.139, and -0.161 mm^2/mm^2, respectively for 2, 4, 8, and 16 weeks. Each group had corresponding 95% CIs of [0.001, -0.125], [-0.012, -0.239], [-0.006, -0.273], and [-0.101, -0.221]. The MAT area had mean differences of +0.008, +0.009, +0.012, and +0.027 mm^2/mm^2 with corresponding 95% CIs of [0.014, 0.002], [0.027, -0.009], [0.055, -0.032], and [0.039, 0.016], for the same timepoints, respectively (Fig. 2B). The 95% CIs not including zero for the mean difference indicated that knees immobilized for 4, 8, 16 weeks had significantly less trabecular bone area than contralateral knees, while knees immobilized for 2 and 16 weeks had significantly greater MAT area than the contralateral knee. The mean difference in trabecular bone and MAT area gradually increased with duration of immobilization but did not reach statistical significance ($P>0.05$).

Effect of remobilization after immobilization on trabecular bone and MAT area.

Descriptive statistics of the trabecular bone and MAT area after unassisted remobilization for 8 weeks following immobilization for 2, 4, 8, and 16 weeks are displayed by boxplots in Fig. 3A. The trabecular bone area between immobilized and contralateral knees had mean differences of -0.045, -0.070, 0.016, and -0.053 mm^2/mm^2, respectively. Each group displayed corresponding 95% CIs of [0.033, -0.124], [0.021, -0.161], [0.107, -0.075], [0.030, -0.136]. The MAT area had mean differences of +0.005, +0.029, +0.018, and +0.052 mm^2/mm^2 with corresponding 95% CIs of [0.013, -0.003], [0.076, -0.019], [0.083, -0.046], and [0.104, -2.697E-4], for the same timepoints,

respectively (Figure 3B). All 95% CIs included zero for the mean difference indicating that remobilization reversed the difference in trabecular bone and MAT areas caused by immobilization. The changes in mean differences of trabecular or MAT area with increasing durations of immobilization were not statistically significant ($P>0.05$).

The effect sizes of remobilization on trabecular bone and MAT area are listed in Table 1. Remobilization had a larger effect on trabecular bone than on MAT, particularly after longer durations of immobilization at 8 and 16 weeks, as shown by CIs not including zero (bold) (Table 1). Representative changes of trabecular bone and MAT areas after immobilization and remobilization are illustrated by histology images in Fig. 4.

Relationship between the changes in trabecular bone and MAT area

The strength of the relationship between the change in trabecular bone and MAT area were analyzed. With all durations of immobilization pooled together, there was a significant correlation between trabecular bone area loss and MAT area increase ($r = -0.47$; $P<0.05$). However, after 8 weeks of remobilization, the correlation between the two variables were no longer statistically significant ($r= -0.33$; $P>0.05$).

Discussion

In this study, we report how knee immobilization altered the bone marrow composition in the epiphysis of the proximal tibia. Immobilization created a reciprocal decrease in trabecular bone and increase in MAT area compared to the contralateral tibia. We also show that remobilization abolished and resolved this reciprocal effect. Unassisted remobilization for 8 weeks restored trabecular bone area to a level comparable to the contralateral knee and the size of the effect was

greater after longer durations of immobilization. Although the differences in MAT area between immobilized and contralateral knees were eliminated by remobilization, MAT area continued to progress with ageing in both immobilized and contralateral knees. These results confirmed our hypotheses.

Trabecular bone loss and MAT increment with immobilization

In comparison to visceral and subcutaneous fat depots, where expansion of the compartment is possible, the marrow space is a rigid compartment of finite volume. Therefore, the expansion of marrow soft tissue must occur at the expense of trabecular bone. Within the tibial epiphysis bone marrow, we report a significant reciprocal relationship between the change in trabecular bone (decreased) and MAT (increased) composition. Previous investigators have used models of rodent hindlimb unloading (microgravity or tail suspension) to study trabecular bone and/or MAT (Jee et al., 1983; Keune et al., 2017). The reduction in trabecular bone observed in our study is consistent with both studies and is in agreement with previous rat knee unloading studies (Jian Li et al., 1990; Turner and Bell, 1986), but the increase in MAT was only reported in rats by Jee et al. (Jee et al., 1983). In comparison to our longitudinal study, both of these studies were limited to a single and short duration of unloading (2 weeks (Keune et al., 2017) and 2.5 weeks (Jee et al., 1983)).

Trabecular bone in the tibia epiphysis is highly responsive and challenged in maintaining its structure and function with changes in mechanical environments. Prolonged skeletal disuse has been shown to result in bone loss (Burr et al., 2002; Ozcivici et al., 2010; Swift et al., 2013) and decreases in bone strength depending on skeletal sites (Thomsen et al., 2012). Bone adynamia in response to unloading can result in an increase of catabolic activity and encourage

osteoclastogenesis (Ozcivici et al., 2010). Furthermore, studies on astronauts have revealed bone losses were restricted to weight-bearing bones and more pronounced in the trabeculae, especially of the tibia (Collet et al., 1997; Vico et al., 2002). Our results showed that a model of rigid knee immobilization in flexion for more than 2 weeks significantly reduced trabecular bone area in comparison to the contralateral knee akin to other models of hindlimb unloading. One consideration is that contralateral knees possibly compensated for the immobilized knees during the post-operative period of both surgeries (1- internal fixation and 2- removal of immobilization device). This effect may have transiently accrued mechanical forces on the contralateral knee. But, a general decrease in activity of the rats post-operatively affected both hindlimbs. Consistent with the validity of the model, the difference in trabecular area not only persisted but increased with longer durations of immobilization.

The role of MAT and the interactions between fat, bone, and hematopoietic tissues, the triad of the bone marrow, are actively being researched (Naveiras et al., 2009). Several *in vitro* studies have suggested an inverse relationship between bone and MAT with decreased or increased mechanical stimuli (David et al., 2007; Kostenuik et al., 1997; Yen et al., 2009; Zayzafoon et al., 2004). This effect has been attributed to a differentiation "switch" on a common mesenchymal stem cell (Yen et al., 2009). We report a significantly higher MAT area in the immobilized compared to the contralateral knees after 2 weeks of immobilization which continued to increase with longer immobilization durations. The statistically significant difference observed as early as 2 weeks may be attributed to the potential overcompensation of the contralateral leg shortly after immobilization surgery. An increase in mechanical stimuli through the contralateral knee could have created a greater difference in MAT area between immobilized and contralateral knees. Nonetheless, the increase of MAT area in the immobilized knee is reciprocal to the decreased

trabecular bone area, further supporting the inverse relationship between bone and fat during immobilization. The accumulation of MAT within the confined marrow compartment must result in the reduction of other components within the space, such as trabecular bone or hematopoietic tissue. In contrast, subcutaneous and visceral fat depots are far less restricted in their expansion due to skin extensibility. Altogether, the changes of marrow composition in response to prolonged immobilization emphasizes the adaptation of musculoskeletal tissues to changes in the mechanical environment (Ozcivici et al., 2010).

Unassisted remobilization restored trabecular bone loss and MAT increments caused by immobilization.

Recovery from trabecular bone loss may take much longer than the time required to induce the initial reduction (Vico et al., 2002). The response of bone to immobilization and exercise are not exact opposites; a study in a rat has shown that while bone loss occurred early with immobilization, bone accrual required longer and sustained input (Yeh et al., 1993). Nevertheless, short exercise regimens have produced enhanced resistance to fracture (Warden et al., 2005). Our model provided temporal insight on the natural recovery after knee immobilization is relieved. Remobilization abolished the differences in trabecular bone between immobilized and contralateral knees that were created by incremental durations of immobilization. Mechanical stimuli has been identified as a key determinant for bone mass and morphology, and even low-magnitude mechanical stimulation has been shown to have an anabolic effect on bone (Rubin et al., 2002b). Remobilization also abolished the immobilized to contralateral difference in MAT area caused by immobilization but did not reverse the age-induced accumulation of MAT (Horowitz et al., 2017; Justesen et al., 2001). Future investigation on the morphology and

cellularity of marrow adipocytes during immobilization and subsequent remobilization is necessary to provide a mechanistic understanding on the dynamic changes of MAT accumulation.

To accommodate for the demands of different mechanical environments, changes in bone mass and morphology can occur. The study of resident bone cell populations (osteoblasts, osteoclasts, and osteocytes) in response to mechanically derived signals have provided insight to the cells and molecular mechanisms responsible for the homeostasis of bone remodeling (Ozcivici et al., 2010). The interconnected distribution of osteocytes throughout the bone matrix enables them to sense external mechanical loads and orchestrate the adaptation of bone mass and structure through osteoblast and osteoclast function (Klein-Nulend et al., 2013; Oftadeh et al., 2015). Bone anabolism can be induced by increased mechanical demand, in which osteoblast recruitment and activity is increased to strengthen the bone (Klein-Nulend et al., 2013). Consistent with the reciprocal relationship between trabecular bone and MAT, mechanical signals have also been shown to bias mesenchymal stem cell differentiation towards osteoblastogenesis and away from adipogenesis (David et al., 2007; Rubin et al., 2007). In the context of mechanical stimulation, there is a likely mechanistic relationship between bone and MAT (Pagnotti and Styner, 2016). Despite extended periods of knee immobilization, unassisted remobilization provided sufficient mechanical stimuli for the restoration of marrow composition.

Ground reaction forces and range of motion

The reversibility of trabecular bone loss and MAT area provides novel insight to combat the negative effects of prolonged joint immobilization. Bone loss due to prolonged disuse, as well as exposure to microgravity, has been attributed to hypodynamia and hypokinesia (Vico et al.,

2002). Our investigation addressed the question: is return to transmission of normal ground reaction forces or return to normal ROM more important to restore bone marrow integrity?

We have previously reported in the same rat cohort that immobilization of the knee in flexion resulted in extension deficits that increased the longer the immobilization duration and of irreversible knee extension deficits despite remobilization (Trudel et al., 2014). In the current study, despite irreversible knee extension deficits, the trabecular bone losses were reversed, and the MAT accrual was attenuated with remobilization. Transmission of ground reaction forces through the knee in our model was reduced, but not eliminated during immobilization, as the rat paw still contacted the ground in a closed kinetic chain, allowing for the transmission of forces through the knee joint (see Supplementary Fig. 1, illustrating distribution of ground reaction forces). We did not quantify the load reduction caused by the rigid internal fixation but the transmission of ground-reaction forces through the knee was indicated by preserved articular cartilage in the middle and posterior regions of the tibia, contacted by the femur in knee flexion (Campbell et al., 2018). Restoration of mechanical forces through the knee during remobilization restored bone marrow composition. This suggests that return to transmission of ground reaction forces through the knee played a more important role than return to normal ROM in restoring trabecular bone and MAT in the marrow. The marrow restoration proceeded despite the ROM deficit. These results further reinforce the importance of mechanical stimuli in maintaining trabecular bone structure and also highlight the sensitivity of bone to external forces.

Clinical relevance

Knee immobilization for duration of 2, 4, 8 weeks or longer are most common in sports medicine, orthopaedic, and general clinical practices. The trabecular bone losses point to a period

of bone fragility after immobility. The reciprocal increase in MAT may negatively modulate bone properties and hematopoiesis (Naveiras et al., 2009). The results of this study demonstrate plasticity in the modulation of bone marrow composition. The restoration of bone marrow constituents to contralateral levels in this model is encouraging as to the reversible nature of the significant deleterious effects of immobilization. Even after immobilization for 16 weeks, the proximal tibia epiphyses maintained the potential to return to contralateral levels. Whether various programs of assisted remobilization could accelerate this recovery is an open question.

Limitations

Inherent to histological analysis, we are limited to a 2-dimensional representation and we did not quantify bone or fat volume in 3 dimensions. The entire epiphysis compartment was not assessed, but representative FOVs were positioned across the entire anteroposterior axis. Although the differences in trabecular bone and MAT areas caused by immobilization and remobilization were measured in both legs of the same rats, the longitudinal changes were measured on different groups of rats. This is different from a design where one could longitudinally monitor the changes in marrow composition of the same rats. When calculating differences with contralateral knees, the output included both the immobilized marrow gain/loss and contralateral gain/loss. Lastly, this study was carried out in male rats, which may exclude sex-specific differences.

Conclusion

This longitudinal *in vivo* study measured reciprocal trabecular and MAT changes in the bone marrow, with increasing durations of immobilization. Unassisted remobilization of the knee

restored trabecular bone and MAT to contralateral levels. This study supports an inverse mechanosensitive regulation for trabecular bone and MAT.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

Conception and design of research: OL, GT. Acquisition of data: KA. Data analysis: KA, OL, HZ. Interpreted results of experiments: OL, GT, HZ. Prepared the figures and drafted the article: OL, HZ. Edited and revised the article: OL, GT, HZ. Approved final version of the article: KA, OL, GT, HZ.

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Figures and Tables

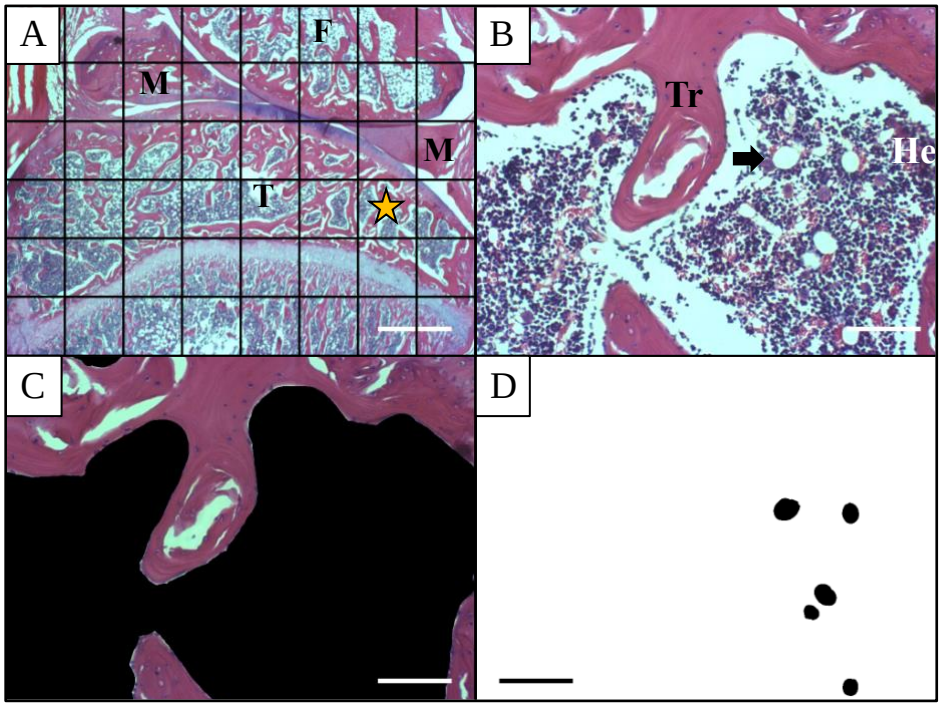

Fig. 1 Method of FOV selection for analysis and output of trabecular bone and MAT area measurements. Micrographs of a sagittal section in the medial mid-condyle of a rat knee immobilized for 2 weeks and remobilized for 8 weeks**. A)** An adjustable grid was superimposed using ImageJ to create equal distances between 6 FOVs. The gold star represents the FOV illustrated in panels B-D. T: Tibia; F: Femur; M: Meniscus; anterior and posterior horns. **B)** Micrograph of the FOV indicated by the gold star in panel A. Arrow points to adipocytes within the bone marrow. Tr: trabecular bone. He: hematopoietic tissue. **C)** Processed image of the FOV measuring the trabecular bone area. **D)** Processed image of the FOV measuring adipose tissue area showing output of the processed image from the particle analyzer function in ImageJ. Adipocytes were masked with black overlays and their combined area was measured. The images were obtained at a magnification of 3.3X (A) and 33X (B-D); scale bar represents 1mm in panel A and 0.1mm in panels B-D.

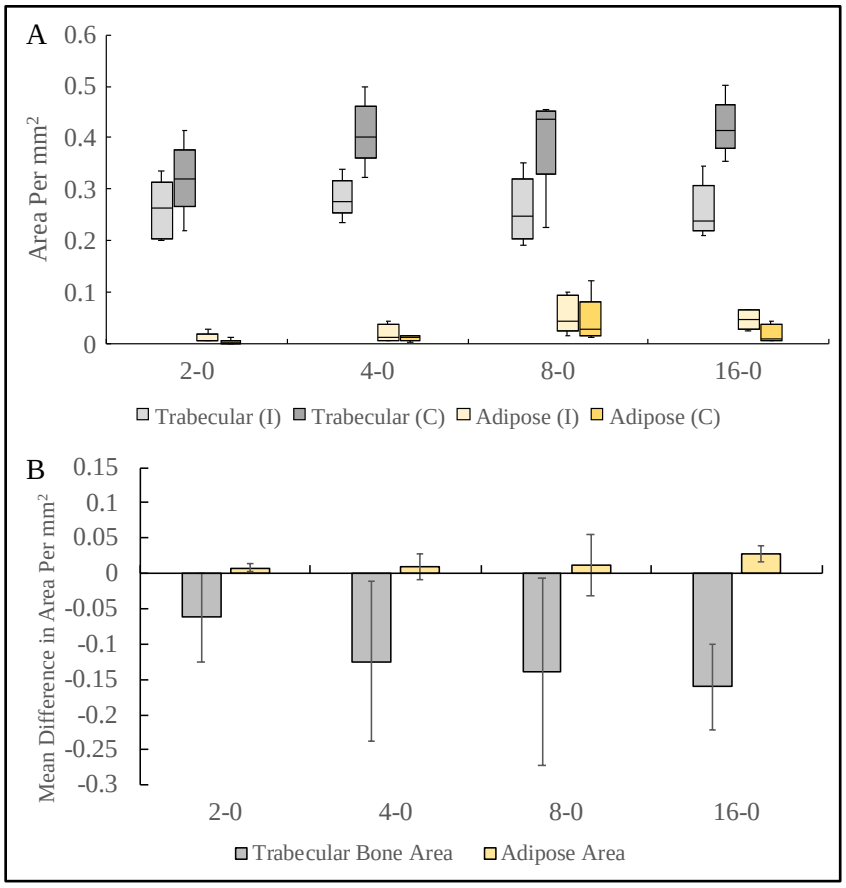

Fig. 2 Distribution of trabecular bone and MAT areas and mean differences between immobilized and contralateral tibiae after unilateral knee immobilization for 2, 4, 8, and 16 weeks. A) Trabecular bone and MAT areas. The median is represented by a horizontal line within each box. I: Immobilized knee. C: Contralateral knee. Each boxplot represents n=5 knees. B) Mean differences in trabecular bone and MAT area between immobilized and contralateral tibiae from the same animal. A negative difference indicates smaller area in the immobilized knee, and a positive difference indicates a larger area in the immobilized knee. A statistically significant difference could be concluded with 95% confidence if the mean difference ± margin of error did not include zero (the null value of the mean difference): trabecular bone area (4-0, 8-0, and 16-0) and MAT area (2-0 and 16-0). Each bar graph represents mean differences between n=5 paired knees. Error bars indicate 95% confidence intervals.

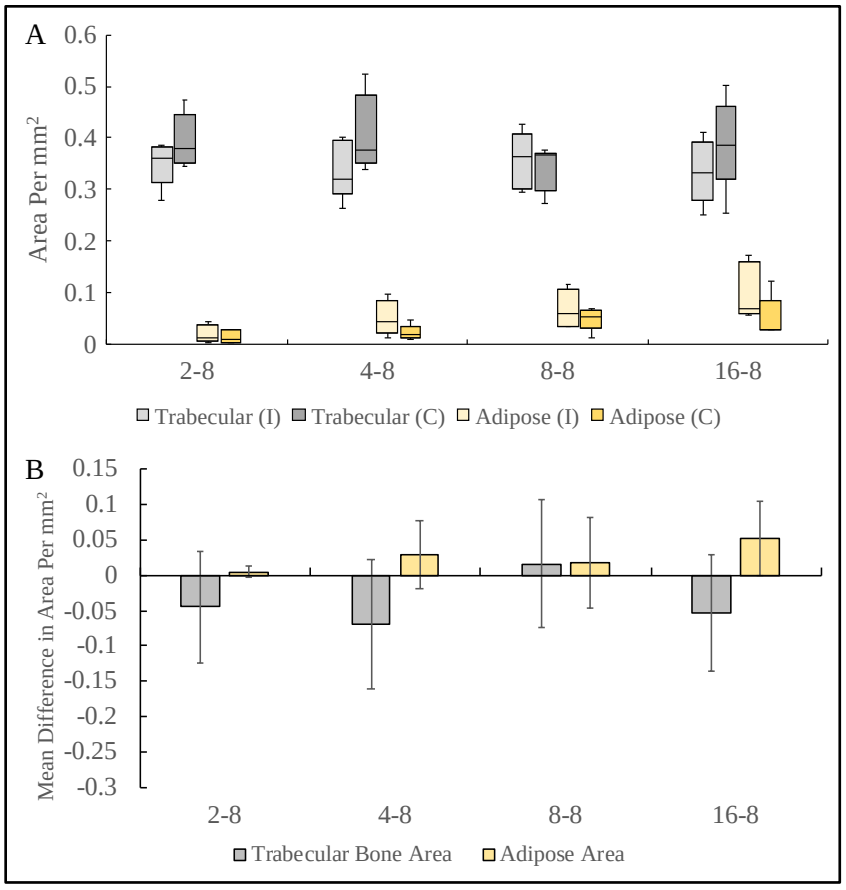

Fig. 3 Distribution of trabecular bone and MAT areas and mean differences between immobilized and contralateral tibiae after unilateral knee immobilization for 2, 4, 8, and 16 weeks followed by 8 weeks of remobilization. A) Trabecular bone and MAT areas. The median is represented by a horizontal line within each box. I: Immobilized knee. C: Contralateral knee. Each boxplot represents n=5 knees. B) Mean differences in trabecular bone and MAT area between immobilized and contralateral tibiae from the same animal. A negative difference indicates lower area in the immobilized knee, and a positive difference indicates more area in the immobilized knee. A statistically significant difference could be concluded with 95% confidence if the mean difference ± margin of error did not include zero (the null value of the mean difference). Each bar graph represents mean differences between n=5 paired knees. Error bars indicate 95% confidence intervals.

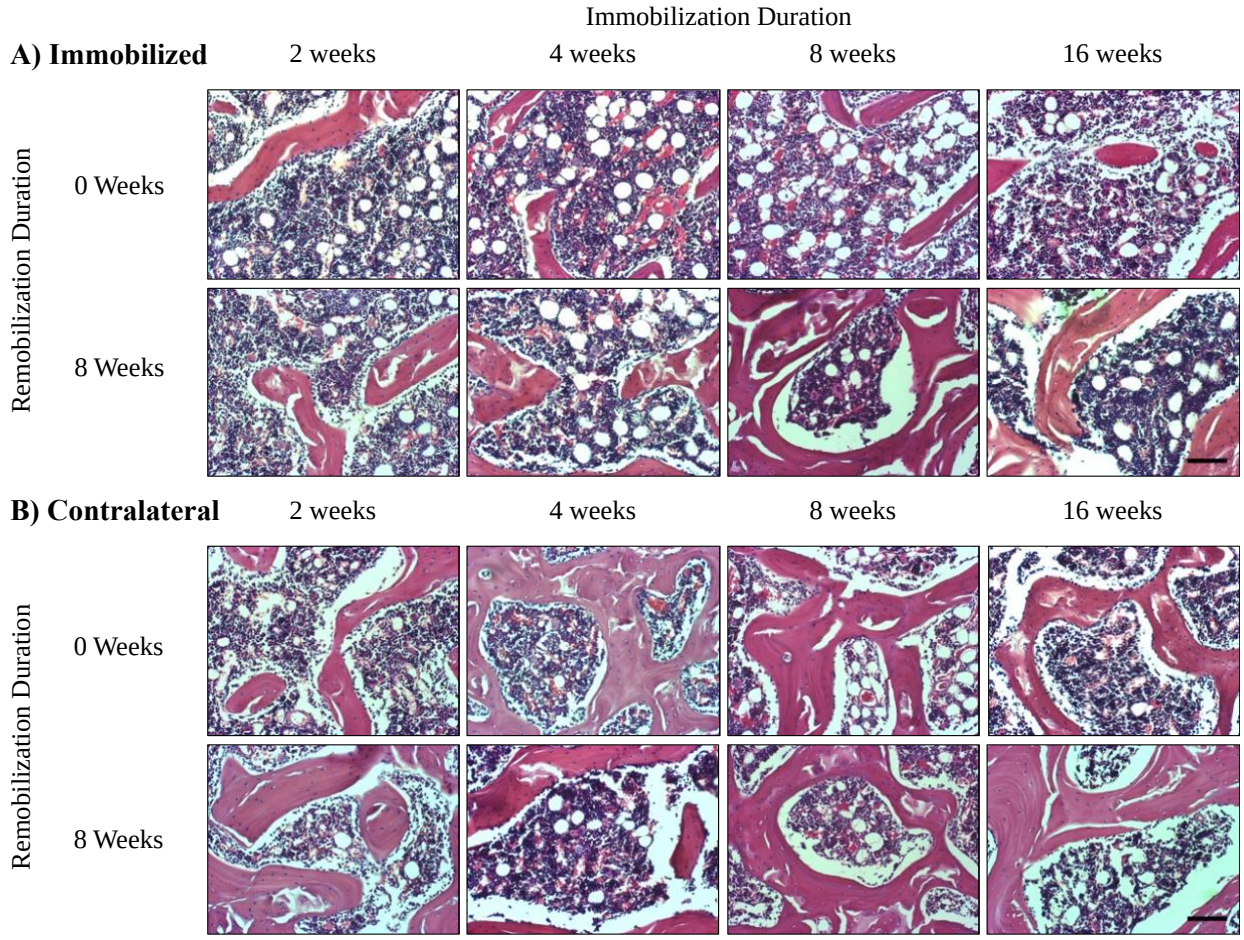

Fig. 4 Effect of immobilization and remobilization on the trabecular bone and adipose tissue areas in the proximal epiphysis of the rat tibia versus contralateral. Micrographs of individual FOVs at 33X magnification in the proximal tibia epiphysis of **A)** Immobilized and **B)** Contralateral knees after 2, 4, 8, and 16 weeks of immobilization, followed by 0 or 8 weeks of remobilization. Scale bar represents 0.1mm.

Table 1. Effect size of remobilization for 8 weeks on the mean difference between immobilized and contralateral knees in trabecular bone and MAT area. Comparisons were made between immobilization-only (n=5) and remobilization groups (n=5) for each respective duration of immobilization.

Immobilization (weeks)	Remobilization (weeks)	Trabecular Effect Size	95% CI	MAT Effect Size	95% CI
2	8	0.29	[-0.955, 1.537]	-0.57	[-1.833, 0.696]
4	8	0.66	[-0.609, 1.938]	0.67	[-0.605, 1.943]
8	8	1.69	**[0.246, 3.134]**	0.15	[-1.093, 1.389]
16	8	1.86	**[0.375, 3.341]**	0.81	[-0.480, 2.099]

Chapter 5

Hyperplasia and accelerated hypertrophy of marrow adipocytes with knee immobilization were sustained despite remobilization

Journal of Applied Physiology

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Keywords: Adipocyte; adipogenesis; marrow adipose tissue; joint immobilization; histomorphometry.

Abstract

Skeletal disuse can cause an accumulation of bone marrow adipose tissue (MAT) characterized by a combination of marrow adipocyte hyperplasia and/or hypertrophy. The malleability of MAT accumulation and of the hyperplasia and hypertrophy upon remobilization is unknown. In this study, we showed extensive hyperplasia and accelerated hypertrophy of bone marrow adipocytes in the proximal tibia epiphysis of rat knees immobilized for durations between 1 and 32 weeks. Similar histomorphometric measures of adipocytes carried out in unoperated controls allowed distinguishing the effects of immobilization from the effects of aging. While both knee immobilization and aging led to adipocyte hypertrophy, adipocyte hyperplasia was the hallmark signature effect of immobilization on MAT. Both bone marrow adipocyte hyperplasia and hypertrophy were sustained despite knee remobilization for durations up to 4 times the duration of immobilization. These results suggest that adipocyte hyperplasia is the predominant mechanism explaining MAT accumulation in skeletal disuse. In this model, the changes were unremitting for the investigated time points. Investigating the cellular and molecular mechanisms of marrow adipocyte mechanoregulation will be important to better understand how adipocytes adapt to changes in mechanical environments.

Keywords: Adipocyte; adipogenesis; marrow adipose tissue; joint immobilization; histomorphometry.

New & Noteworthy

This longitudinal study elucidates the response of marrow adipose tissue adipocytes in weight-bearing joints to changes in different mechanical environments and we provide insight on the malleability of the changes over time. In a rat animal model, knee immobilization induced hyperplasia and accelerated the age-dependent hypertrophy of adipocytes. Changes in adipocyte number and size were sustained despite unassisted remobilization. Multimodal distributions of cell size were characteristic of bone marrow adipocytes.

1. Introduction

Adipose tissue is widely distributed throughout the body and commonly categorized based on the localization of the fat depot; subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), inter/intramuscular adipose tissue, and marrow adipose tissue (MAT) (31). Adipocytes are described according to their morphology; and alterations in cell sizes have been associated with metabolic dysfunctions and impaired cell function (28). To that end, adipocyte morphology has been investigated in SAT and VAT depots, but similar analyses for MAT are limited, possibly because of its relative inaccessibility. Currently, there is a knowledge gap regarding adipocytes in MAT and their morphological changes in response to physiological or pathological situations (15). The physiological role of MAT is still being uncovered and increases in MAT have been associated with altered hematopoiesis, low bone density, and hibernation (4, 11, 19, 21).

MAT accumulation has been described in conditions of reduced mechanical stimuli such as during prolonged disuse or exposure to microgravity (17, 37). In contrast, exercise in mouse models suppressed MAT accumulation induced by high-fat diets (30) and exposure to high-frequency low magnitude mechanical signals suppressed adipogenesis (25). Mechanical stimuli play an important role in the regulation of musculoskeletal tissue, but whether a reduction in mechanical stimuli encourages a specific morphological phenotype of MAT is unclear (21). Furthermore, knowledge on the malleability of marrow adiposity after prolonged skeletal disuse remains limited even though it is a common occurrence in patients after orthopedic injuries or prolonged stays in hospital (17), and is observed in nature for hibernating animals (4). Investigating the malleability of marrow adiposity is pertinent for further understanding the biology of adipose tissue and its response to changes in the mechanical environment.

MAT accumulation can result from hyperplasia (an increase in adipocyte number) and/or hypertrophy (an increase in adipocyte size) (31). Current technologies now enable quantification of MAT in the bone marrow, but the characterization of individual adipocytes *in vivo* have been limited to a few studies (21, 35). Imaging methods such as MRI are reliable for the relative fatty acid signal quantification in the bone marrow, but cannot inform on adipocyte number or size (29). Thus, histomorphometric analyses are necessary to assess changes in adipocyte morphology. We have previously quantified the changes of adipocyte size and number induced by knee immobilization (for durations ranging from 2 to 16 weeks) in the epiphysis of the proximal tibia in a rat model (35). In comparison to sham-operated controls, a short duration of immobilization (2 weeks) showed a hyperplasia of adipocytes, whereas a longer duration of immobilization (16 weeks) showed hypertrophy. In the current study, we investigated the morphology of adipocytes in the proximal tibia epiphysis in response to various durations of knee immobilization ranging from 1 to 32 weeks and as a natural progression with age. Additionally, we investigated the malleability of the changes in adipocyte size and number after remobilization for periods up to 4 times the duration of immobilization. We hypothesized that 1) knee immobilization over time will accelerate the effect of aging on marrow adipocytes through hyperplasia and hypertrophy and 2) knee remobilization will suppress the accumulation of MAT.

2. Materials and Methods

2.1. *Experimental methods*

The experimental model and study design for this project was previously described (32) and approved by the Institutional Animal Care Committee (ME-2461) (Ottawa, Ontario, Canada). In this study, 145 male Sprague Dawley rats (10 weeks old, 350g) were used for which 145 knees

were analyzed. Rats were housed at room temperature with consistent light/dark cycles to maintain consistent diurnal rhythms and had access to a standard rat chow diet and water *ad libitum*. A Delrin® plate (DuPont Engineering Polymers, Wilmington, DE, USA) spanning the proximal femur and distal tibia was secured with screws and used as an internal fixation device to rigidly immobilize the knee joint in flexion at a 45° angle for various durations: 1, 2, 4, 8, 16 or 32 weeks. The surgical fixation alternated between knees. Following immobilization, the plate was surgically removed, and the rats were allowed unrestricted movement within their cage for various durations of unassisted remobilization (simply referred to as remobilization): zero, one, two or four times the immobilization duration. The exceptions to this rule were for the 16-week immobilization group (0, 8, 16 and 32 weeks of remobilization) and for the 32-week immobilization group (0, 16, 32 and 48 weeks of remobilization). Experimental groups were identified by immobilization-remobilization, expressed in weeks. Following remobilization, rats were sacrificed by carbon dioxide inhalation, the passive range of motion in extension of each knee was measured using an automated arthrometer, and the results were reported in a previous publication (32). Each experimental group had a sample size of n=5 knees. Contralateral knees were excluded from analysis due to compensation and potentially increased mechanical stimuli through the contralateral leg. To minimize the number of animals required and to isolate the effect of aging, 11 unoperated rat groups (n=5 per group) with ages ranging from 10-86 weeks were used instead of sham-operated controls. Knees from unoperated rats were harvested at an age approximate to the experimental rats at the end of the remobilization period and were used as the control group (Table 1). For longer durations of immobilization, the age of the control group could differ up to 4 weeks in comparison to experimental groups; approximate ages were paired with multiple experimental groups to minimize redundancy and unnecessary use of additional animals.

2.2. *Tissue processing and staining*

Adipocytes in the bone marrow of the proximal tibia epiphysis were analyzed by histomorphometry. Knee joints were harvested *en bloc,* fixed in Bouin's solution (Sigma Aldrich Canada, Oakville, Ontario, Canada) for 24 hours, decalcified in 10% Tris-ethylenediaminetetraacetic acid solution 0.49M at pH 7.0 (Fisher Scientific Limited, Ottawa, Ontario, Canada) for 2 months, and embedded in low melting point paraffin (Oxford Labware, St. Louis, MO, USA) (34). Standardized 7µm sections at the mid-medial condylar level were cut in the sagittal plane. Tissue sections were stained with hematoxylin & eosin (H&E) by an automated system from the Histology Core Facility at our institution (Ottawa, Ontario, Canada).

2.3. *Histomorphometric analysis*

Sections were visualized by light microscopy with an Olympus BH-2 (Tokyo, Japan) microscope at low magnification (3X) and captured using a Marlin F080C digital camera (Allied Vision Technologies) with AVT Smartview 1.5.1 software. Images were processed using ImageJ (NIH, Bethesda, MD) and the user was blinded to groups. A standardized method of measurement was applied to individual knee sections by positioning 6 fields of view (FOVs) of equal and fixed ($0.3mm^2$) areas every ~15% of the length in an anteroposterior line across the epiphysis. Individual FOVs were captured at 33X magnification. The paintbrush tool was used to manually remove non-adipocyte cells and acellular interstitial tissue. For all sample analyzed, the image type was converted to a 16-bit binary image, and noise was removed with the Despeckle and Remove outliers' functions. Watershed function was also applied in order to dissociate individual adipocytes. To count and measure adipocyte area, the Particle Analyzer function was used:

parameters were set as following: Size = 10 – Infinity, Circularity = 0.3 – 1. All outputted data was recorded in an Excel document.

2.4. *Calculations*

To measure adipocyte hyperplasia, the number of adipocytes in each of the 6 FOVs for one knee were summated and the average number was calculated from all knees per timepoint. Data was normalized to provide a per mm^2 value of total tissue area. As for hypertrophy, the average cross-sectional area (CSA) of individual adipocytes was taken across all 30 FOVs in a timepoint (6 FOVs/knee). Incomplete adipocytes positioned at the borders of FOVs were considered for adipocyte number but not for CSA and frequency distribution graphs.

2.5. *Statistical analysis*

Adipocyte number and CSA for all knees of each group were reported as the mean ± SD. Non-parametric Kruskal-Wallis tests were performed to assess the effect of time on adipocyte number and individual adipocyte CSA for both the experimental and control groups independently. The earliest timepoint served as the comparator for all subsequent timepoints. P values < 0.05 were considered statistically significant. Descriptive statistics with frequency distribution graphs of the CSA for individual adipocytes were created to demonstrate the spread of adipocyte size within each group with bin sizes of 135.6 μm^2. Statistical analysis of all data was completed with IBM's SPSS version 24. Effect size calculations were completed using Hedges' g coefficient which was calculated as the difference in means between the experimental and control group divided by a pooled standard deviation. Effect size was calculated to quantify the difference between averages

for the experimental and the control groups in the immobilization-only timepoints for both adipocyte number and CSA.

3. Results

3.1. *Effect of Knee Immobilization on Marrow Adipocyte Number and Size*

The effect of increasing durations of knee immobilization on the number and CSA of adipocytes located in the epiphysis of the proximal tibia is shown in Fig. 1. Corresponding unoperated controls to experimental groups are listed in Table 1. A statistically significant increase in the number of adipocytes was observed after 8 and 32 weeks of immobilization in comparison to 1 week of immobilization (Fig. 1A; $P<0.05$). After 16 weeks, the hyperplasia did not reach statistical significance compared to 1 week of immobilization ($P=0.052$). The effect of aging on adipocyte hyperplasia was assessed in the unoperated controls (10-40 weeks old) and no difference was observed at any time point in comparison to the 10-week old group. (Fig. 1A; $P>0.05$). Knee immobilization in flexion also impacted the size of marrow adipocytes. The CSA of adipocytes in the proximal tibia epiphysis significantly increased after 4, 8, 16, and 32 weeks of immobilization compared to 1-week of immobilization (all $P<0.05$). Aging alone increased the size of marrow adipocytes and CSA was significantly higher in control rats aged 18 weeks or older (corresponding to 8, 16, and 32 weeks of immobilization) compared to 10-week old rats (corresponding to 1 week of immobilization) (Fig. 1B; $P<0.05$). Thus, knee immobilization for 4 weeks accelerated the age-dependent hypertrophy of adipocytes.

The strength of the association between knee immobilization and marrow adipocyte hyperplasia and hypertrophy was evaluated with effect size calculations which compared

standardized mean differences between immobilized and control knees. Hedges' g values for adipocyte number and size calculated at the different time points are presented in Table 2. Larger effect size for adipocyte number compared to size indicated that knee immobilization caused MAT accumulation through adipocyte hyperplasia more than hypertrophy.

The effect of duration of immobilization on adipocyte hyperplasia and hypertrophy was displayed using frequency distribution graphs along with representative histological images after immobilization for 1 and 32 weeks (Fig. 2). One week of knee immobilization produced more adipocytes of all sizes compared to controls (Fig. 2A). Thirty-two weeks of immobilization also produced markedly more adipocytes of all sizes compared to control knees (Fig. 2B). The larger number of adipocytes of all sizes caused by immobilization held true despite older rats displaying a much broader spectrum of adipocyte size including very large adipocytes in the marrow of the proximal tibia epiphysis (Fig. 2B).

3.2. *Malleability of Adipocyte Hyperplasia and Hypertrophy by Remobilization*

Rat groups where knee immobilization caused significant increases in adipocyte number (groups 8-0, 16-0, and 32-0) and size (group 4-0) from Fig. 1 were further studied for the potential effect of remobilization and results are presented in Figs. 3 and 4, respectively. Regardless of the duration of immobilization, the effects on adipocyte hyperplasia were sustained, despite remobilization (Fig. 3A-C; $P>0.05$). Similarly, number of adipocytes was not significantly different among the controls despite their advanced age ($P>0.05$). Malleability of adipocyte hypertrophy with remobilization after 4 weeks of immobilization was studied (Fig. 4). Four weeks of remobilization produced a significant reduction in mean adipocyte size followed by significant increases after 8 and 16 weeks of remobilization compared to 4-0 (Fig. 4; all 3 $P<0.05$). The age-

dependent increase in adipocyte size in controls continued during remobilization (Fig. 4; all 3 $P<0.05$).

Malleability of adipocyte hypertrophy after 4 weeks of immobilization was displayed using frequency distribution graphs (Fig. 5). After 4 weeks of remobilization, there were more smaller adipocytes in the proximal tibia compared to the control group (Fig. 5B). As the duration of the remobilization increased, the tibia of the rat knees that were immobilized contained more adipocytes of all sizes compared to controls (Fig. 5C, D).

4. Discussion

Accumulation of MAT in states where mechanical stimuli is reduced, such as during prolonged disuse, have previously been reported (17). However, the morphological adipocyte changes underpinning MAT accumulation are unclear. In addition, the malleability of these morphological changes after immobilization had not, to our knowledge, been investigated. In the current study, we determined that adipocyte hyperplasia was the hallmark of MAT accumulation after knee joint immobilization for 8 and 32 weeks, which supported the first hypothesis. Moreover, knee joint immobilization for 4 weeks accelerated the age-dependent MAT adipocyte hypertrophy. The effects of immobilization were unremitting as remobilization for extended durations did not alter these changes, showing that remobilization does not overcome the accumulation of MAT.

4.1. Impact of mechanical environment on adipocyte hyperplasia/hypertrophy

The bone marrow contains a combination of hematopoietic red marrow and non-hematopoietic yellow marrow into bony limits. This microenvironment experiences an age-dependent conversion from red to yellow marrow where yellow marrow eventually predominates

(7, 24). Lack of mechanical input has been shown to accelerate this red to yellow conversion by driving MAT accumulation (17, 33, 37). The epiphysis of the proximal tibia served as a valid model to study the effects of skeletal disuse on marrow adipocyte morphology since it was shown to be sensitive to mechanical environments such as treadmill running, microgravity, and unloading (36). Given the location of MAT within bone, it may be influenced by the mechanical forces exerted on the skeleton. Ground-reaction forces transmitted to the tibia during weight-bearing can help maintain bone marrow homeostasis through cross-talks between osteoblastogenic and adipogenic differentiation pathways (18, 22). In this study, we showed that a change in the knee joint mechanical environment through prolonged knee joint immobilization promoted adipocyte hyperplasia and accelerated adipocyte hypertrophy, in comparison to controls over time. The effect size for hyperplasia was much larger than for hypertrophy (Table 2). Notably, hyperplasia was uniquely featured in immobilization groups. Aging alone increases MAT content (7, 11, 12, 24). In this study, aging of 10-week-old rats for up to 40 weeks (in rats that typically have a lifespan of 2-3 years) had no significant effect on the number of adipocytes. Adipocyte hypertrophy was initially accelerated by immobilization but became a feature shared by both immobilization and control groups with aging.

Tandon et al. (2018) suggested that the statistical treatment of adipocyte morphology using parametric statistics may inaccurately represent morphometric changes of adipocyte size in the fat depots (31). Numerous studies in SAT and VAT have described bi-modal and tri-modal distributions of adipocyte size (5, 28, 38). Examining the distribution of adipocytes according to their size allows for more representative insights into the changes occurring in fat depots than just averages. In the current study, comparison of adipocyte frequency distribution after different durations of immobilization identified that the distribution of adipocytes broadened with time in

both control and experimental knees and included adipocytes of very large sizes (Fig. 2). However, the increased frequency of adipocytes was most striking in the immobilized group. For most size bins, the proximal tibia of animals whose knees were immobilized for 32 weeks showed a 3 to 4-fold increase in the number of adipocytes. The comparison with control animals also allowed attributing adipocyte hyperplasia to the experimental condition and distinguishing it from an effect of aging only. These results identified adipocyte hyperplasia as a hallmark feature of MAT accumulation secondary to joint immobilization.

4.2. *Malleability of adipocyte hyperplasia/hypertrophy*

The anabolic response of bone and muscle mass to mechanical stimuli and the parallel suppression of MAT have been reviewed (20, 22). The ability of mechanical stimuli to inhibit adipogenesis was supported by numerous *in vitro* studies (3, 26, 39). Previous research in a mouse model has demonstrated the systems-interdependent benefit of mechanical stimuli for inhibiting adipogenesis and its anabolic potential for bone and muscle (25). Exercise in mice suppressed the accelerated accumulation of MAT induced by high fat diets (30) and resulted in a decrease in adipocyte number and size (29). However, it remained unclear whether hyperplasia/hypertrophy of marrow adipocytes induced by prolonged immobilization was malleable by remobilization. In the current study, despite durations of remobilization up to 4 times the duration of knee immobilization, no significant alterations in adipocyte number or size was measured when compared to immobilization only. These data suggested that remobilization was ineffective in affecting the adipocyte hyperplasia induced by prolonged immobilization. Our study described the natural history of remobilization after immobilization with no active regimen of remobilization.

Studies using high impact loading regimens and exercise training were effective in reducing marrow adiposity (23, 27) and suggest the next logical step for future experiments.

The frequency distribution graphs provided interesting insights into adipocyte size upon remobilization after immobilization and the case of animals with knees immobilized for 4 weeks was reviewed. After 4 weeks of immobilization, there were more adipocytes of all sizes compared to controls (Fig. 5A). After 4 weeks of remobilization, only smaller adipocytes were in excess numbers compared to controls (Fig. 5B), which decreased the mean size of adipocytes compared to controls (Fig. 4). However, after 8, then 16 weeks of remobilization, there were more adipocytes of all sizes including larger adipocytes in the experimental groups. This pattern may represent a transient effect of remobilization to reverse the hypertrophy of adipocytes, but eventually aging of all adipocytes occurred. The mechanical stimulus of knee remobilization may have led to a transient inhibition of adipocyte hypertrophy.

4.3. *Regulating adipocyte number and size*

The cellular and molecular mechanisms regulating marrow adipocytes *in vivo* are unknown (11), but the cellular mechanosensation was reported to largely contribute to the composition of the bone marrow and to impact adipocyte differentiation (21, 22, 26, 39). Marrow adiposity may involve a competitive balance between osteoblast and adipocyte differentiation (1), where mechanical signals dictate whether adipogenesis or osteoblastogenesis is favoured (25, 39). The current data suggested that knee immobilization reduced mechanical stimuli in the tibia sufficiently to encourage the differentiation of adipocytes within the marrow space, but this required an extended period of immobilization (8 weeks). Although this is the proposed mechanism, the methods used cannot distinguish between hyperplasia of adipocytes resulting from

increased proliferation of adipocyte precursor cells, and increased differentiation of precursor cells into the adipogenic lineage; both of which are regulated by distinct signalling pathways and mediators (8). In contrast, knowledge on mechanisms regulating adipocyte size, or even cells in general, is far more limited (14, 31). Hypertrophy of marrow adipocytes has previously been reported with age (6, 9, 16). In the current study, the hypertrophy of adipocytes with time in both immobilized and control groups are consistent with these previous findings. Zoncu et al. (2011) suggested that defects in growth control and cell size homeostasis are more commonly associated with aging (40). However, the accelerated increase in adipocyte size after 4 weeks of immobilization and its transient inhibition after 4 weeks of remobilization supported that transduction of mechanical stimuli, at least in part, regulated the hypertrophic capacity of marrow adipocytes (14, 31). The regulation of MAT adipocyte differentiation, proliferation, and size is highly complex, but the current findings clearly demonstrate that mechanical stimuli play an important role with a predominant effect on hyperplasia.

4.4. *Implications of MAT accumulation*

MAT has various metabolic and thermogenic roles along with its interactions with bone (10). MAT accumulation can increase bone resorption, interfere with bone repair, disrupt the differentiation for mesenchymal and hematopoietic stem cells, suppress hematopoietic activity and weaken immune and regenerative responses (2, 19, 22). Changes in the morphology of adipocytes in SAT and VAT depots have been implicated in various metabolic disorders such as insulin resistance, obesity, and cardiovascular diseases (13, 28, 31). Whether the changes in morphology of MAT adipocytes we described in this study are responsible for the metabolic alterations attributed to MAT remains to be elucidated. In the current study, the frequency distribution graphs

showed a multimodal distribution with more adipocytes of smaller size. In metabolic disorders such as obesity, bi-modal distributions of sub-cutaneous adipocytes have commonly been reported (31), which we did not observe after knee immobilization in the bone marrow. The frequency distribution of adipocyte size in MAT in the current study is consistent with our previous work (35) and suggested a different profile of MAT adipocyte sizes compared to SAT and VAT depots.

5. Conclusion

Rat knee immobilization for up to 32 weeks caused MAT adipocyte hyperplasia and accelerated age-dependent hypertrophy, the former being the most important contributor to MAT accumulation. In this model, these changes were unremitting for the investigated time points; remobilization of the knee joint for long durations after immobilization did not alter the changes in MAT adipocytes. These findings identified adipocyte hyperplasia as the hallmark feature explaining MAT accumulation after joint immobilization. Investigating the cellular and molecular mechanisms of marrow adipocyte mechanoregulation will be important to better understand how adipocytes adapt to changes in mechanical environments.

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Competing Interests

No competing interests declared.

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Author Contributions

Conception and design of research: OL, GT. Acquisition of data: KA. Data analysis: OL, JT, HZ. Interpreted results of experiments: KA, OL, GT, JT, HZ. Prepared the figures and drafted the article: OL, JT, HZ. Edited and revised the article: KA, OL, GT, JT, HZ. Approved final version of the article: KA, OL, GT, JT, HZ.

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Figures and Tables

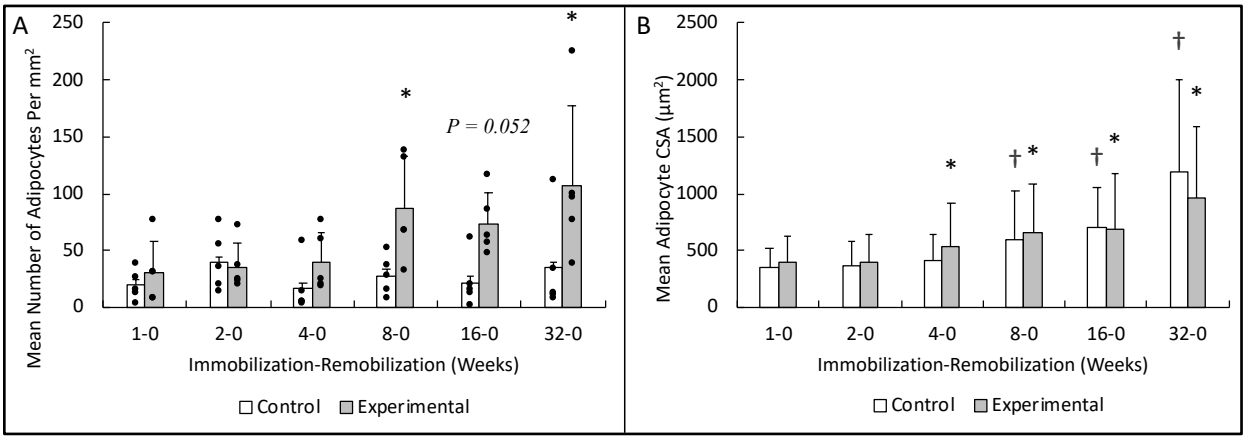

Figure 1. Knee immobilization induced adipocyte hyperplasia and accelerated adipocyte hypertrophy in the epiphysis of the proximal tibia. (A, B) One knee was immobilized in flexion for durations of 1, 2, 4, 8, 16 and 32 weeks. Corresponding knees from control rats are listed in Table 1. (A) Mean ± SD of adipocyte number corresponded to the summation of numbers from the 6 FOVs of the same knee and averaged for the 5 rat knees from the same experimental group. Individual data points (n=5) are presented as black dots for each group. (B) Mean ± SD of adipocyte CSA corresponded to averages from 30 FOVs for the 5 rat knees in the same experimental groups. Statistical significance was determined by the Kruskal-Wallis non-parametric test comparing the groups of immobilized knees and control rats separately. Immobilization increased the mean number of adipocytes, statistically significant after 8 and 32 weeks. Both age and immobilization increased the mean adipocyte size, but statistically significant hypertrophy was accelerated; arose 4 weeks earlier in the experimental group. *: $P<0.05$ compared to the 1-0 group. †: $P<0.05$ compared to the 10-week-old rats (control group to the 1-0 group; Table 1).

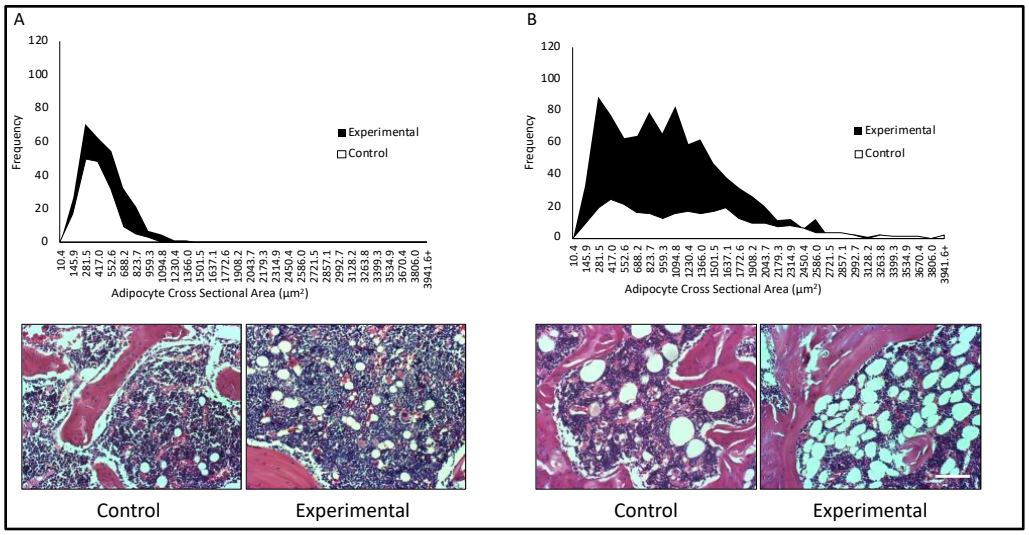

Figure 2. Frequency distribution graphs of adipocyte cross-sectional areas after 1 and 32 weeks of immobilization with representative histological images of adipocyte hyperplasia and hypertrophy in the epiphysis of the proximal tibia. Knees immobilization for durations of 1 (A) or 32 (B) weeks. CSAs of all adipocytes are displayed in bins of sizes increasing by 135.6 μm^2 and ranging between 10.4 and 8153 μm^2 (largest adipocyte measured). These graphs show more adipocytes in immobilized proximal tibia epiphyses compared to unoperated controls at the same approximate age. After 32 weeks, control knees contained a wider range of adipocyte sizes that included larger adipocytes. Despite the change in adipocyte distribution with age, the immobilized tibia contained 3 to 4-fold more adipocytes. Micrographs of H&E stained sagittal sections of the rat knee at the mid-medial condylar level. Sections were visualized by light microscopy with an Olympus BH-2 (Tokyo, Japan) microscope at a magnification of 33X and captured using a Marlin F080C digital camera (Allied Vision Technologies) with AVT Smartview 1.5.1 software. (A) Unoperated control (10 weeks) contained few small adipocytes, while knee immobilized for 1 week contained more small adipocytes compared to control. (B) Unoperated control (40 weeks) contained few larger adipocytes, whereas knee immobilized for 32 weeks contained more large adipocytes compared to controls. Scale bar represents 0.1mm.

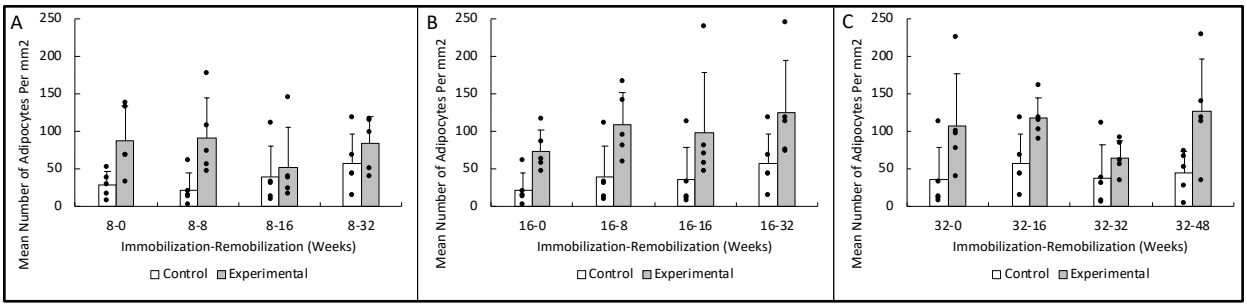

Figure 3. Adipocyte hyperplasia caused by immobilization were sustained despite remobilization. For each group (n=5 rats), one was knee immobilized in flexion for durations of 8 (A), 16 (B), or 32 (C) weeks. Experimental groups and corresponding knees from control rats are listed in Table 1. At the end of the immobilization period, the internal fixation device was removed surgically, and rats were returned to their cages for various durations of remobilization corresponding to 8, 16 and 32 weeks (groups immobilized for 8 and 16 weeks) and for 16, 32, or 48 weeks (groups immobilized for 32 weeks). Mean ± SD of adipocyte number corresponded to the summation of numbers from the 6 FOVs of the same knee and averaged for the 5 rat knees from the same experimental group. Individual data points (n=5) are presented as black dots for each group. Differences in the number of adipocytes in the remobilization groups compared to the immobilization-only groups were statistically tested using the Kruskal-Wallis non-parametric tests and all were $P>0.05$. Differences in the number of adipocytes between control rats were similar and all were $P>0.05$.

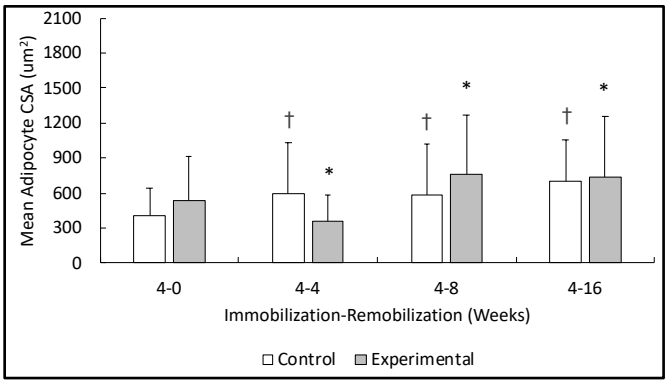

Figure 4. Four weeks of remobilization transiently reversed hypertrophy of adipocytes induced by 4 weeks of immobilization. For each group (n=5 rats), one knee was immobilized in flexion for 4 weeks and at the end of the immobilization period, the internal fixation device was removed surgically, and rats were returned to their cages for various durations of remobilization: 4, 8, or 16 weeks. Corresponding knees from control rats are listed in Table 1. Mean ± SD adipocyte CSA corresponded to averages from 30 FOVs for the 5 rat knees from the same experimental group. Statistical significance was determined by the Kruskal-Wallis non-parametric test comparing the groups of immobilized knees and control rats separately. Adipocyte hypertrophy at 4-0 (see Fig. 1) was transiently reversed after 4 weeks of remobilization (4-4 group) before resuming at 4-8 and 4-16. Adipocyte hypertrophy proceeded with aging in the control groups. *: $P<0.05$ compared to the 4-0 group. †: $P<0.05$ compared to the 14-week-old rats (control to the 4-0 group; Table 1).

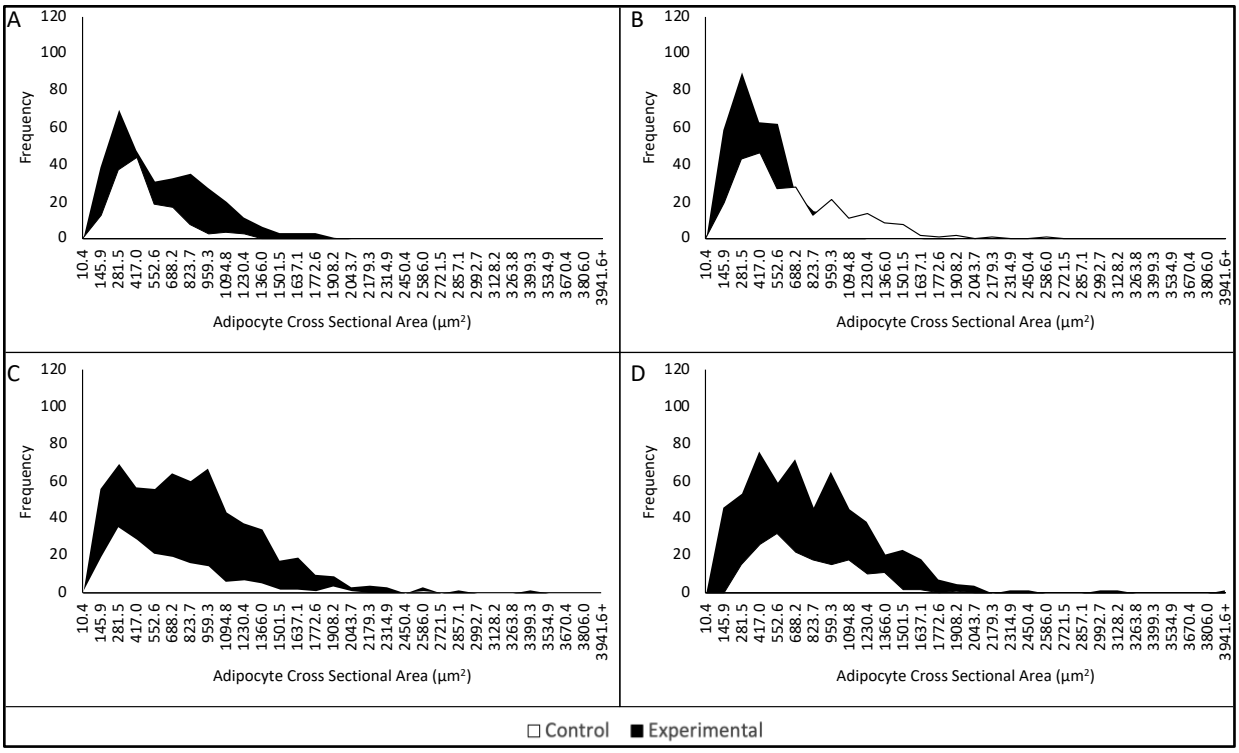

Figure 5. Frequency distribution graphs of adipocyte cross-sectional areas after 4 weeks of immobilization with or without remobilization. (A, B, C, D) In groups of 5, one knee was immobilized in flexion for a duration of 4 weeks. At the end of the immobilization period, the internal fixation device was surgically removed, and rats were returned to their cages for various durations of remobilization: 0 (A), 4 (B), 8 (C), or 16 weeks (D). Unoperated rats were used as controls (Table 1). CSAs of all adipocytes are displayed in bins of sizes increasing by 135.6 μm^2 and ranging between 10.4 and 8153 μm^2 (largest adipocyte measured). These graphs show more adipocytes in immobilized proximal tibia epiphyses compared to controls (A, B, C, D). After 4 weeks of remobilization (B), hypertrophy was transiently reversed, and remobilized rat proximal epiphyses contained only more smaller adipocytes. With aging, control knees contained a wider breath of adipocytes sizes that included large adipocytes (B, C, D). Longer duration of remobilization (C, D) showed that the immobilized tibia contained 2-3-fold more adipocytes than controls across the wider adipocyte distribution.

Table 1. Unoperated control groups with corresponding experimental groups. The corresponding unoperated control groups associated with the immobilization-remobilization experimental groups. Each group contained 5 rat knees.

Age of unoperated controls (weeks)	Corresponding experimental groups
10	1-0
12	2-0
14	4-0
18	4-4, 8-0
22	4-8
30	4-16, 8-8, 16-0
38	8-16, 16-8
40	16-16, 32-0
54	8-32, 16-32, 32-16
70	32-32
86	32-48

Table 2. Effect size of duration of immobilization on the mean difference between immobilized and control knees for number and cross-section area of adipocytes. The Hedges' g statistic was obtained by calculating the differences between the means of immobilized and controls groups divided by the pooled standard deviation for each time point and for both adipocyte number and CSA measurements.

Groups	Effect Size (g)	
	Adipocyte Size	Adipocyte Number
1-0	0.28	0.50
2-0	0.10	-0.20
4-0	0.39	0.92
8-0	0.14	1.70
16-0	-0.04	2.01
32-0	-0.34	1.23

Discussion

Many factors can contribute to the development of knee joint contractures, but prolonged periods of immobilization are a common denominator. The duration of immobilization is linked to the progressive stages of joint contractures: initiation, progression, and severity [5]. In turn, the temporal experimental design with our animal model of immobility-induced knee flexion contractures has provided us a comprehensive overview of the various stages of this joint disease and our findings showed clear biological differences between short and long durations of immobilization. Furthermore, with durations of remobilization up to 4 times the duration of immobilization, the studies presented in this thesis provide novel data on the reversibility of changes to tissue structures induced by joint immobilization and the dynamic nature of individual tissue response to unassisted remobilization.

Joint contracture pathophysiology

Through histomorphometric analysis, my findings showed a progressive decrease in posterior capsule length measured between tibia and femur insertion points as duration of immobilization increased. Despite extended periods of remobilization, there was no recovery in capsule length for durations of immobilization beyond 2 weeks. The findings also showed a significant positive correlation between posterior capsule length and ROM (i.e., a shorter capsule length was associated with a greater deficit in knee extension), further reinforcing the contribution of the posterior capsule to arthrogenic contractures. These results have reaffirmed the involvement of the posterior joint capsule as a key articular structure and highlights the functional importance of reduced capsule length in the limitation of knee extension after prolonged durations of immobilization [28,29,71–73]. However, the mechanisms for capsule shortening remain to be fully

elucidated. It has been suggested that the lack of tension in the posterior capsule during knee flexion permits the loose synovial layer to fold and adhere to one another [3,28]. The importance of these synovial adhesions should be recognized as they persisted despite remobilization beyond 2 weeks of immobilization. Findings from Chapter 1 show that the shortening of the posterior capsule length occurred predominately in the femoral part of the capsule, as the femoral side of the posterior capsule is anatomically longer than the tibial side, allowing for more synovial folds to coalesce.

The adhesion of synovial folds prompted the temporal investigation of synoviocytes type A and B within the femoral side of the posterior capsule to provide insight on potential contributions to capsule shortening. To understand cellular interactions during various joint pathologies, determining the cell type ratios within the capsule was proposed as an important measure [37]. The ratio of cell types from immunostaining of serial sections were compared to previously published mRNA expression data of the posterior joint capsule following knee immobilization [74]. Eliminating knee motion up to 2 weeks increased the synoviocyte A:B staining ratio (due to a decrease in type B staining) and differences were restored by remobilization. Gene expression data of CD68 and CD55 (markers for type A and type B synoviocytes, respectively) after knee immobilization corroborated the changes seen at the protein level [74]. Since type B synoviocytes are involved in the production of hyaluronan (a lubricant secreted into the joint cavity), the decrease in expression is biologically relevant to the well-described decrease of hyaluronan concentration in the synovial fluid of immobilized joints [75]. The production of hyaluronan by type B synoviocytes may play an important role in preventing adhesion formation and maintaining a non-adherent tissue surface to allow stretching of the capsule and movement. In cases of immune-mediated joint pathologies such as arthritis where joint stiffness is commonly

reported, the synoviocyte profile may also be attributed to the accompanying mechanical effects. A decrease in type B synoviocytes along with a relaxed and folded posterior capsule during knee immobilization may facilitate adhesion of synovial folds that can result in a shortened capsule and ultimately an overall reduction in ROM in extension. Results from Chapter 2 support a mechanosensitive modulation of the synovium that can contribute to anatomical changes of the posterior joint capsule when combined with knee immobilization in flexion.

The findings from mechanical joint stiffness in Chapter 3 revealed distinct differences from that of ROM in the same rat knees. Restrictions in ROM were identifiable as early as 1 week of immobilization [11], but stiffness didn't arise until 8 weeks of immobilization in comparison to the contralateral knee. An increase in stiffness indicates that more force in extension is required to achieve the same displacement in ROM. This temporal difference may be explained by muscular restrictions associated with short-term immobilization and articular structures during long-term immobilization. Muscles are much more pliable to stretching than articular connective tissues [76]. Consequently, ROM could be restricted with unaltered stiffness. Furthermore, stiffness continued to increase during remobilization of knees that were immobilized beyond 8 weeks, while ROM did not worsen. The irreversibility of ROM in joint contractures beyond 4 weeks of immobilization may have reduced overall mobility of the rats, thus extending the immobilized-like state, despite remobilization of the knee joint. The calculation of mechanical stiffness introduced a new measurable outcome in the evaluation of knee joint contractures in our experimental model and suggested that biomechanical properties of joint tissue structures are changing in addition to changes in anatomical structure, such as capsule length.

Consistent throughout the literature, the joint capsule is recognized as a key articular structure involved in the pathogenesis of articular stiffness induced by joint immobilization

[11,18,26,36,73,77,78]. The reduction in capsule length play a significant role in reducing ROM and increasing knee stiffness, which can critically impact joint performance [79,80]. How this structural change contributes to the biomechanical properties of the capsule remain unknown. Despite a tapering in the reduction of capsule length for longer durations of immobilization, joint stiffness continued to increase. The temporal differences between a reduction in ROM and increased joint stiffness during knee immobilization suggest that the biomechanical behaviour of the capsule is negatively impacted by eliminating joint motion and warrants further investigation [11,79]. Individual tissue mechanical testing of the posterior capsule has yet to be conducted, but such quantitative data can provide insight to how biomechanical characteristics of individual joint tissues, in addition to structural changes, can contribute to the limitation in knee function. Biomechanical property changes of individual tissues and timing of dynamic tissue changes in the context of the period of immobilization must be considered in treatment design. During prolonged durations of knee immobilization, the capsular tissue adopts an important role in joint kinematics and continues to be a connective tissue of interest for the treatment of articular stiffness.

Effects of disuse on bone marrow composition

Weight-bearing structures such as the knee joint are susceptible to changes in mechanical stimuli from both loading and muscle extensor forces, which can then result in remodeling by removing or adding bone [81,82]. Internally, the composition of bone marrow has shown a reciprocal relationship between trabecular bone and MAT during circumstances of skeletal disuse; increased MAT and decreased trabecular bone [54,68,69]. Findings in Chapter 4 reflected a similar profile in dynamic change of marrow composition during knee immobilization, showing a significant negative correlation between trabecular bone loss and MAT increase. Remobilization

of the knee joint for 8 weeks abolished all the differences in both trabecular bone and MAT between the contralateral knee after joint immobilization up to 16 weeks. The effect size of remobilization was significant and largest for trabecular bone of joints that were immobilized for 8 and 16 weeks. Our model sheds light on the natural recovery following incremental durations of knee immobilization. The observed changes in marrow composition in response to immobilization and remobilization emphasizes the adaptation of trabecular bone and MAT to changes in the mechanical environment and highlights mechanical stimuli as a key determinant [50].

The accumulation of MAT with age can be accelerated by skeletal disuse [83–85]; this process can be characterized by a combination of marrow adipocyte hyperplasia and/or hypertrophy. Despite the ability of mechanical stimuli to mitigate the accelerated accumulation of MAT in the bone marrow, the conversion of hematopoietic red marrow to nonhematopoietic yellow marrow is an age-dependent process [86,87]. The investigation of aging in our experimental model is valid as the period of immobilization and remobilization can total up to 1.5 years in rats that typically have a lifespan of 2 years. Findings from Chapter 5 showed an acceleration of age-induced adipocyte hypertrophy after 4 weeks of immobilization, while hyperplasia was exclusive to immobilized knees. Additionally, the effect size of knee immobilization was greater for adipocyte hyperplasia. Both hyperplasia and hypertrophy of adipocytes within the marrow microenvironment were sustained despite extensive periods of remobilization. These results suggested adipocyte hyperplasia to be the predominant mechanism explaining MAT accumulation during skeletal disuse and is consistent with lack of reversibility after remobilization: adipocyte hypertrophy can be addressed with exercise, but hyperplasia may not [88]. Using our methods of histomorphometric analysis, we could not distinguish whether the hallmark signature effect of hyperplasia observed was due to an increase in the number of precursor cells or due to the increased

differentiation of precursor cells into the adipogenic lineage. The complexity of marrow adipocyte mechanoregulation and potential interaction with surrounding trabecular bone has yet to be fully elucidated, but our study showed a clear implication of mechanical stimuli in the regulation of adipocyte number and, in part, size. Changes of adipocyte morphology in other fat depots such as visceral and subcutaneous adipose tissue have been implicated in conditions such as insulin resistance, obesity, and cardiovascular disease [88–90]. Although MAT accumulation can interfere with bone repair and disrupt the differentiation of mesenchymal and hematopoietic stem cells [54,70,91], it is not clear whether the change in MAT adipocyte morphology induced by joint immobilization can be attributed to the mentioned metabolic disorders.

A previous study described that bone loss during prolonged disuse and exposure to microgravity could be attributed to both hypodynamia and hypokinesia [92]. Our experimental model provides a unique perspective into whether a return to normal ground reaction forces or a return to normal ROM contributes more to the restoration of bone marrow integrity. It has previously been described in our animal model that passive ROM in knee joints that were immobilized beyond 2 weeks were irreversible despite remobilization [11]. For the time points studied, the restoration of marrow composition was achieved despite limitations in ROM. Therefore, the restoration of ground reaction forces during remobilization that were reduced by the rigid internal fixator during immobilization had a greater contribution to the recovery of trabecular bone and MAT in the marrow space. As a reduction in trabecular bone can increase the susceptibility to fractures, the restoration of trabecular bone and MAT comparable to the contralateral knee is encouraging for the reversibility of detriments caused by joint immobilization. Moreover, the mitigation of continued hyperplasia of marrow adipocytes during remobilization shows the malleability and sensitivity of the bone marrow microenvironment in weight-bearing

structures such as the knee joint. The findings from our study continues to reinforce one of the oldest tenets in musculoskeletal biology, “use it or lose it” (also known as Wolff’s law) and highlights the importance of mechanical stimuli in the regulation of musculoskeletal tissue and maintaining function.

Implications and Applications

In current clinical practices, joint immobilization is sometimes unavoidable and necessary for the management of acute knee injuries to protect damaged tissues and alleviate pain [7,8]. Current rehabilitation treatments for developed contractures include sustained stretching and exercises to increase ROM, while severe contractures require surgical intervention [26,93]. A 2-year follow-up of 375 patients that had undergone 500 total knee arthroplasties found that most patients experienced difficulty performing functional activities and achieving better ROM was an important factor for increasing satisfaction [94]. Joint contractures are involved in a number of other musculoskeletal diseases, such as rheumatoid arthritis, and although they are not directly evaluated, it is commonly identified that lack of complete joint mobility is a desired area of improvement, only secondary to pain [95].

The findings from Chapters 1-5 all suggest that the duration of immobilization should be reduced as longer periods have shown greater severity in the changes of measurable outcomes with limited reversibility (e.g., posterior capsule length, joint stiffness, and MAT accumulation). Shortening the duration of immobilization presents a challenge as most contractures are diagnosed when they have become chronic and are no longer responsive to physiotherapy, further adding complications towards treatment [26]. Although previous work has demonstrated reambulation/exercise after immobility to be effective in alleviating muscle atrophy [96], knee

flexion contractures remain in some cases and limits mobility. My findings provide evidence that unassisted remobilization was an insufficient method of intervention to achieve significant reversal of other detriments induced by immobilization. This suggests alternative and deliberate methods of treatment are required early on to restore joint mobility. By evaluating quantitatively, the onset and extent by which the posterior capsule limits knee extension, temporal data provides guidance for the design of interventions with a focus on capsular changes and its contribution to limiting knee extension and increased stiffness. Function remains a top priority for the knee joint.

Future Direction

The use of animal models to study the effects of joint immobilization has been well established in the literature. Currently, the focus has shifted towards investigating the reversibility of articular stiffness induced by prolonged immobilization (Appendix III). The natural recovery of joint function through unassisted remobilization of the knee has been shown to be ineffective following durations of immobilization beyond 2 weeks for the rat knee [11]. In turn, studies are now being designed to assess the efficacy of different treatment modalities (such as exercise [31], stretching [97], and pharmacological interventions through intraarticular injections [98–100]) to address the severity of articular stiffness and limitation in ROM induced by prolonged immobilization. The current state of the literature suggests that a combination of exercise and/or pharmacological intervention may be needed to treat severe contractures. As for the bone marrow, additional investigations on the cellular and molecular mechanisms of the reciprocal relationship between trabecular bone and MAT in response to mechanical stimuli will be important for understanding the mechanoregulation of these tissues within the marrow space and how they adapt to changing mechanical environments.

Conclusion

The comprehensive temporal overview of our animal model of immobility-induced knee flexion contractures has shown distinct biological differences between short and long durations of immobilization. The reversibility of the reduction in capsule length is limited despite extended periods of remobilization. Quantitative data further supports the contribution of the posterior joint capsule to the limitation of knee ROM in extension. Elucidation of the synoviocyte profile within the synovium during immobilization provided insights to the potential mechanisms of synovial fold adhesions and ultimately capsule shortening. In addition to anatomical changes, measures of joint stiffness suggest alterations in biomechanical properties of joint tissues are also contributing to the limitation in passive ROM. Lastly, the malleability of trabecular bone loss and MAT accumulation (predominately through adipocyte hyperplasia) following joint immobilization emphasizes the sensitivity of the bone marrow microenvironment to mechanical stimuli and lack thereof. This compilation of studies accentuates the vast detriments of joint immobilization and the limited ability to ameliorate these changes through unassisted remobilization.

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Appendix I

Joint contractures and acquired deforming hypertonia in older people: Which determinants?

Annals of Physical and Rehabilitation Medicine

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Abstract

Joint contractures and acquired deforming hypertonia are frequent in dependent older people. The consequences of these conditions can be significant for activities of daily living as well as comfort and quality of life. They can also negatively affect the burden of care and care costs. However, etiological factors and pathophysiologic mechanisms remain only partly understood. As a result, preventive interventions and treatments focus entirely on controlling symptoms rather than the causes. Moreover, the effectiveness of these interventions remains to be validated. The purpose of this position paper is to present current data on etiological factors contributing to the development of joint contractures and acquired deforming hypertonia in older people. The pathophysiologic mechanisms of joint contractures in animal models are also presented.

Keywords: Contractures; acquired deforming hypertonia; etiological factors; pathophysiology

1. Introduction

The aging process starts as early as 25 years of age and inevitably continues until death. Thus, humans spend approximately 70% of their life undergoing age-related decline. Aging is a multifactorial process often characterized by progressive degeneration of organ systems and tissues (1). The physiological state during aging is widely influenced by genetics and exposure to environmental factors.

Beyond physiological aging, several diseases can be associated with loss of autonomy. Alzheimer disease and associated disorders, ischemic and hemorrhagic stroke, extrapyramidal diseases, degenerative diseases of the musculoskeletal system, and diabetes and its vascular and neurological complications are the main pathologies increasing the dependence level in older people. Loss of autonomy in older people is typically associated with decreased mobility, which results in further functional decline. Moreover, abnormal postures or joint deformities affecting the upper and lower limbs can develop when combined with motor, articular, or periarticular disorders (2). Usually, the term “joint contractures” is used to describe the development of joint deformities in this context.

Although the decrease in joint range of motion (ROM) is the most commonly reported clinical characteristic of contractures, this term lacks a standard definition. For some authors, contractures are only the result of changes in periarticular tissues, whereas for others, both intra- and extra-articular components are involved (3, 4). The lack of consensual definition and the imprecision of assessment criteria to determine the presence of contractures can explain the wide prevalence range reported in the literature, from 20% to 75%, in older people (5, 6).

Recently, a new terminology, acquired deforming hypertonia (ADH), was proposed to replace the term joint contracture, with a more restrictive and precise definition. ADH was defined as any joint deformity with decreased ROM and increased resistance to passive movements, regardless of the cause, that promotes functional impairments, discomfort or any other limitation in activities of daily living (ADL). In the ADH survey in France, a multicenter cross-sectional study conducted in 39 geriatric institutions, 22% of 3,145 institutionalized older patients had ADH (2).

ADH can cause serious consequences for older people. Indeed, it increases the level of mobility limitation of the upper or lower limbs, thus maintaining the risk of new deforming hypertonia or contractures. In the ADH survey, one-third of patients had more than 5 ADH locations in the upper and/or lower limb and two-thirds had bilateral ADH (2).

ADH development can represent the harbinger of a cascade of decompensations that can exacerbate the functional decline, affecting social participation and quality of life of these older adults (7).

ADH is often associated with pain, which can be spontaneous or provoked by mobilization. This consequence, sometimes very intense, can generate or amplify behavioral symptoms, which may lead to increased use of antalgic or psychotropic drugs, with additional iatrogenic risk. Pressure ulcers are another consequence, especially for hands with clenched fist deformities, and represent a common reason for medical consultation. To a lesser extent, maceration of skin folds (elbows, armpits, palms of hands etc.) is also frequently associated with ADH. This situation can undermine skin and lead to chronic skin wounds. Indeed, in a vicious cycle, cutaneous lesions increase pain, which can worsen the initial hypertonia by acting as a major noxious stimulus.

Basic ADL are altered by ADH. For the upper limbs, ADH of the shoulder, elbow or wrist and finger can interfere with the ability to assume self-care, eat or dress without assistance. Regarding the lower limbs, difficulties in getting dressed and putting on shoes as well as getting up and walking independently represent the negative impact of ADH. Furthermore, ADH can be an important source of difficulties for positioning in a bed or chair, with a risk of discomfort and pressure ulcers. Access to the perineum or axilla may be impeded, which can cause pain during mobilization, maceration and hygiene problems.

For caregivers, ADH increases care demands in all ADL and basic care (toilet, cutting of nails, transfers, positioning etc.). Finally, ADH also increases care costs in nursing homes and long-term care facilities (8, 9).

However, despite the important prevalence and possibly major consequences of ADH for dependent older people, its risk factors and pathophysiology remain only partially identified. Moreover, no therapeutic guideline or clear preventive strategy is currently available.

In this position paper, we present current data about etiological factors potentially involved in the development of contractures and ADH and the pathophysiologic mechanisms, including mechanical, histological, cellular and molecular changes observed during joint immobilization.

2. Etiological factors of ADH in older people

Muscular hypertonia in older people is most often multifactorial. Upper motor-neuron syndrome, extrapyramidal dysfunction and paratonia are frequently incriminated and associated, thus resulting in “mixed” hypertonia. The exact causes of each hypertonia often remain unknown, and currently, treatments proposed address the symptom rather than the cause. Behavioral and

psychological symptoms of dementia (BPSD) and environmental factors can also contribute to the development of contracture mainly by favoring a decrease in mobility or activity.

2.1. *Upper motor-neuron syndrome*

The components of upper motor-neuron syndrome are both positive and negative signs, including, among others, spasticity, spastic dystonia and motor weakness (10). Immobilization and hypo-mobility induced by motor weakness and spasticity are the main contributors to the development of contracture. Spasticity is defined as a "disordered sensori-motor control ... presenting as intermittent or sustained involuntary activation of muscles" (11). In older people, the most common etiology is cerebrovascular disease. The characteristics of spastic hypertonia in older people are the same as in the general population (12). However, the burden of this condition is all the greater because this population is often already affected by several other comorbidities and physical limitations (13). Furthermore, spasticity can be aggravated by concomitant medical conditions frequently encountered in older people, such as pressure ulcers, skin infections, constipation or urinary tract infections (14).

2.2. *Extrapyramidal system dysfunction*

Extrapyramidal lesions are source of muscular hypertonia and joint contractures. The main etiologic classes are neurodegenerative disorders (e.g., Parkinson disease), cerebrovascular disease and iatrogenic causes (medication). Clinically, damages observed in the striatum generally correspond to rather distal hypertonia (hands and feet) (15), whereas thalamic locations lead to more dystonic manifestations.

Although extrapyramidal syndrome is generally associated with Parkinson disease and parkinsonian syndromes, people with Alzheimer dementia and other dementia such as frontotemporal lobal degeneration or Lewy body disease also seem to present these symptoms (16, 17). In older people with major cognitive impairment, extrapyramidal symptomatology is correlated with the severity of cognitive decline and functional limitations. The most common signs are rigidity and bradykinesia, which have a direct impact on motor skills and increase the risk of functional impoverishment (17). Striatal deformities are often observed in older people because these deformities are more common in advanced stages of Parkinson disease (15).

Moreover, neuroleptics are by far the most frequent medication associated with extrapyramidal syndrome (18). In older people, their use is common in the treatment of neuropsychiatric and behavioral symptoms associated with dementia (19).

2.3. *Paratonia*

Beyond pyramidal and extrapyramidal hypertonia, paratonia represents the most singular part of the hypertonia observed in older people. Paratonia is a motor disturbance seen mostly in people with cognitive impairment. In 2006, a small group of international experts proposed a consensus definition of paratonia: “hypertonia with an involuntary variable resistance during passive movement” in any direction (20).

Paratonia is a common problem. In a recent study, paratonia was found in 58% of people with severe dementia, and its prevalence seems to increase with the progression of cognitive impairment (21). Another study suggested a prevalence of paratonia of up to 100% in people with

severe dementia (22). Paratonia has been described in degenerative, vascular and mixed dementias but also in head trauma, anoxia and depression (22, 23).

As previously mentioned, several motor signs are described in dementia, whose appearance and nature vary according to the type of dementia (24). These symptoms can evolve, contribute to a loss of mobility and promote the development of contractures (22, 25). The prevalence and severity of paratonia as well as that of rigidity and hypomimia are increased in people with Alzheimer disease with more severe functional impairment (25). However, paratonia was the most consistently present form of hypertonia seen across all stages of functional impairment, with an overall prevalence of 85.7% in people with moderately severe and severe dementia. Follow-up of people with subcortical vascular disease over 6 years identified paratonia as an independent significant predictor of decline in instrumental ADL (26).

The pathogenesis of paratonia remains poorly understood, and more studies are needed to clarify the changes underlying motor signs in dementia. Some findings suggest vascular cerebral damage, and medical conditions such as diabetes that may promote vascular disease, may be part of the answer (21). Paratonia is also frequently associated with frontal release signs, which suggests a frontal lobe dysfunction (23-25).

2.4. *Behavioral and psychological symptoms associated with dementia*

BPSD encompass a wide variety of clinical manifestations that directly and indirectly affect mobility. they may contribute to contracture development if mobility and functional level are significantly reduced.

Apathy, whose core feature is a loss of or diminished motivation in comparison to the person's previous state (27), is a highly prevalent neuropsychiatric symptom in Alzheimer disease, affecting more than half of the individuals at some point during the disease (28). It has also been documented frequently in older adults with Parkinson disease and other atypical parkinsonian syndromes (29), frontotemporal lobar degeneration, cerebral vascular disease (stroke and vascular dementia) and traumatic brain injury (28, 30). In most dementias, the severity of apathy increases with the disease progression (30). Apathy has been found associated with several negative outcomes.

Agitation and aggression contribute to the burden of care and caregivers' distress, which leads to the increased use of both chemical and physical restraints for older individuals. Several studies report the extensive use of antipsychotics for agitation and aggressive behaviors, whether in community, hospitalized or long-term care patients (31). Their effectiveness is modest (32), but the harmful consequences of their use are well known. The many side effects associated with antipsychotics include extrapyramidal symptoms, gait disorders and orthostatic hypotension (31), which can all lead to mobility limitations. Other drugs used to control BPSD may also have side effects with similar consequences. Benzodiazepines and other hypnotics can cause sedation and confusion and increase the risk of falling. Selective serotonin reuptake inhibitors and serotonin and norepinephrine reuptake inhibitors may cause extrapyramidal symptoms.

Physical restraints are mainly used in long-term care facilities for patients with impaired mobility function, presumably to limit the risk of falling, and in those with disruptive behaviors (33). However, a study of 264 068 nursing-home residents showed that outcomes including

behavior issues, falls, walking dependence, ADL, pressure ulcers and contractures were significantly worse when physical restraints were used (34).

When in pain, people with dementia may become more agitated or aggressive (35). However, other behavioral changes and modifications in body activity such as refusal to move, decreased spontaneous motor activity and presence of muscle rigidity can also indicate the presence of pain. Pain can contribute to the appearance of contractures probably in part via these changes in body activity (9).

2.5. *Environmental factors*

Environmental factors may contribute to ADH development. Care given to older patients in long-term care or nursing homes often focuses on doing things for rather than with the person (36). This philosophy of care, present all over the world, prevents older people from using their remaining abilities and can lead to further deconditioning and loss of function (37).

Beyond environmental factors, older people are vulnerable to acute medical conditions with the accumulation of deficits and comorbidities with age (38). Immobility induced by those conditions constitutes a supplementary risk of developing joint contractures and ADH (39).

3. Aging and potential pathophysiologic mechanisms implied in ADH development

3.1. *Connective tissue*

The following sections will first synthesize the knowledge of connective tissue (CT) and present an overview of the literature on the aging process of joint CT that can contribute to joint stiffness and ADH. Joint flexibility depends on an integrated system involving the viscoelastic

properties and neurophysiological mechanisms of the tissues that cross that joint. Age-related modifications to this system can interfere with the ability of these tissues to lengthen or to deform. This section focuses on the non-contractile component of this system.

3.1.1. *Macro structure of connective tissue*

CT consists of cells and fibres immersed in a ground substance, also known as the extracellular matrix (ECM). On the basis of specific morphological and functional characteristics, CT can be differentiated into subtypes, proper CT being one of them. Proper CT is a very large group of tissues comprising both loose and dense CT (40). Dense CT is primarily located in fibrous load-bearing tissues such as tendons, ligaments, capsules and fasciae (40). The cells, namely fibroblasts, can be considered an "active" component of CT because they provide the metabolic properties of the tissue. Their stimulation by mechanotransduction leads to synthesis activities when the tissue experiences physiological loading or to degradation activities when the tissue experiences unloading or overloading (41, 42). In the past few decades, a particular type of fibroblast called a myofibroblast has been found in tendons, fasciae and scar tissues (43). These cells have actin fibres in their cytoplasm that allows them to contract. Some authors have hypothesized that long-lasting isometric contraction plays a role in pathological fascial contractures such as Dupuytren disease, plantar fibromatosis or frozen shoulder (44-46).

The ECM and its contents (the "inert" component) provide the mechanical and viscous properties of the tissue. It contains approximately 60% to 80% water and 20% to 40% solid material, but these distributions vary by anatomical region, tissue type, tissue function and age (47, 48). The ECM is an interlaced network that distributes mechanical stresses on the CT and provides support for the cells. The main components of the ECM are the ground substance and several types

of fibres, the principal ones being collagen and elastin. The ground substance is composed of water, extracellular proteins, proteoglycans and glycosaminoglycans (GAGs). The ground substance is responsible for providing nutrition to the cells. It also provides hydration by attracting water, which creates a viscous gel that acts as a lubricant. The ground substance also creates space between the collagen fibres at points where they cross, so that they can glide on each other when tensile force is applied on the tissue. The inter-fibrillar space may also prevent excessive cross-link formation that could decrease tissue mobility and deformation (49), two mechanisms contributing to the development of ADH. In general, each collagen fibre is made up of thread-like subunits called collagen fibrils. Each fibril, in turn, is made up of collagen molecules linearly arranged in an overlapping head-to-tail fashion. The fibril's strength is due to intra- and inter-molecular bonds. Elastic fibres are thinner and arranged in a complex 3D branching network according to the tensile force imposed on the CT. Therefore, the concentration of elastin is reflective of the amount of mechanical strain imposed on the CT and the need for that tissue to return to its original state (40). In summary, collagen and elastin fibres provide the mechanical properties of the tissue, which explains their role as being complementary from a functional point of view. Although the main role of collagen is to resist tensile stress, it is interwoven with elastin fibres to prevent tearing and facilitate the tissue's return its pre-deformed state. Finding an effective intervention that allows collagen to be deformed without being injured is one of the challenges clinicians encounter in the conservative management of ADH.

3.1.2. *Age-related changes of CT*

Although age-related mechanisms and their impact on joint flexibility are specific to each type of CT, some more general processes can be applied to most CT (for reviews see (41, 47)).

For example, enzymatic modification of the structural proteins collagen and elastin following their synthesis has been reported. Acquired imbalance between the synthesis and breakdown of the ECM has been related to decreased tissue volume and accumulation of degraded molecules that affect the CT state or "quality". Collagen, elastin and the ground substance undergo changes that affect the viscoelastic properties of the tissues and therefore modify their responses to tensile stress, their ability to deform, their force transmission capacity and also, responses of cells (41) to the mechanical loading.

With aging, an increase in intramuscular lipid concentrations has been reported (50, 51). The total amount of collagen fibres of the tendon (52, 53), the capsule (54) and the intramuscular muscle's CT also increases (55, 56). More complex intermolecular cross-links between tropocollagen molecules have been reported, and all these modifications lead to more stable cross-links (57). Moreover, the wave length of the crimp structure of the collagen fibril increases and the wave-crimp angle decreases (58). Therefore, when the collagen fibril is exposed to a load, these changes can be expressed from a temporal standpoint by using the load-deformation curve model. The fibrils spend a shorter time in the toe region of the curve to reach the linear region sooner, and once in this zone, the load increases at a faster rate. The clinical meaning of these changes is increased tissue stiffness and CT that attains its limits of deformation sooner. The reduced content of elastin fibre also interferes with the tissue's ability to deform.

Another mechanism that could explain stiffness resulting from aging is related to changes of the ground substance. Ground substance content has been shown to decrease with age (59, 60), thereby resulting in reduced hydration and a lower gel-to-fibre ratio. A decrease in this ratio can potentially reduce the space between the collagen fibrils, interfering with their ability to glide and

promote binding between collagen and the GAGs that surround the fibre, thus interfering with CT lubrication. These modifications can increase the friction between the different layers of CT that surround or cross a joint and contribute to joint stiffness during aging and possibly to the development of contractures or ADH. To summarize, aging CT features greater collagen content and less water, elastin and ground substance. These histological changes contribute to CT stiffness and in some cases, ADH.

The stiffness of CT tissue and its reduced ability to deform results in decreased flexibility as people age. In turn, older people have difficulty moving joints into ranges of motion required for daily activities (e.g., reaching overhead, standing up from a chair, walking). This is particularly true for older individuals with a medical condition (e.g., stroke, dementia) that limits their capacity to move even more. The CT of individuals no longer able to move one or more joints by a previously available ROM (relative immobilization) can become unloaded. This under-stimulation causes changes in the CT that mimic those observed during absolute joint immobilization (e.g., post-injury, casting, etc.).

In summary, age-related modifications in the CT can lead to CT stiffness and loss of flexibility. When joints are no longer used to their maximum capacity, as with conditions of relative immobilization, the CT might become unloaded and under-stimulated, which can induce changes in CT. Therefore, aging and immobilization are two factors that induce decreased ROM in older individuals. In some individuals, the pathological changes in the CT will add to those changes associated with aging and immobilization, thereby resulting in the development of ADH and thus a vicious cycle that is very difficult to break.

3.2. Molecular and cellular aspects of joint contractures and ADH

Clinical research investigating molecular and cellular mechanisms of aging and immobility on human articular tissue is rare (61) and is limited by ethical issues and various factors that complicate clinical aging research (62). Animal models continue to contribute basic new data to aging research (Table 1). For ADH, the rat model of knee immobilization in flexion has allowed for studying the progression of joint contractures throughout aging, with up to 32 weeks of immobility and up to an additional 48 weeks of mobility in animals with a lifespan of 2 to 3 years (63). A temporal study on the reversibility of knee flexion contractures determined that recent-onset contractures were primarily due to muscular structures and were reversible, whereas long-lasting contractures were primarily due to articular structures and were irreversible after unassisted mobilization (63). The animal models allow for comparing and contrasting joint contractures caused by immobility or secondary to trauma, inflammatory joint diseases and central or peripheral neurological conditions causing muscle imbalance around a joint (64). In this section, we review the cellular and molecular changes associated with aging and immobility in ADH and joint contractures.

3.2.1. Histological and cellular changes

Sensory input provokes spasticity and can contribute to upper motor-neuron signs leading to continuous muscle activity, lack of volitional command, and lack of phasic stretch (65). The onset of spasticity may contribute to the plastic rearrangement of the central nervous system, which can result in over-active muscles and exaggerated reflex responses to external stimuli (66). Histological changes in paretic and immobilized muscles are atrophy, loss of sarcomeres, accumulation of intramuscular connective tissue and increased fat content (67). Immobilization in

a shortened position further aggravates muscle contractile properties and ultimately results in myogenic joint contractures (67). Pyramidal or extrapyramidal diseases modify the neurogenic sensory and motor environment around the joint, but the resistance to passive movements can only be attributed to non-neurogenic tissues (2). Several histomorphological and cellular changes accompany the pathophysiology of joint contractures. The capsule is an important articular structure that limits ROM (63, 64). The rat model of immobilization in flexion has demonstrated a significant reduction in posterior synovial intima length (Figure) and synoviocyte proliferation, which suggests synovial adhesions rather than pannus proliferation during contractures (68). Prolonged immobilization in the same model is characterized by the replacement of articular cartilage by bone in the non–weight-bearing region of the tibia, possibly mediated by chondral vascularization (69). Additionally, fat deposition in the bone marrow is induced by joint immobilization, characterized by hyperplasia of small adipocytes in recent-onset contractures and adipocyte hypertrophy in chronic contractures (70).

3.2.2. *Molecular changes*

Immobilized joints contain higher amounts of type I collagen and lower amounts of type III collagen in the joint capsule of immobilized legs as compared with sham treatment, which suggests that the contracture process is caused by fibrosis (71). An initial disorganization of collagen fibres followed by an increase in advanced glycation end products during immobilization is a potential mechanism contributing to the chronic stiffness of the capsule (72). Characteristic modulation of specific biochemical pathways identified by a temporal gene expression profile during immobilization-induced contracture in rat knees revealed that the joint capsule was sensitive to immobility (Table 2) (73). Along with mechanical factors in the environment of the

joint, a study of different inbred rat strains provided evidence that intrinsic genetic factors contribute to the development and susceptibility to joint contractures (74). These experimental results provide insight into molecular changes relevant to the pathophysiology of joint contractures in the context of ADH.

4. Conclusion

An increasing number of dependent older people are living at home or in long-term care facilities. At least 20% of this population is at risk of developing joint contractures or ADH. These conditions can have significant consequences. They can contribute to functional limitations, discomfort and decreased quality of life. However, little is known about preventing and treating ADH. Better knowledge of the etiological factors and pathophysiologic mechanisms of ADH is needed and might help improve patient care and lead to new therapeutic approaches.

Figures and Tables

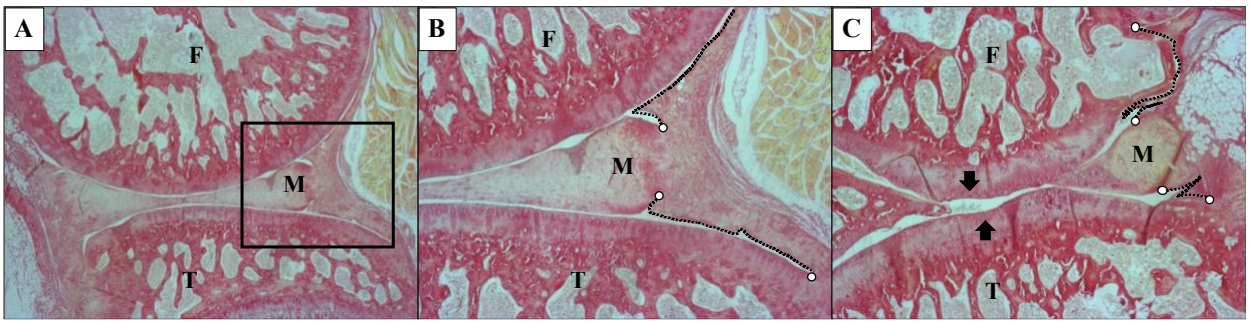

Figure 1. Histomorphological changes in the posterior side of the rat knee joint after 16 weeks of immobility. Microphotograph of a sagittal section of the rat knee joint at the medial mid-condylar plane stained with Alcian blue and direct red. A markedly reduced posterior capsule length is shown in the immobilized knee caused by obliteration of the joint recess and synovial folds. Irregularity and degeneration of articular cartilage is noticeable on both the femur and tibia as compared with the smooth and uniform articular cartilage on the contralateral knee. A) 3.3X magnification of contralateral knee. B) 6.6X magnification enlargement of boxed region in A. C) 6.6X magnification of knee immobilized for 16 weeks. F, femur; T, tibia; M, meniscus. Dotted lines measure synovial intima length of the posterior capsule. Open circles indicate measurement from the medial horn of the meniscus to the synovial cartilage junction. Arrows indicate the degeneration and irregularity of the articular cartilage. Magnification 6.6X (Olympus BH2).

Table 1. Animal models used to study the effects of aging and immobility.

Model	Benefit	Drawback
Aging and immobility		
Senescence-accelerated mouse [a]	Premature onset of aging-related bone and joint disease. Rapid testing of hypotheses about the causes and treatments of aging-related orthopaedic complications.	Mechanisms by which senescent cells promote degeneration of bone and joint tissues is unknown.
Rat knee immobilisation [b]	Investigation of joint contractures secondary to immobility and study of its temporal progression and reversibility.	Direction of limitation of range of motion is unidirectional, and natural standing position of rat knees is in flexion.
Rabbit knee post-traumatic contractures [c]	Modelling post-traumatic joint contracture with a fracture that exposes the joint environment to a hemarthrosis with bone-marrow elements. Joint stability or congruity are not compromised.	Model is limited to flexion contractures.
Aging		
Inbred mice [d]	Genetic similarity minimizes confounding factors attributing differences to environmental or treatment effects.	Genetic uniformity of inbred strains is not representative of the human population. Limited in range of pathology.
Outbred and F1 mice [d]	Hybrid vigor, long life span, rapid growth, large size. Considered to be more representative of human population.	Heterogeneity limits the assessment of the benefits of an intervention.

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Table 2. List of differentially expressed genes and pathways in the posterior joint capsule during immobilization-induced joint contracture [73].

Gene Ontology: biochemical pathways	**Genes** [3]
Lipid biosynthetic and metabolic process [1]	Fasn, Ggat2, Acaca, Gpd1, Elov1, and Acsl1.
Extracellular structure and organization[2]	MMP3, MMP9, MMP13, Col2a1, Col10a1, Col11a1, Agt, Alb, Cdh1, Cdh2, Cfd, Chad, Ibsp, Ky, Myh11, Obp3, Pcsk6, Tf, Tnfrsf11b, Tnn, and Vit.

[1] All 7 genes showed reduced expression over the immobilization time course [93].

[2] All metalloproteinase genes (MMPs) showed a decreased expression profile over the immobilization time course [89].

[3] Fasn: fatty acid synthase; Ggat2: glutamate-glyoxylate aminotransferase 2; Acaca: acetyl-CoA carboxylase alpha; Gpd: glycerol-3-phosphate dehydrogenase 1; Elov1: elongation of very long chain fatty acids protein 1; Acs11: 1-aminocyclopropane-1-carboxylate synthase 11; MMP: metalloproteinase genes; Col: collagen type alpha chain; Cdh: cadherin; Cfd: complement factor D; Chad: chondroadherin; Ibsp: integrin binding sialoprotein; Ky: kyphoscoliosis peptidase; Myh11: myosin heavy chain 11; Obp3: odorant bind protein; Pcsk6: proprotein convertase subtilisin/kexin type 6; Tf: transferrin; Tnfrsf11b: TNF receptor superfamily member 11b; Tnn: Tenascin N; Vit: vitrin.

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Appendix II

Embryonic movement stimulates joint formation and development: Implications in arthrogryposis multiplex congenita

BioEssays

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Keywords: joint formation; embryonic movement; mechanical stimuli; immobilization; arthrogryposis.

Abstract

Arthrogryposis multiplex congenita (AMC) is a heterogeneous syndrome where multiple joints have reduced range of motion due to contracture formation prior to birth. A common cause of AMC is reduced embryonic movement *in utero*. This reduction in embryonic movement can perturb molecular mechanisms and signaling pathways involved in the formation of joints during development. The absence of mechanical stimuli can impair joint cavitation, resulting in joint fusion, and ultimately eliminate function. In turn, mechanical stimuli are critical for proper joint formation during development and for mitigating AMC. Studies in experimental animal models have provided a greater understanding on the molecular pathophysiology of congenital contracture formation as a consequence of embryonic immobilization. Elucidation of how the mechanical signaling environment is transduced to initiate a biological response will be necessary to gain a deeper understanding of how mechanical stimuli are intertwined in the molecular regulation of joint development.

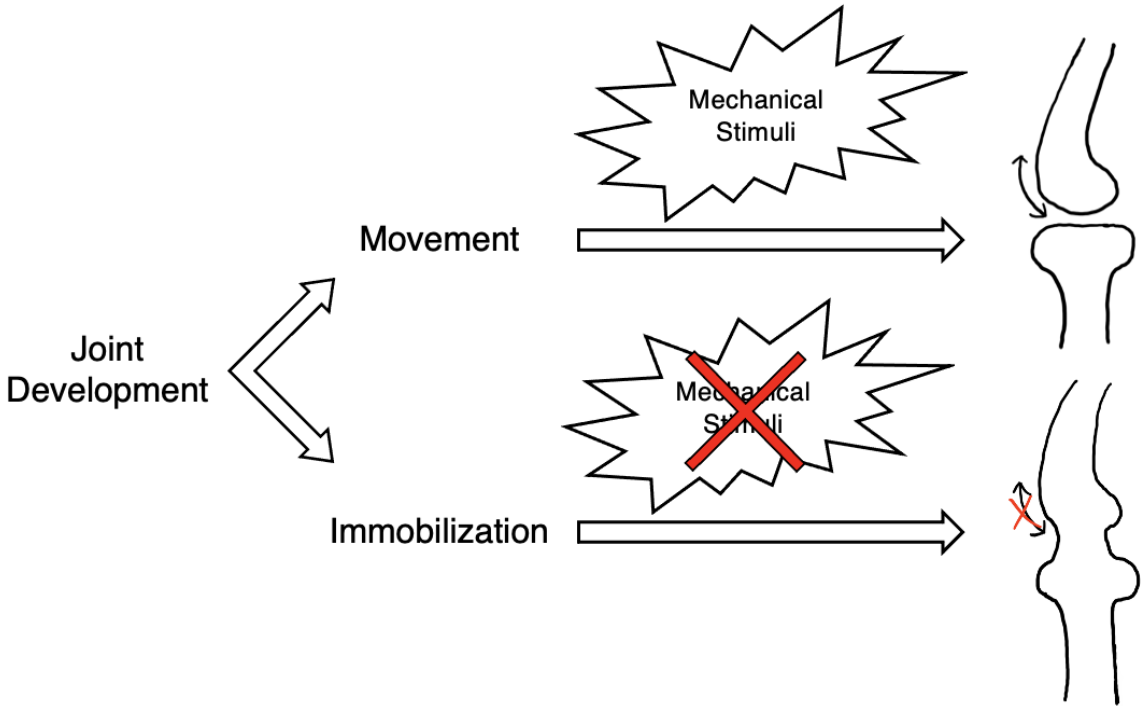
Abbreviation list:

AMC	Arthrogryposis multiplex congenita
BMP	Bone morphogenic protein
GDF5	Growth differentiation factor 5
IHH	Indian hedgehog
ROM	Range of motion
SFRP2	Secreted frizzled-related protein 2
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
Wnt	Wingless-related integration site

Keywords: joint formation; embryonic movement; mechanical stimuli; immobilization; arthrogryposis.

Graphical Abstract

Embryonic movement is critical for joint development. Lack of mechanical stimuli from reduced movement during development perturbs many molecular mechanisms necessary for joint formation and cavitation. Improper joint formation limits function by restricting range of motion, and this is reflected in the clinical case of arthrogryposis multiplex congenita.



Introduction

ROM is a critical component for the proper function of joints. A limitation in the passive ROM of affected joints is termed a joint contracture, and this can be detrimental for joint performance.[1,2] The development of contractures can arise as a consequence of injury/disease (requiring extended periods of immobility),[2–6] or due to treatment (prolonged use of bracing).[7] The pathophysiology of joint contractures is progressive in nature, where increases in the duration of immobilization increases the severity of ROM loss.[8] Alternatively, contractures can also be formed prior to birth. AMC is a heterogeneous syndrome where two or more joints have developed contractures prior to birth.[9] Another name for AMC is multiple congenital contractures.[10,11] In the clinical classification of AMC, arthrogryposis is separated into 3 groups: 1) disorders with limb involvement 2) disorders with limb involvement and other body area(s) and 3) disorders with limb involvement and dysfunction of the central nervous system.[9,12] Moreover, AMC can be classified between amyoplasia, distal arthrogryposis, and arthrogryposis involving central nervous system etiologies.[13] Amyoplasia is characterized by minimal muscle growth, resulting in contracture formation among most joints. Distal arthrogryposis primarily involves the distal parts of limbs (consistently with hands and feet) without influence by neurological factors or muscle disease. Lastly, developmental abnormalities that impact activation of spinal cord motor neurons can contribute to decreased fetal movement, and ultimately AMC. [13] The prevalence of AMC has been estimated to be roughly 1 in every 3000 live births - amyoplasia being the most common form - and can arise as a result of a wide range of disorders.[12]

The etiologies of AMC are broad and can be categorized into intrinsic and extrinsic conditions. Intrinsic conditions include the improper development or function of components within the embryo/fetus, such as disorders arising from muscle, metabolism, and the central

nervous system.[9] Alternatively, extrinsic conditions correspond to maternal effects (uterine structural anomalies, illnesses, teratogens) or other environmental problems (drugs, infections, crowding of uterus).[9] Relationships between maternal physical activity and fetal movement remain inconclusive and ambiguous.[14] Regardless of the cause, they all share a commonality of reducing embryonic/fetal movement (Figure 1).[9,15] Furthermore, a direct relationship has been found between the early onset of decreased fetal movement and contracture severity.[15–17] This draws many parallels to regular joint contractures in that AMC develops secondary to decreased movement (mimicking immobilization).[9] However, contractures that arise from AMC are typically nonprogressive after birth, contrasting the progressive nature of contractures that arise due to prolonged immobilization.[12]

Movement, and ultimately mechanical stress, play a critical role in musculoskeletal development.[18–24] In AMC, the lack of movement within the embryo/fetus may perturb many aspects of tissue differentiation and maturation that are guided by mechanical forces during development.[22] Understanding the role of mechanical stimuli in regulating various processes during differentiation and morphogenesis is of growing interest in the field of developmental biology. This review seeks to synthesize the current literature on the role of joint formation/development in the pathogenesis of arthrogryposis that primarily affects the limbs. More specifically, this review will be guided by the following research question: What is the effect of reduced embryonic movement on joint formation and development? In turn, what are the implications of this in AMC? This review does not examine all the causes that can contribute to decreased embryonic movement, but rather how decreased embryonic movement impacts proper joint development, which can result in joint fixation and ultimately AMC.

Experimental animal models used to study arthrogryposis

Observations of AMC in animals were initially documented in bovine strains, where neonatal calves had developed contractures in their limbs and was termed "crooked calf disease."[11] In order to study arthrogryposis experimentally, animal models were developed to mimic the reduced embryonic and fetal movement observed in AMC. The primary goal was to eliminate motion of joints *in utero*. Since muscular contractions are the main source of movement, experimental models sought to eliminate movement by inducing muscular paralysis. Embryonic immobilization was achieved through the administration of neuromuscular blocking agents or viral infectious agents to the embryo in the early experimental models of AMC.[23,25,26] Exposure to a wide variety of other teratogens such as chronic maternal illness and maternal ingestion of drugs/toxins have also been reported with AMC.[27] Additionally, injection of anti-acetylcholine receptor antibodies into pregnant mice have also been shown to be a viable model to study AMC and other developmental abnormalities.[28] Animal models that do not require the administration of an agent to induce muscle paralysis include a mouse model of hereditary peroneal muscular atrophy and, more recently, mouse models with reduced-muscle and muscle-less phenotypes.[22,29,30] Nonetheless, the preferred model organism is the chick embryo, due to the ease of drug/viral agent delivery *in ovo* during various stages of development.[22]

Initial experimental models established the fact that reduced movement due to a lack of muscle contraction indeed caused AMC, which in turn highlighted the observations that embryonic movement was essential for joint morphogenesis.[23,25] Given that the structure of joints has been adapted for motion, it was hypothesized that movement was required to complete the formation of joints. In a chick embryo model that examined 3 methods of muscle paralysis, Drachman and Sokoloff concluded that skeletal muscle contractions were essential for a number of elements: joint

cavity formation, the appearance of the plantar tarsal sesamoid cartilages and intra-articular ligaments, and proper formation of articulating surfaces.[23] The process in which joint cavitation occurs appear to act in conjunction with joint motion, and is preceded by a cell-related phenomenon during interzone formation.[31] Without mechanical stimuli induced by movement, it has been suggested that there is insufficient alteration of extra-cellular matrix components along the interzone to allow for joint cavitation.[32] However, muscle contractions were not essential for the formation of the interzone and stages prior to cavity formation, suggesting that joint differentiation was able to proceed to a certain extent without movement.[23] Research in this field has since shifted to investigate the role of mechanical stimuli in joint formation and elucidate how the molecular mechanisms are perturbed due to a lack of embryonic movement.

Molecular regulation necessary for key stages of joint development and formation

The function of a joint is dependent on its ability to allow for articulation and transfer of mechanical loads between two opposing skeletal elements.[32] This function is supported by articular structures such as the articular cartilage, ligaments, and joint capsule, which encapsulates the synovial fluid within the joint cavity. Prior to the appearance of these structures during the later stages of joint morphogenesis, two main events must occur during joint development: 1) joint specification and the emergence of an interzone at the joint site within a pre-cartilage tissue (transient cartilage) and 2) joint cavitation at the center of the interzone, where the two opposing skeletal elements separate (Figure 2).[32,33] Improper separation of the skeletal elements and inability to form a cavity would be detrimental to the primary function of a joint. To that end, proper joint development is essential to mitigate AMC.

The overall process for the development of skeletal elements in the appendicular skeleton is referred to as endochondral ossification and is characteristic of long bones in vertebrates.[33,34] In brief, this process involves the replacement of cartilaginous tissue, differentiated from mesenchymal cells, by bone.[35] During this process, the proliferation and differentiation of chondrocytes is largely mediated by IHH signaling originating from pre-hypertrophic chondrocytes [36,37]. In turn, this upregulates parathyroid hormone related protein signaling, which inhibits the differentiation of proliferating chondrocytes into pre-hypertrophic chondrocytes; creating a feedback loop to maintain chondrocyte proliferation.[36,37] Additionally, signaling of the BMP family has been identified as key players in the processes of chondrogenesis and osteogenesis.[38] However, BMPs are able to induce apoptosis or differentiation of cells depending on history of the cell. This is termed *context dependency* and is critical for the formation of joints.[35] BMPs were originally recognized for their ability to induce new bone formation, but the current understanding is that they can also act as mediators during the pathophysiology of joints.[38]

During joint development, interzone emergence and joint cavitation are heavily regulated by various molecules and signaling pathways such as GDF5, Wnt, IHH, and BMP to ensure proper joint formation and are considered to be a part of the early stages of joint generation and development.[33] Interzone emergence is first recognized when flattened cells appear at the presumptive joint site, establishing the origin of the joint.[33] At this stage, BMP signaling is negatively regulated by BMP antagonists, such as noggin and chordin.[33] Within the presumptive joint site, the phosphorylation of SMADs (part of BMP signaling pathway) is not observed.[18] Instead of the original expression of chondrocyte marker genes by the cells in the transient cartilage, the cells gathered at the interzone then acquire expression of GDF5.[39] Moreover, Wnt4, Wnt9a, Wnt16, ETS transcription factor ERG, doublecortin, and GLI family zinc finger are representative

markers also expressed in the interzone.[40] Although not expressed in the interzone, IHH signaling contributes to the development of the interzone by recruiting flanking cells into the interzone.[41] In IHH-deficient mice, there is lack of an interzone, and GDF5 positive cells instead surround the uninterrupted joint site.[42] Without GDF5, skeletal malformations such as brachydactyly have been shown to develop, suggesting that GDF5 is essential for proper joint development.[43] Expression of GDF5 outside of the interzone in mice with brachypodism also suggest it is necessary for generation and maintenance of the interzone.[44] Although GDF5 is deemed to be important for the interzone, it is unlikely to contribute to articular cartilage formation, a notion that is corroborated by its diminished expression before appearance of the articular cartilage.[39] Wnt signaling aids GDF5 in the interzone formation by suppressing chondrogenesis in the regions flanking the interzone and has been shown to be an important factor for articular cartilage differentiation.[45,46] The suppression of chondrogenesis is demonstrated by the suppression of markers of transient cartilage (SOX-9 and collagen type II alpha 1) by ß-catenin.[45]

Following interzone emergence, joint cavitation is able to occur in the middle of the interzone and subsequently form the synovial joint.[33] During this intermediate stage between joint specification and cavitation, cells found at the interface co-express both transient cartilage and articular cartilage markers.[47] The formation of the cavity itself has been suggested to be mediated by the filling of the interzone with hyaluronan, a glycosaminoglycan found as a structural component in extracellular matrices and in synovial fluid as a lubricant for opposing skeletal elements.[48] Upregulation of hyaluronan related factors such as hyaluronan synthase and uridine diphosphoglucose dehydrogenase were specific to the intermediate zone throughout the process of cell segregation.[49–51] Hyaluronan synthase deficient limbs have been shown to result in defective cavitation and joint formation.[48] *In vitro* studies have shown that the process of losing tissue

cohesion between joint elements to form cavities during development is largely regulated by the activation of extracellular-regulated kinase 1/2 through the mitogen-activated protein kinase pathway.[52] The lack of extracellular-regulated kinase 1/2 activity within the intermediate zone during embryonic immobilization suggest that this process is dependent on mechanical stimuli.[52] At this stage of joint cavitation, mechanical stress plays a crucial role,[18–24] but how the stimuli are transduced to intracellular signals remains to be elucidated.[33] After joint cavitation, the later stages involve differentiation of articular cartilage, which involves noggin expression within the epiphyseal cartilage to prevent BMP ligands from diffusing into the joint region,[47] and maturation of the rest of the joint to complete morphogenesis.[38] The molecular mechanisms and signaling pathways involved in the late stages of articular cartilage differentiation and maturation remain to be fully understood.[33]

Embryonic movement stimulates joint development

Considering all these steps in joint formation and development, the importance of movement and mechanical stress is emphasized during joint cavitation. Joint progenitor cells require mechanical stimuli to maintain their cell fate, otherwise chondrocyte proliferation continues and prevents the formation of a joint.[53] Interzone emergence does not appear to be affected due to a lack of embryonic movement,[23] but it has been shown in the developing jaw joint of zebrafish that when muscle-induced strain is removed, interzone size is significantly affected; contributing to asymmetrical joint development and disrupting joint integrity.[54] Interzone emergence remains a critical checkpoint for the segmentation of skeletal elements. Continuous long bones lacking joints were observed in chick embryos when the interzone was removed.[55] Current research now investigates how mechanical stimuli generated by embryonic

movement can guide particular molecular events for joint formation.[19] In the context of AMC, improper joint development due to lack of cavitation and/or segmentation simply results in a loss of joint function by limiting the ROM. There are numerous intrinsic and extrinsic conditions that can reduce embryonic movement, but the reduction in mechanical stimuli is shared among the different causes. Mechanical stimuli generated by movement plays a key role in joint cavitation, and ultimately proper joint formation. How a lack of mechanical stress perturbs the molecular mechanisms and signaling involved in stages leading up to and during joint cavitation are discussed in the following sections.

Research of vertebrates in recent years has suggested that transduction of mechanical stimuli is capable of mediating cellular processes responsible for organogenesis.[56] Pertaining to musculoskeletal development, the forces that act on skeletal elements originate from muscle contraction. Mechanical forces contribute greatly to bone morphology and many functional aberrations can arise throughout the various stages of development due to a lack of mechanical stimuli: 1) impairment of bone elongation due to reduced chondrocyte proliferation and improper chondrocyte organization into columns, 2) absence of bone eminences, 3) lack of differential appositional growth, and 4) impaired joint formation during embryonic development.[56] Multiple aspects of joint patterning and morphogenesis become altered in the absence of mechanical stimuli. Using finite element analysis, Roddy et al., have shown within the chick knee joint how a typically dynamic pattern of stimuli is replaced by a static stimuli environment during embryonic immobilization.[20] Within the modeled simulation, the consequences of altered shape and structure of the joint and associated tissues may have been due to a combination of the absence of dynamic stimuli and/or reduced stimuli overall.[20] It's suggested that the local patterns of biophysical stimuli provide specific positional information necessary for the correct patterning of emerging

tissues in the joint.[20] In a muscle-deficient mouse model, abnormalities in bone and joint development was attributed to a complex interaction between mechanical forces and location-specific regulatory factors.[29,30] The mechanical stimuli generated from movement guides numerous aspects of tissue differentiation and maturation during development,[22] but here we focus on the impact movement has on the events leading up to and during joint cavitation.

The seminal research by Drachman and Sokoloff revealed the importance of embryonic movement in the development of functional joints in chicks and paved the way to investigate how molecular mechanisms and signaling pathways in joint development were mediated by mechanical stimuli.[23] Genome-wide microarray and RNA-sequencing methods have been used to identify mechanosensitive genes that were differentially expressed between muscle-less and normal embryonic humerus tissue during skeletal development of mice.[57] Of the genes that were differentially expressed, 680 independent genes were downregulated, and 452 genes were upregulated in the muscle-less embryos in comparison to controls. Gene ontology annotations of the differentially expressed set of genes in muscle-less embryos affected biological processes associated with development and differentiation, cytoskeletal architecture, and cell signaling. Of the genes associated with cytoskeletal architecture, 84 genes showed a downregulation in muscle-less embryos, suggesting a potential set of mechanosensitive genes involved in mechanotransduction. Regarding the genes associated with cell signaling pathways, Wnt signaling was heavily affected; upregulation of Wnt4 and SFRP2 in the muscle-less group. Moreover, a target gene by Wnt, CD44, was no longer expressed in muscle-less embryos. CD44 encodes for a glycoprotein that binds proteoglycan and hyaluronan, and this interaction has been shown to be implicated in joint cavitation.[58] Roddy et al., previously described a loss of tissue organization in the interzone of knee joints in immobilized chick embryos and CD44 was one of many gene

expression patterns that were affected.[20] Given the sensitivity of CD44 to mechanical stimuli and its involvement as a regulator in joint formation, it may be one of many important mediators of mechanical stimuli.[57] This study by Rolfe et al., highlighted important regulatory genes involved in development that were impacted by embryonic immobilization, and provided insight to the potential molecular mechanisms perturbed in response to a lack of mechanical stimuli.[57] These results provided a strong resource for understanding the mechanistic basis of mechanoregulation during joint development.

Mechanical stimuli regulate the molecular mechanisms of joint development

In the recent decade, advancements in knowledge on the mechanoregulation of joint development have started emerging. Although a majority of studies focus on the soluble molecules that regulate cell differentiation, Kahn et al. investigated the effect embryonic movement had on joint progenitor cells in a mouse model.[53] Their results agreed with the notion that interzone formation was unaffected due to a lack of mechanical stimuli, and that an intermediate stage between joint specification and cavitation was perturbed.[53] With a lack of muscle contraction, the suppression of chondrogenesis during joint formation by GDF5 and Wnt signaling was lost, and expression of chondrogenic markers such as SOX-9 and collagen type II alpha 1 were maintained, preventing joint cavitation.[53] This provided insight to how joint progenitor cells required mechanical stimuli to maintain its identity and to suppress chondrogenesis. Interestingly, it was also reported that different joints did not respond the same to a lack of muscle contraction, which may be compensated by joint-specific genetic components, and presents a challenge in identifying joint-specific mechanisms.[56] This discrepancy may be explained by differences in experimental methods between research groups or in the species used (e.g. mice vs. chicks) and not just between

joints. The initial rationale by Kahn et al. relied heavily on differences in ß-catenin activity between joints to justify their claims, but we now know the mechanical regulation of joint development to be much more complex. To address this, Singh et al. sought to investigate whether the absence of mechanical stimulation during embryonic development was consistent across species.[18] In their investigation of the canonical Wnt signaling and BMP signaling during embryonic immobilization of vertebrate species, they concluded that the molecular mechanisms during movement-induced development of various joints were conserved between mice and chicks.[18]

Singh et al. went on to further describe the mechanosensitive response and interplay between BMP and Wnt signaling in defining joint territories during embryonic immobilization. The findings from their study showed that immobilization affected the expression of SFRP2, an antagonist of Wnt signaling, and SMURF1, an antagonist of BMP signaling that insulates the joint region, both of which are involved in articular cartilage differentiation.[18] The upregulation of SFRP2 prevented the suppression of chondrogenesis and concurrent downregulation of SMURF1 allowed for the ectopic activation of BMP signaling. Taken together, this prevented the creation of a permissive environment for articular cartilage differentiation and joint cavitation. As a result, joint-specific markers were lost, and transient cartilage markers were maintained.[18] Although it was not described how SMURF1 was regulated through mechanical signaling, the study recognized that the mechano-dependent expression of SMURF1 played a key role in mediating the interplay between BMP and Wnt signaling during joint development.[18] The influence of mechanical stimuli on the interplay between canonical Wnt and BMP signaling during embryonic immobilization was further investigated in the developing elbow and shoulder joint; particularly regarding Wnt signaling.[59] During immobilization, Wnt signaling restricted to the developing

joint region was lost, but activity was maintained in scattered cells in the subchondral region. In comparison, BMP signaling had a reciprocal effect, supporting the coordinated effects immobilization has on the two signaling pathways. In contrast to immobilization, exogenous activation of the Wnt signaling pathway via electroporation of the limbs with a ß-catenin fusion protein was able to suppress chondrogenesis and expand the joint territory. These results suggest that mechanical stimuli are required to properly coordinate spatial territories of Wnt and BMP signaling and to influence contribution of joint progenitor cells.[59] Otherwise, improper formation of joint-specific tissues and inappropriate tissue differentiation can occur, preventing the separation of skeletal elements to form a joint cavity.

Movement during development is essential for proper joint formation, and these findings suggest that the molecular events are largely regulated by the activation of Wnt signaling and downregulation of BMP signaling. The studies conducted thus far set the stage for further investigation of the regulatory dynamics between Wnt, BMP, and mechanical stimuli.[59] Exact mechanisms of how mechanical stimuli are transduced to regulate gene expression remains to be explained, but these studies have identified key candidates that are dysregulated during joint development by embryonic immobilization.

How is the transduction of mechanical stimuli regulated?

The importance of mechanical stimuli in joint development is clear. However, the ability to study mechanotransduction pathways is challenging *in vivo,* and often relies on *in vitro* studies to understand these pathways at the cellular level.[22] The activation of biological processes that are regulated by mechanical stimuli during development are dependent on the successful detection by cells to initiate a biological process. The transduction of mechanical signals may be regulated

by three mechanisms: 1) cell to extracellular matrix transduction, 2) cell to cell interactions, and 3) primary cilium sensing.[22] Both cellular forces and forces generated by tissue are able to guide different aspects of differentiation and maturation during development, but the challenge in this area of research is the bridging of events at the microscale and macroscale.[22] Elucidating how mechanical stimuli are integrated into molecular mechanisms of joint development will be essential for understanding the mechanical signaling environment.[19]

The microenvironment of the extracellular matrix provides many cues that are essential for cell homeostasis. A few key roles include providing cellular scaffolding, and sequestration of bioactive factors, which have been shown to influence cell shape, proliferation, migration, and differentiation.[60] The dynamics of the extracellular matrix are constantly changing during development, and microenvironmental cues initiates many of these changes. The mechanical properties of the extracellular matrix have been shown to be potential regulators of stem cell differentiation; cells differentiated according to the elasticity of the underlying substrate.[61] Thus, if the microenvironment of the extracellular matrix were disrupted during embryonic immobilization, the ability to maintain cell and tissue-specific phenotypes through extracellular matrix to cell mechanotransduction may be compromised.[22] The transmission of force is largely mediated by transmembrane integrins that are bound to extracellular matrix proteins and are relayed to the focal adhesion complex.[22] The ensuing cascades after reaching the focal adhesion complex is highly complex, but it is clear that the transduction of mechanical signals between cell and extracellular matrix can be instrumental in understanding how lack of movement during development alters basic cell biology and signaling pathways involved in joint formation.

Similarly to the transduction of mechanical stimuli between cell and extracellular matrix, cadherins are transmembrane proteins that transmit intracellular-generated forces from cell to

cell.[22] The mechanisms of cadherin signaling have yet to be explained, but have been shown to be involved in activation of Wnt signaling pathways during embryonic development.[62,63] In addition to the role of mediating cell to cell adhesion, cadherins have been involved in various aspects of tissue morphogenesis such as cell recognition and sorting, boundary formation in tissues, and coordination of cellular movements.[62] Taken together, the intracellular signaling pathways initiated by cell to cell mechanical cues may largely be in response to extracellular interactions.[22,62] That being said, there may be a great deal of overlap between cell to extracellular matrix and cell to cell interactions during joint development that could be dysregulated due to a lack of mechanical stimuli.

Lastly, primary cilium sensing has been recognized as another form in which cells can detect mechanical stimuli and is also an important organelle for various functions during development.[64] Primary cilia are able to sense mechanical signals such as tensile strain, fluid flow, and osmotic pressure, all of which are present throughout various musculoskeletal tissues.[65] The downstream signaling pathways associated with primary cilia sensing, however, are not entirely clear. Hedgehog signaling is one of the few pathways that have been established to be coordinated by primary cilia.[66] Wnt, transforming growth factor beta 1, and calcium signaling are among other signalling pathways implicated with primary cilia, but it has yet to be established whether these pathways are directly activated by primary cilium sensing.[64] Whether primary cilia is implicated in other components of joint formation through mechanotransduction remains unknown, but its association with hedgehog signaling implicates its role during endochondral ossification, as long bone growth is regulated by IHH.[36,37]

Conclusions and outlook

Movement is an essential source of mechanical stimuli during musculoskeletal development, and this is reflected in the clinical syndrome of AMC. The molecular mechanisms involved in the transduction of mechanical stimuli to generate a cellular response during joint cavitation has yet to be fully understood, but the involvement of mechanical stimuli during joint formation is clear. Molecular mechanisms associated with embryonic immobilization have recently started emerging and this provides insight to how lack of mechanical stimuli can perturb the regular processes during joint development and result in joint fusion. Although Wnt signaling has been identified as mechanosensitive and an important component during joint development, the inhibition of Wnt signaling alone results in less severe abnormalities in the joint region when compared to immobilization.[18] In the context of joint development, this suggests that multiple molecular mechanisms are mediated by mechanical stimuli and warrants further investigation. Moreover, the spatial territories of Wnt and BMP signaling, along with the contribution of joint progenitor cells, have been suggested to be important components of joint formation, and their regulatory mechanisms associated with mechanical stimuli require further investigation.[59] Experimental animal models have identified key molecular regulators that are perturbed during embryonic immobilization and this has emphasized the importance of mechanical stimuli in regulating musculoskeletal development. Understanding how tissue-generated and cellular forces are able to guide tissue differentiation will be critical in advancing our knowledge on the molecular regulation of musculoskeletal development as a whole.[22] Nevertheless, great strides have been made to identify potential molecular regulators of joint development that are sensitive to mechanical stimuli, and this establishes a promising starting point for future research.

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Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the author.

Figures

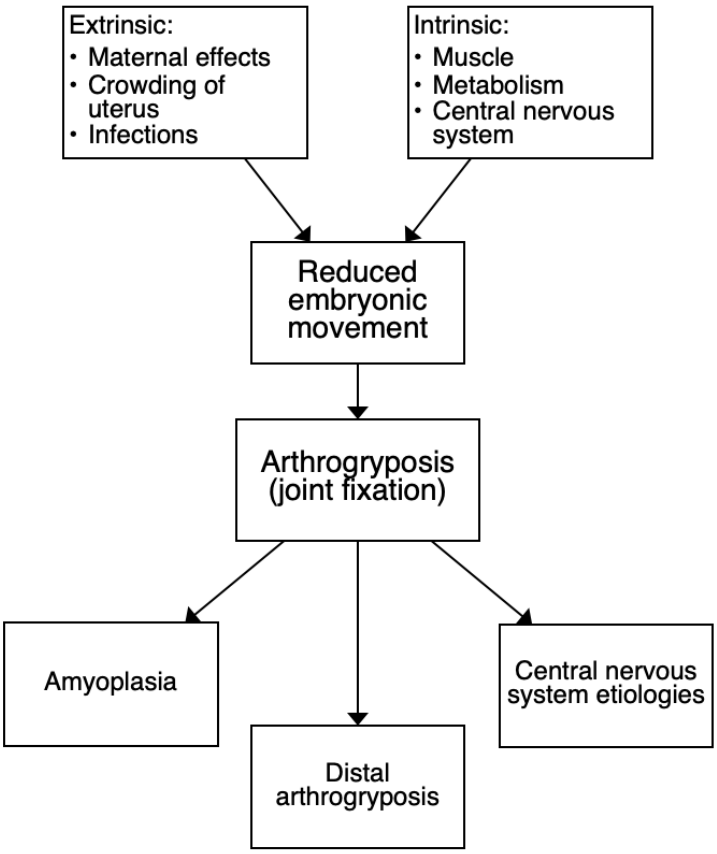

Figure 1. Overview of AMC. Extrinsic and intrinsic conditions both share a commonality of reducing embryonic movement, and in turn causes contracture formation during fetal development. AMC can then be categorized into 3 main groups: amyoplasia, distal arthrogryposis, and etiologies involving the central nervous system. [12,13]

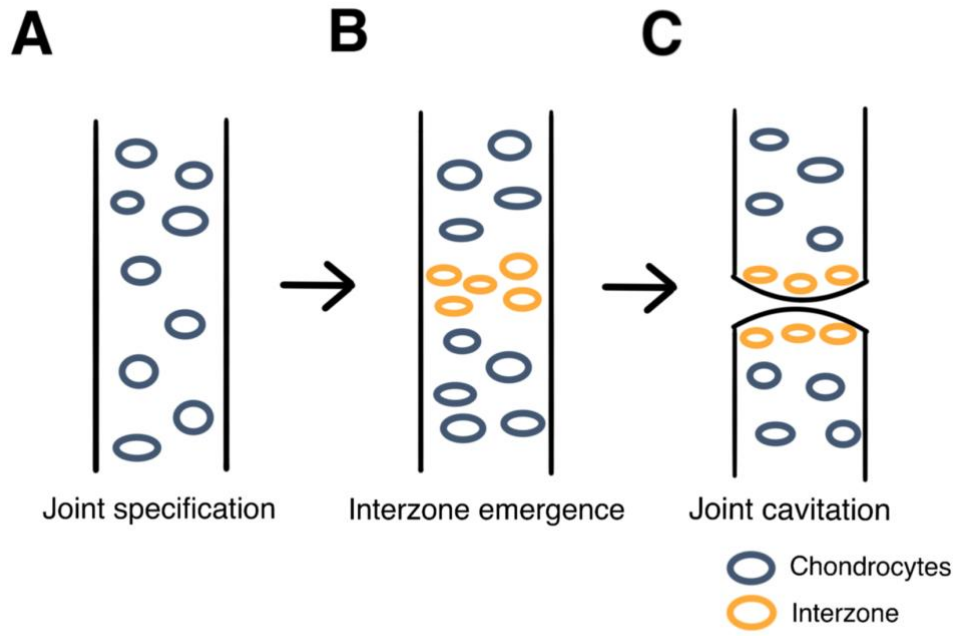

Figure 2. Simplified overview of joint specification and cavitation. (A) The early stages of joint formation start within a continuous pre-cartilaginous tissue characterized by chondrogenic markers. (B) Interzone cells gather at the presumptive joint site and chondrogenesis is suppressed. (C) The separation of opposing skeletal elements paves the way for the remainder of joint morphogenesis to proceed.

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Appendix III

Articular stiffness related to immobilization

Sauramps Medical

In: Julia M, Perrey S, Dupeyron A, Herisson C, eds. Le deconditionnement locomoteur: mécanismes, prevention et contre-mesures.

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Laneuville O, Zhou H, Campbell M, Uhthoff HK, Trudel G. Articular stiffness related to immobilization. In: Julia M, Perrey S, Dupeyron A, Herisson C, eds. Le déconditionnement locomoteur: mécanismes, prévention et contre-mesures. Collection: Pathologie locomotrice et médecine orthopédique. Sauramps Medical, 2022: 53-68. ISBN: 9791030302592.

Abstract

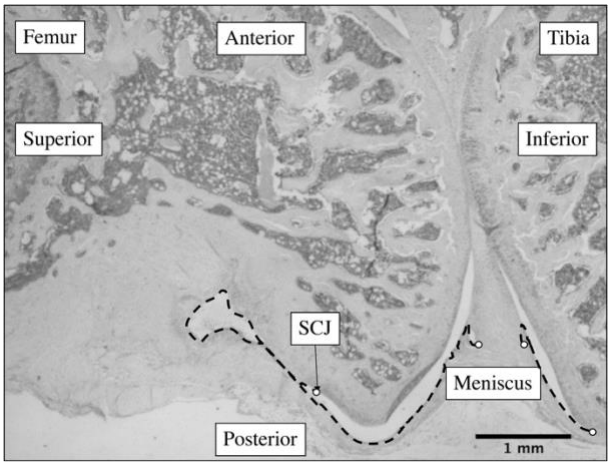
Experimental research on joint immobilization started more than 150 years ago. However innovative and effective approaches to preventing and treating joint contractures are still lacking. The development of novel research models and standardized techniques have allowed quantifying the salient features of articular stiffness related to immobilization. The articular capsule undergoes significant and irreversible changes, limiting the joint range of motion and participates in joint stiffness. Cartilage degeneration ensues with a characteristic histological presentation. Gene expression and protein products in both the capsule and cartilage changing as the duration of immobilization progresses can help identifying the modulators of these changes. New therapeutic approaches targeting these modulators can be tested in existing models and their effect measured with current techniques. New and effective clinical treatments are anxiously awaited by patients with articular stiffness arising from a number of conditions sharing the common denominator of joint immobilization.

Keywords: Contracture, immobilization, cartilage, capsule, reversibility, gene expression

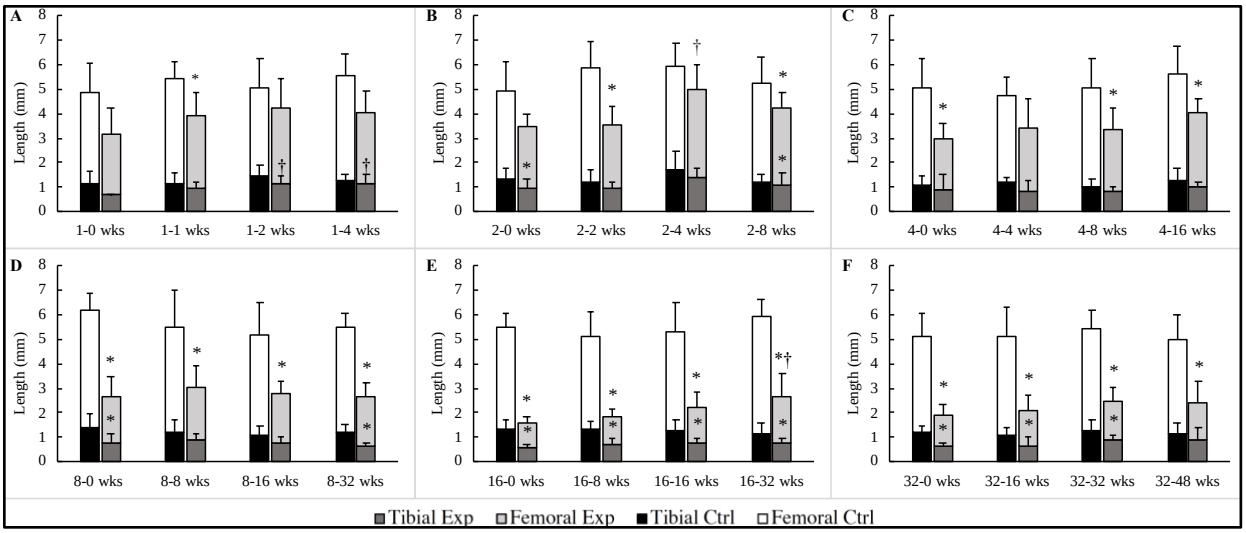
Appendix IV

Supplementary Material for Published Articles

Chapter 1: *Medicine & Science in Sports & Exercise*



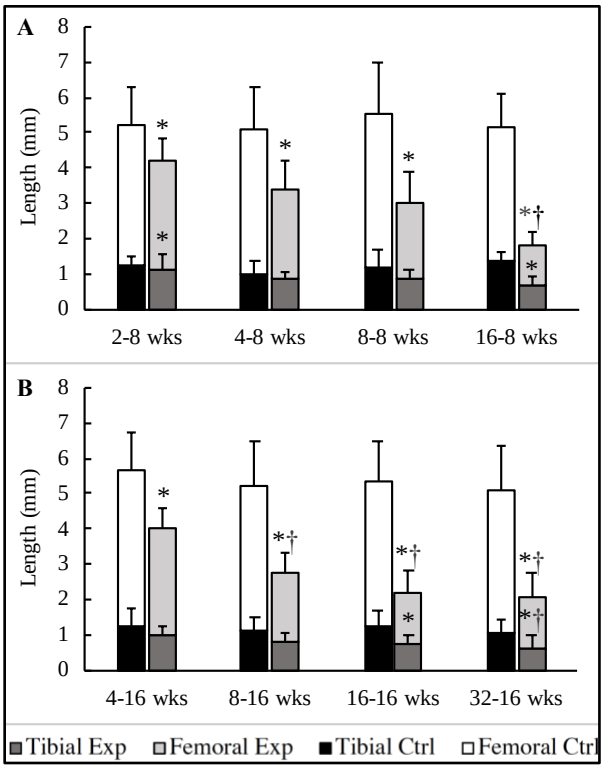
Supplementary Figure 1. Anatomical structures and orientation of the rat knee joint. 7µm section of the rat knee at the medial mid-condylar level were made in the sagittal plane. The segmented lines delineate the posterior capsule length measured from superior and inferior posterior horns of the meniscus to the femoral and tibial synovio-cartilage junction (endpoints represented by open circles). Note the longer femoral posterior capsule length compared to tibial. SCJ: synovio-cartilage junction. Magnification of 3.3X.



Supplementary Figure 2. Femoral and tibial posterior capsule length (mm) of rat knee joints following a fixed duration of immobilization and increasing durations of remobilization. Bar graphs represent femoral and tibial posterior capsule lengths, which are stacked on top of one another to equal the posterior capsule length. A-D) Immobilization of 1, 2, 4, or 8 weeks, followed by remobilization that were one, two, and four times the immobilization duration. E-F) Immobilization of 16 and 32 weeks, followed by remobilization that were one-half, one, and two times the immobilization duration, with the exception of group 32-48. *: significant difference compared to contralateral knee ($P<0.05$). †: significant difference compared to no remobilization ($P<0.05$). Corresponding statistical and significance values are listed in supplementary Tables 1 and 2.

Supplementary Table 1. Summary of statistics comparing posterior capsule length of different groups of experimental and contralateral knees with a fixed duration of immobilization and increasing durations of remobilization; Fig. 3, and Supp. Fig. 2. One-way ANOVA and Tukey post-hoc analysis was conducted. Post-hoc analysis was only conducted for the significantly different experimental group. *: (P<0.05).

Time Group	Exp (n)	Ctrl (n)	ANOVA – Experimental Significance			ANOVA – Contralateral Significance			Tukey Post-Hoc – Experimental		
			Total	Femoral	Tibial	Total	Femoral	Tibial	Total	Femoral	Tibial
1-0	6	8	0.412	0.758	0.026*	0.616	0.543	0.574	-	-	-
1-1	8	7							-	-	-
1-2	10	8							-	-	1-0 (0.034)
1-4	9	6							-	-	1-0 (0.044)
2-0	8	9	0.006*	0.018*	0.043*	0.285	0.264	0.209	-	-	-
2-2	10	9							2-4 (0.013)	2-4 (0.040)	-
2-4	10	9							2-0 (0.012)	2-0 (0.029)	-
2-8	7	8							-	-	-
4-0	7	9	0/268	0.179	0.815	0.420	0.450	0.518	-	-	-
4-4	10	9							-	-	-
4-8	8	10							-	-	-
4-16	11	11							-	-	-
8-0	11	11	0.690	0.908	0.237	0.366	0.544	0.574	-	-	-
8-8	10	9							-	-	-
8-16	9	10							-	-	-
8-32	10	9							-	-	-
16-0	10	9	0.029*	0.028*	0.366	0.386	0.169	0.752	-	-	-
16-8	9	8							-	-	-
16-16	10	9							-	-	-
16-32	10	9							16-0 (0.025)	16-0 (0.025)	-
32-0	10	7	0.376	0.803	0.139	0.852	0.931	0.621	-	-	-
32-16	14	13							-	-	-
32-32	9	10							-	-	-
32-48	11	10							-	-	-



Supplementary Figure 3. Femoral and tibial posterior capsule length (mm) of rat knee joints following increasing durations of immobilization with a fixed duration of remobilization. A-B) Bar graphs represent femoral and tibial posterior capsule lengths, which are stacked on top of one another to equal the posterior capsule length. Remobilization of 8 and 16 weeks, each with durations of immobilization that were one-quarter, one-half, one, and two times the duration of remobilization. *: significant difference compared to contralateral knee ($P<0.05$). †: significant difference compared to earliest time point ($P<0.05$). Corresponding statistical and significance values are listed in supplementary Tables 2 and 3.

Supplementary Table 2. Summary of statistics comparing posterior capsule lengths between experimental and contralateral rat knees within the same time groups. In reference to Figs. 3, 4, and Supp. Figs. 2, 3. Paired t-test analysis was conducted. *: (P<0.05).

Time Group	Paired Knees (n)	Paired t-test		
		Total	Femoral	Tibial
1-0	5	0.261	0.284	0.242
1-1	5	0.121	0.047*	0.705
1-2	8	0.065	0.337	0.172
1-4	6	0.110	0.115	0.764
2-0	7	0.066	0.152	0.011*
2-2	9	<0.001*	<0.001*	0.107
2-4	9	0.108	0.219	0.281
2-8	6	0.031*	0.045*	0.004*
4-0	6	0.010*	0.003*	0.380
4-4	8	0.092	0.098	0.157
4-8	8	0.005*	0.001*	0.463
4-16	10	0.011*	0.020*	0.269
8-0	11	<0.001*	<0.001*	0.015*
8-8	9	0.002*	0.004*	0.111
8-16	9	<0.001*	<0.001*	0.071
8-32	9	<0.001*	<0.001*	<0.001*
16-0	8	<0.001*	<0.001*	<0.001*
16-8	7	<0.001*	<0.001*	<0.001*
16-16	9	<0.001*	<0.001*	0.012*
16-32	8	<0.001*	<0.001*	0.003*
32-0	7	<0.001*	0.003*	<0.001*
32-16	13	<0.001*	<0.001*	0.005*
32-32	7	<0.001*	<0.001*	0.004*
32-48	8	<0.001*	<0.001*	0.067

Supplementary Table 3. Summary of statistics comparing posterior capsule lengths of different groups of experimental and contralateral knees with a fixed duration of remobilization and increasing durations of immobilization. In reference to Fig. 4 and Supp. Fig. 3. One-way ANOVA and Tukey post-hoc analysis was conducted. Post-hoc analysis was only conducted for the significantly different experimental group. *: (P<0.05).

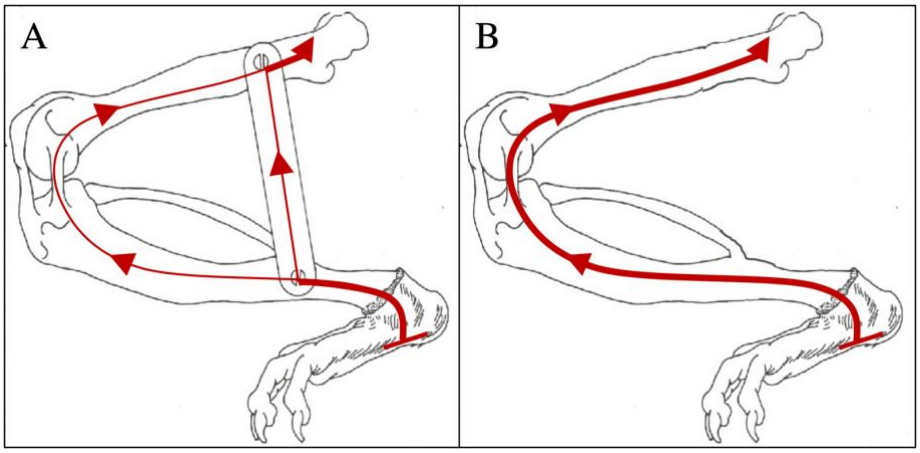
Time Group	Exp (n)	Ctrl (n)	ANOVA-Experimental Significance			ANOVA-Contralateral Significance			Tukey Post-Hoc - Experimental		
			Total	Femoral	Tibial	Total	Femoral	Tibial	Total	Femoral	Tibial
2-8	7	8	<0.001*	<0.001*	0.068	0.905	0.871	0.338	-	-	-
4-8	8	10							-	-	-
8-8	10	9							-	-	-
16-8	9	8							2-8 (<0.001)	2-8 (<0.001)	-
4-16	11	11	<0.001*	<0.001*	0.035*	0.828	0.915	0.668	-	-	-
8-16	9	10							4-16 (0.003)	4-16 (0.004)	-
16-16	10	9							4-16 (<0.001)	4-16 (<0.001)	-
32-16	14	13							4-16 (<0.001)	4-16 (<0.001)	4-16 (0.020)

Supplementary Table 1. Distribution of knee sample sizes for all time points between experimental and contralateral knees.

Groups	Experimental (n)	Contralateral (n)	Paired (n)
1-0	10	10	10
1-1	9	8	8
1-2	10	10	10
1-4	10	9	9
2-0	10	8	8
2-2	10	10	10
2-4	9	10	9
2-8	9	10	9
4-0	9	10	9
4-4	11	11	11
4-8	10	10	10
4-16	10	10	9
8-0	11	11	11
8-8	10	10	10
8-16	10	10	10
8-32	10	11	10
16-0	9	9	9
16-8	10	10	10
16-16	11	11	11
16-32	12	16	12
32-0	11	11	11
32-16	9	11	8
32-32	10	11	10
32-48	10	10	10

Supplementary Table 2. Distribution of rat and knee sample sizes for age-matched unoperated control groups with corresponding experimental groups.

Age of unoperated controls (weeks)	Number of rats (n)	Number of knees (n)	Corresponding experimental groups
12	4	6	1,0 1-1, 2-0.
14	5	10	1-2, 2-2, 4-0.
18	5	10	1-4, 2-4, 4-4, 8-0.
22	5	10	2-8, 4-8.
30	5	10	4-16, 8-8, 16-0.
38	4	8	8-16, 16-8.
42	3	6	8-32, 16-16, 32-0.
86	6	12	16-32, 32-16, 32-32, 32-48.



Supplementary Fig. 1. Illustration of the ground reaction force distribution during immobilization with an internal fixator and after hardware removal. A) During immobilization, part of the ground reaction forces is borne by the tibiofemoral joint and part is conveyed by the internal fixation plate and screws. The net result is a decrease in ground reaction forces through the proximal tibia and distal femur possibly explaining the bone marrow alterations in the proximal tibia epiphysis. B) After removal of the immobilization hardware, all the ground reaction forces are conveyed through the knee joint. The net result is a restoration of the ground reaction forces going through the proximal tibia and distal femur possibly explaining the bone marrow restoration in the proximal tibia epiphysis. A thicker line in the figure indicates a larger amount of force being conveyed. Note that the permanently reduced range of knee extension concentrates the forces to a reduced femorotibial contact area.

Appendix V

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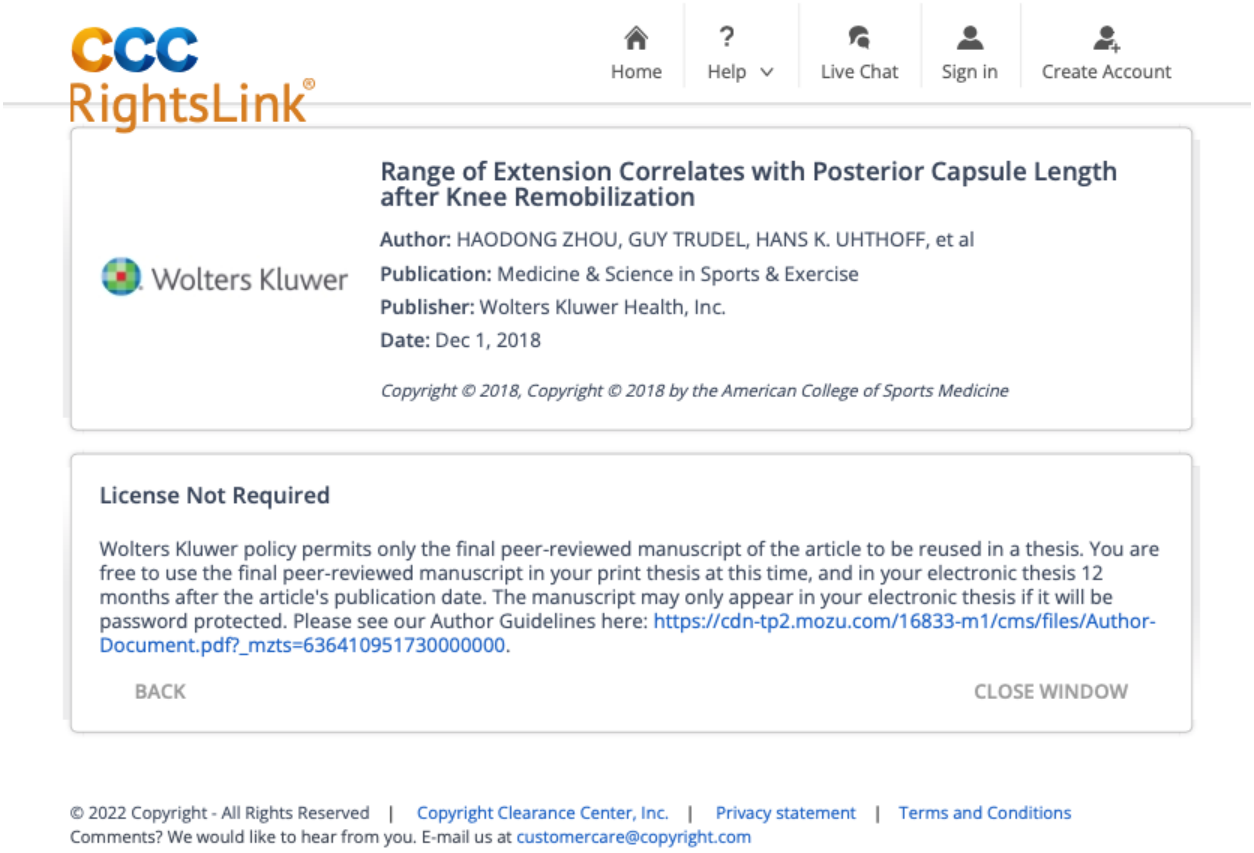

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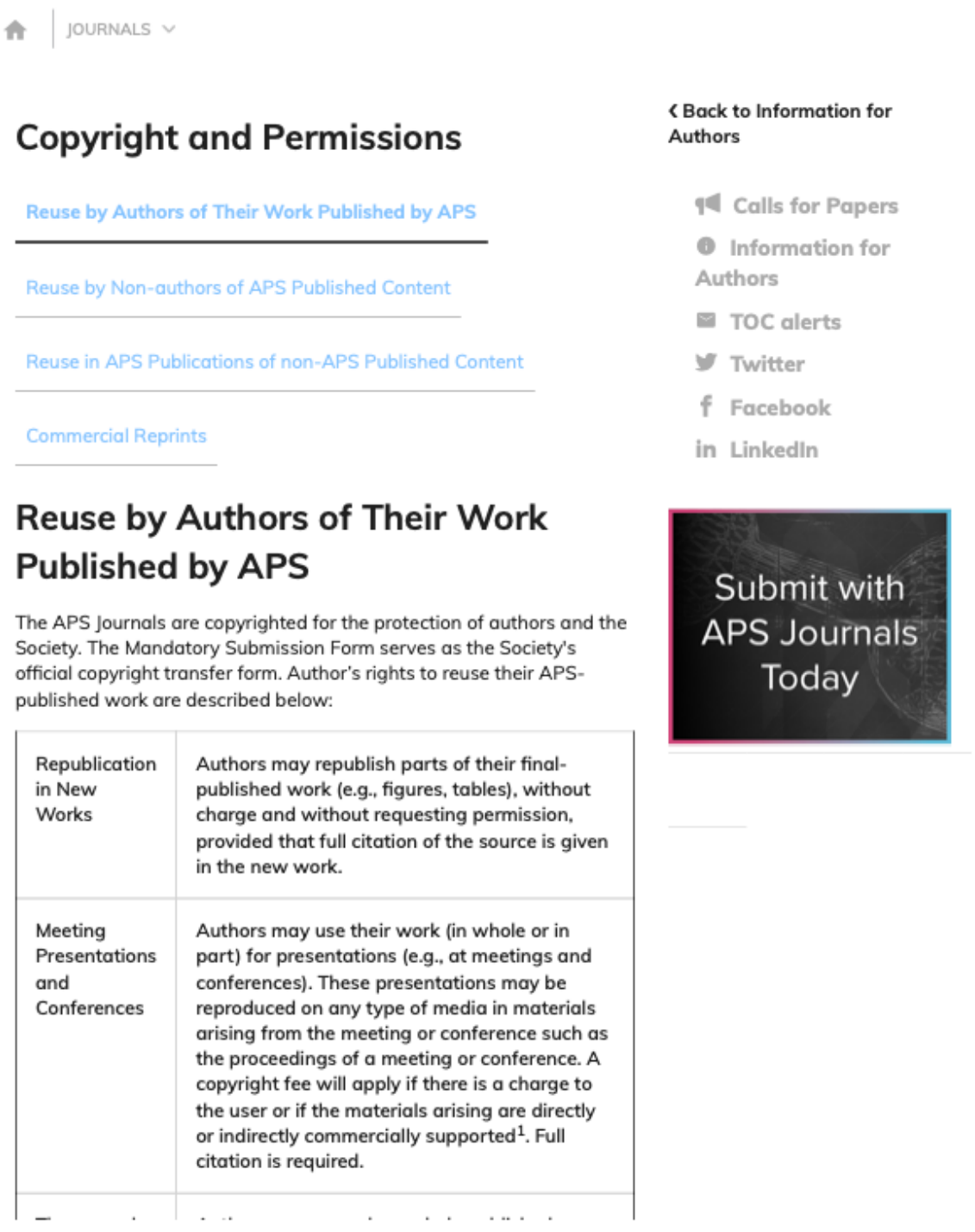

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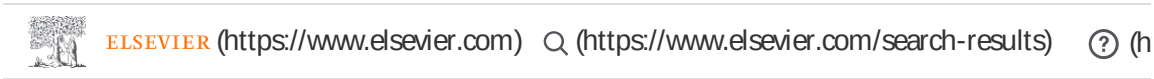

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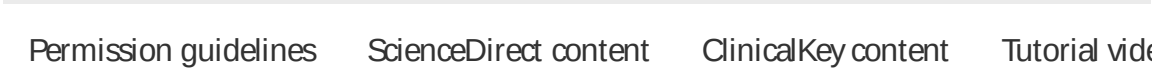

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BioEssays

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Protocol Detail Report

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Report Comments

Protocol Information

Version # 1

Reference Number	2461		
Protocol Number	ME-2461		
Protocol Type:	Original		
Principal Investigator:	Trudel, Guy	**Approval Date:**	7/23/2015
Submittal Date:	6/26/2015	**Effective Date:**	7/23/2015
Author:	Nie, Winnie	**Renewal Date:**	7/23/2016
Status:	Approved	**Next Review Date:**	7/23/2016
Inactive Date:		**Expiration Date:**	7/23/2019

Overview 1

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Protocol Number 1.4

ME-2461