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Increased lubricin/proteoglycan 4 gene expression and decreased modulus in medial collateral ligaments following ovariohysterectomy in the adult rabbit: Evidence consistent with aging

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ABSTRACT

This study investigated whether ovariohysterectomy (OVH) surgery to induce menopause resulted in changes to modulus, failure strain and lubricin/proteoglycan 4 (PRG4) gene expression in rabbit medial collateral ligaments (MCLs), similar to aging (Thornton et al., 2015a). The MCLs from adult rabbits that underwent OVH surgery as adolescents (15-week-old) and adults (1-year-old) were compared by evaluating mechanical behaviour (adolescent OVH, $n=8$; adult OVH, $n=7$; normal, $n=7$), gene expression (adolescent OVH, $n=9$; adult OVH, $n=8$; normal, $n=8$), and collagen and glycosaminoglycan (adolescent OVH, $n=9$; adult OVH, $n=8$; normal, $n=8$) and water (adolescent OVH, $n=9$; adult OVH, $n=8$; normal, $n=8$) content. Mechanical behaviour evaluated cyclic, static and total creep strain, and ultimate tensile strength, modulus and failure strain. The RT-qPCR assessed mRNA levels for matrix regulatory genes. Adult OVH MCLs exhibited increased cyclic creep and failure strain, and decreased modulus with increased mRNA levels for lubricin/PRG4 and collagen I compared with normal MCLs. Adolescent OVH MCLs exhibited increased cyclic, static and total creep strain with decreased mRNA levels for the progesterone receptor. Lubricin/PRG4 plays a role in the lubrication of collagen fascicles which is likely related to the decreased modulus and increased failure strain observed in ligaments from adult OVH rabbits. Progesterone and its receptor are thought to play a role in the stretching of ligaments in pelvic organ prolapse and pregnancy which is likely related to the increase in creep strain observed in ligaments from adolescent OVH rabbits. Ovariohysterectomy in adult rabbits resulted in changes that were consistent with the aging MCL.

1. Introduction

Female menopause is characterized by a decrease in ovarian hormone production (estrogen and progesterone) and a reciprocal increase in pituitary hormone production (Burger, 1996). In developed countries, the onset of menopause occurs typically between the ages of 50–52 years and brings with it an increased risk of cardiovascular disease, osteoporosis and possibly osteoarthritis (Lobo et al., 2014). Estrogen and progesterone receptors have been identified in ligaments; for example, human anterior cruciate ligament (ACL) (Liu et al., 1996; Sciore et al., 1998), and rabbit ACL and medial collateral ligament (MCL) (Sciore et al., 1998). Correspondingly, ligament mechanical properties may be affected by hormonal changes, such as those associated with menstrual cycles, pregnancy and menopause. Alterations in the mechanical behaviour of ligaments can lead to ligament damage accumulation, abnormal joint loading, decreased joint function and contribute to progression of osteoarthritis.

Previous studies on estrogen and ligament failure properties have examined the effect of ovariectomy on adult animals, thereby exposing the mature ligament to decreased levels of estrogen. In MCLs and ACLs from adult sheep (Strickland et al., 2003), ovariectomy did not affect failure force, stiffness or ultimate tensile strength (UTS). In ACLs from adult monkeys (Wentorf et al., 2006), ovariectomy did not affect failure force, stiffness, failure elongation, UTS, modulus or failure strain. In adult rabbits that underwent ovariectomy surgery as late post-pubertal adolescents (21-week-old), Räsänen and Messner (2000) found no effect of

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ovariectomy on MCL failure force, UTS and modulus. The above studies focused on high-load mechanical behaviour, like failure, rather than low-load mechanical behaviour, like creep.

Our recent study regarding the effect of aging on rabbit MCLs revealed that modulus was decreased and failure strain was increased comparing MCLs from 3-year-old with 1-year-old rabbits. These age-related mechanical changes were associated with increased lubricin/proteoglycan 4 (PRG4) gene expression which may have implications for ligament function through increased collagen fascicle lubrication (Thornton et al., 2015a). We had yet to evaluate the timing of OVH on failure properties other than UTS (Thornton et al., 2015b), like modulus and failure strain, where decreased modulus could indicate accumulation of damage or increased potential for damage.

Collagen fibril diameters in rabbit MCLs achieved the typical bimodal distribution by 14 weeks of age and maintained that bimodal distribution at 1 year of age while MCL volume and weight increased (Lo et al., 2004). The mRNA levels for collagens (I, III, V) and biglycan decreased while those for decorin increased from 14-week-old to 1-year-old rabbit MCLs, suggesting potential roles in arrest of collagen fibril diameter growth (Lo et al., 2004). In rabbits, puberty occurs around 12–14 weeks of age (Achari et al., 2008). Ovariohysterectomy in early post-pubertal adolescents (15-week-old) may affect the MCL at this stage which has not reached its final volume or weight but has reached a bimodal collagen fibril diameter distribution.

In this study, we examined MCLs from adult rabbits that had undergone OVH surgery as early post-pubertal adolescents (15-week-old) or adults (1-year-old). Our purpose was to examine the effect of timing of ovariohysterectomy (OVH) on MCLs by comparing creep behaviour (cyclic, static and total creep strain), failure behaviour (modulus, failure strain, UTS), molecular expression of genes of interest (collagens, proteoglycans, hormone receptors, matrix metalloproteinases and their inhibitors), and biochemical assessments (water, collagen and glycosaminoglycan content). Our first hypothesis was that MCLs from adult OVH rabbits would exhibit increased failure strain, decreased modulus and increased lubricin/PRG4 gene expression compared with MCLs from normal rabbits, similar to the findings associated with aging in this pre-clinical model. Our second hypothesis was that MCLs from early post-pubertal adolescent OVH rabbits would have no change in modulus or failure strain but would have increased creep strain compared with MCLs from normal rabbits.

2. Methods

The MCLs from female New Zealand White rabbits were used in this study approved by the University of Calgary Animal Care Committee. Two groups underwent ovariohysterectomy (OVH) surgery where the ovaries and uterus are removed because both affect estrogen levels in rabbits. In the adolescent OVH group, the OVH surgery was performed on 15-week-old rabbits and assessments were performed at a minimum of 48 weeks of age. In the adult OVH group, the OVH surgery was performed on 1-year-old rabbits and assessments were performed at a minimum of 14 weeks post-OVH. The MCLs from seventeen adolescent OVH rabbits underwent mechanical ($n=8$), molecular ($n=9$), collagen and glycosaminoglycan (GAG) content ($n=9$) and water content ($n=9$) assessments. The MCLs from eighteen adult OVH rabbits underwent mechanical ($n=7$), molecular ($n=8$), collagen and GAG content ($n=8$) and water content ($n=8$) assessments. The MCLs from fifteen normal (1-year-old) rabbits underwent mechanical ($n=7$), molecular ($n=8$), collagen and GAG content ($n=8$) and water content ($n=8$) assessments as previously reported (Thornton et al., 2015a). The complete MCL was evaluated for the mechanical and water content assessments. For the molecular and biochemical assessments, the middle portion of the MCL was used for the molecular evaluation, and even distributions of the anterior and posterior portions of the MCL were used for collagen and GAG content evaluations. The MCL used for molecular and biochemical assessments was contralateral to the MCL used for water content assessment except for 2 cases where it was contralateral to the MCL used for mechanical assessments and 3 cases where it was contralateral to an MCL used in a different study. Likewise, the MCL used for water content assessments was contralateral to the MCL used for molecular and biochemical assessments except for 5 cases where it was contralateral to an MCL used in a different study. Also, the remaining MCLs that were contralateral to the MCLs for mechanical assessments were used in a different study.

2.1. Mechanical

Hindlimbs were dissected and the menisci and ligaments were left intact. To accommodate the grips, the femur was cut 30 mm proximal to the MCL insertion and the tibia was cut at the fibula–tibia junction. First, the tibia was cemented (polymethylmethacrylate) in the upper grip before being mounted in series with a 500 N load cell. The MCL was aligned with the load axis of the actuator (MTS Systems Corporation, Minneapolis, MN, USA) and the force was zeroed. Next, the femur was cemented in the lower grip with the knee at approximately 70° of flexion and the displacement was zeroed.

After completing two compression–tension cycles from −5 N to +2 N, the lateral collateral ligament, medial and lateral meniscus, and anterior and posterior cruciate ligaments were removed to isolate the MCL. Two additional compression–tension cycles were completed stopping at +0.1 N to establish "ligament zero" and the displacement was zeroed. The MCL midsubstance cross-sectional area was measured using area calipers (Shrive et al., 1988) and the MCL length was measured using digital calipers. After the environment chamber equilibrated (37 °C and 99% relative humidity), two additional compression–tension cycles were used to re-establish "ligament zero".

The MCL underwent 30 cycles at 1 Hz from 0.1 N to a force corresponding to 4.1 MPa for cyclic creep. The MCL was held at a force corresponding to 4.1 MPa for 20 min for static creep. Following a 20-min recovery period, the MCL was elongated to failure at 20 mm/min.

Strain was deformation divided by MCL length. Cyclic creep strain was the increase in strain from the peak of the first cycle to the peak of the 30th cycle in the cyclic creep test. Static creep strain was the increase in strain from the start to the end of the 20-min static creep test. Total creep strain was the increase in strain from the peak of the first cycle in the cyclic creep test to the end of the 20-min static creep test. Failure strain was deformation at failure force divided by MCL length and UTS was failure force divided by MCL cross-sectional area. Modulus was calculated as the slope of the linear regression of the upper 50% of the failure stress–strain curve.

2.2. Molecular

Tissues were dissected, immediately frozen in liquid nitrogen, and powdered with a Braun Dismembrator (B. Braun Biotech Inc., Allentown, PA, USA). Total RNA was isolated using the TRIspin method (Reno et al., 1997) and quantified on a Turner 450 fluorescence spectrofluorometer (Barnstead/Thermolyne, Dubuque, IA, USA) with a standard of calf liver ribosomal RNA. Total RNA (1 μg) was reverse transcribed using an Omniscript RT kit (Qiagen Inc., Valencia, CA, USA). Aliquots were used in real-time RT-qPCR using primers for the target genes (Table 1). The RT-qPCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad). Values for individual genes were normalized and expressed as relative to 18S.

2.3. Biochemical

2.3.1. Water content

The MCL was weighed immediately on a microbalance to obtain the wet weight and then dried under vacuum until the dry weight ceased to change (Funakoshi et al., 2007). Water content was calculated as the difference in ligament wet weight and dry weight divided by wet weight and then expressed as a percentage.

2.3.2. Collagen and glycosaminoglycan (GAG) content

2.3.2.1. Collagen assay. The collagen content was determined using the Sircol Collagen Assay (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Following the kit protocol, collagen was extracted from the tissues with a 1.0 mL aliquot of 0.1 mg pepsin/mL 0.5 M acetic acid and the extraction was carried out overnight at 4 °C. The extraction was repeated with an additional 1.0 mL aliquot of the pepsin/acetic acid solution and the two extractions were combined. An aliquot of 1.0 mL of the combined extraction was used for each assay. Aliquots (200 μL) of the final supernatant were transferred to a 96-well plate and analyzed with a micro-plate plate reader at 550 nm (Bio-Rad Benchmark Plus Microplate Spectrophotometer with Bio-Rad Software Microplate Manager 5.2). Collagen content was determined from the standard curve and expressed as μg Collagen/mg wet weight.

2.3.2.2. GAG assay. The glycosaminoglycan (GAG) content was determined using the Blyscan Sulfated Glycosaminoglycan Assay (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Following the kit protocol, tissues were digested with 1.0 mL of papain buffer and the digestion was carried out overnight at 65 °C. An aliquot of 100 μL of the digestion was used to continue as per instructions in the

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