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Effects of a viscosupplementation therapy on rabbit menisci in an anterior cruciate ligament transection model of osteoarthritis

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ABSTRACT

The aim of this study was to evaluate the morphological, microstructural, and mechanical effects of a viscosupplementation therapy on rabbit menisci at an early stage of osteoarthritis (OA). Anterior cruciate ligament transection (ACLT) was performed in twelve male New-Zealand White rabbits on the right knee joint. Six of these twelve rabbits received a mono intra-articular injection of high molecular weight hyaluronic acid (HA) two weeks after ACLT. Six additional healthy rabbits served as controls. Medial menisci were removed from all right knees ($n = 18$) six weeks after ACLT and were graded macroscopically. Indentation-relaxation tests were performed in the anterior and posterior regions of the menisci. Collagen fiber organization and glycosaminoglycan (GAG) content were assessed by biphotonic confocal microscopy and histology, respectively. Viscosupplementation significantly ($p = 0.002$) improved the surface integrity of the medial menisci compared to the operated non-treated group. Moreover, the injection seems to have an effect on the GAG distribution in the anterior region of the menisci. However, the viscoelastic properties of both operated groups were similar and significantly lower than those of the healthy group, which was explained by their modified collagen fiber organization. They displayed disruption of the tie fibers due to structural alterations of the superficial layers from which they emanate, leading to modifications in the deep zone. To conclude, the viscosupplementation therapy prevents macroscopic lesions of the menisci, but it fails to restore their collagen fiber organization and their viscoelastic properties. This finding supports the role of this treatment in improving the lubrication over the knee.

0. Introduction

Menisci are essential for load transmission and shock absorption across the knee (Makris et al., 2011). In healthy joints, these functions are fulfilled thanks to their complex mechanical behavior, which is mainly governed by their particular extracellular matrix (Danso et al., 2015; Tissakht and Ahmed, 1995). In a previous study (Levillain et al., 2017), we have demonstrated that post-traumatic osteoarthritic (OA) menisci were torn at an early stage of disease progression. Moreover, they displayed a disruption of the tie collagen fibers in the posterior region as well as a decrease in the GAG content in the anterior region, leading to modifications of the viscoelastic properties in both regions. These alterations contribute to the progression of OA and often cause cartilage loss and subchondral bone defects (Iijima et al., 2014). Currently, there is no efficient treatment able to restore the meniscal properties in OA knees.

OA knees exhibit a decrease in the concentration and the molecular weight of hyaluronic acid (HA) in the synovial fluid (Moreland, 2003; Watterson and Esdaile, 2000), altering its lubricative properties. Viscosupplementation is a widely used intra-articular therapy for the non-operative management of patients with symptomatic OA, which consists in replacing the lost HA within the joint (Strauss et al., 2009). Several injectable forms of HA are approved by the Food and Drug Administration (Migliore et al., 2010). Among them, Hylan G-F 20 is a high-molecular-weight crosslinked HA composed of hylan A mixed with hylan B, of which insolubility delays its removal from the joint. It is now well-established that HA injections provide pain relief and functional improvement in OA knees (Hemplfling, 2007; McArthur et al., 2012), but their effects on the menisci have been poorly investigated (Hope et al., 1993; Sonoda et al., 2000; Takahashi et al., 2001). Moreover, these

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studies were mostly limited to morphological and biochemical assessments. Thus, the effects of viscosupplementation therapy on the mechanical properties of the menisci are still unknown.

The aim of this study was to evaluate the effects of intra-articular injection of HA on the medial menisci in an anterior cruciate ligament transection (ACLT) rabbit model of OA. The surface integrity of the menisci, their collagen fiber organization, their GAG content and their viscoelastic properties were assessed in the anterior and posterior regions through macroscopic grading, biphotonic confocal microscopy, histology, and indentation-relaxation testing, respectively.

1. Materials and methods

1.1. Animal model

All of the experiments and procedures involving animals were approved by the local ethics committee (ComEth Anses/ENVA/UPEC number 16) and were performed in full accordance with European legislation. Eighteen healthy adult male New Zealand White rabbits (six months of age, 3.8 kg in weight on average) that were free of degenerative joint disease (absence of swelling and normal aspect and viscosity of the synovial fluid) were obtained from a licensed vendor (EUROLAP, Gosné, France). After two weeks in acclimatization and quarantine, two groups of six and twelve rabbits were randomly constituted. Experimental OA was surgically induced by ACLT, performed by the same trained veterinary surgeon, in the right knee (stifle) of twelve rabbits. The complete rupture of the anterior (cranial) cruciate ligament was assessed with the anterior drawer sign (manual horizontal dislocation) before the closure of the articular capsule. The contralateral left limb was not operated upon. The right operated limb was not immobilized postoperatively and the rabbits were allowed to move freely in their individual cages after surgery. The remaining six rabbits were not operated upon, and are called "healthy rabbits" hereafter. Two weeks after ACLT, six of twelve operated rabbits received a single 300 μL intra-articular injection of Hylan G-F 20 (Synvisc one®) (concentration: 48 mg/6 mL; molecular weight: $6 \cdot 10^3$ kDa) in the right knee. These six rabbits are called "operated treated rabbits" and the six other operated rabbits are called "operated non-treated rabbits" hereafter. After a six-week observation period, all rabbits were euthanized. The right knees were explanted and carefully dissected, and the medial menisci were detached. Macroscopic changes in the femoral meniscal surface and in the articular cartilages of femoral and tibial condyles, as well as osteophyte production, were graded by two blinded reviewers in the medial compartment of right healthy ($n = 6$), operated non-treated ($n = 6$), and operated treated ($n = 6$) knees using the macroscopic grading system developed by Laverty et al. (2010) and given in Table 1.

1.2. Experimental procedure

The medial menisci ($n = 17$) from the right knee of all rabbits were stored at -20 °C in wet compresses soaked with 10× PBS until ready for use in subsequent biomechanical and micro-architectural analyses. After thawing the menisci for one day at 4 °C, two slices measuring 2 mm in width were cut with a scalpel in the anterior and posterior regions (Fig. 1A), and each sample was cut parallel to the tibial meniscal surface, approximately 1 mm above this surface. Indentation-relaxation tests were performed on the (x, y) horizontal plane in the vertical direction (Fig. 1B). Next, the tibial and femoral surfaces and deep zone of each sample were imaged by biphotonic confocal imaging (Fig. 1C). Finally, the GAG content was quantified on the indented surface of each sample by histology.

Table 1
Grading system used to quantify the meniscus and articular cartilage degradation as well as osteophyte formation (Laverty et al., 2010).

Meniscus (femoral surface)
1: Normal
2: Minimal fibrillation
3: Moderate surface fibrillation but no tears
4: Severe fibrillation, incomplete tears
5: Complete tears, bucket-handle tears or multiple incomplete tears
Articular cartilage (tibial and femoral condyles)
0: Surface smooth with normal color
1: Surface rough with minimal fibrillation or a slight yellowish discoloration
2: Cartilage erosion extending into the superficial or middle layers
3: Cartilage erosion extending into the deep layers
4: Complete cartilage erosion with subchondral bone exposed
Osteophyte formation (tibial plateau, femoral condyles and trochlea)
0: Absence
1: Mild
2: Moderate
3: Severe

1.3. Mechanical analyses

Indentation-relaxation tests were performed on meniscus samples immersed in PBS at 25 °C using a commercial Nanoindenter (Agilent Nanoindenter G200; ScienTec, Les Ulis, France). The indenter was a spherical sapphire tip with a radius of curvature, R, of 0.479 mm. The tibial surface (opposite to the indented surface) was glued onto an aluminium support (glue 3; Loctite®). Indentation tests were conducted on three locations with a minimum spacing of 200 μm between two locations. Each series of tests on three locations was repeated three times. The difference between the calculated moduli on each point was always less than 5%. A constant displacement rate of 5 μm s^{-1} and a penetration of 100 μm were imposed to avoid surface and fiber disruption effects. Contact with the sample was defined from a slope of 5 N/m in the load *vs* displacement curve, corresponding to a sharp change in the contact stiffness. The indenter displacement was then maintained for 400 s until equilibrium was reached. Unloading was carried out at 0.5 μm/s.

Both instantaneous and equilibrium moduli were determined from the resultant force-time data using a previously described method (Levillain et al., 2017). The elastic fraction, f, was calculated according to Eq. (1).

$$f = \frac{E_{eq}}{E_{ins}} \quad (1)$$

where E_{eq} and E_{ins} are the equilibrium and instantaneous moduli, respectively.

The elastic fraction describes the elastic/viscous behaviour of the material: $f = 1$ corresponds to a perfectly elastic material, whereas $f = 0$ corresponds to a perfectly viscous material (Oyen, 2011). These parameters were calculated for each indent and were then averaged for each sample. As classically performed in nanoindentation, a rubber reference material was indented before each series of tests in order to calibrate the device.

1.4. Microscopic imaging and grading

The collagen microstructure was observed by biphotonic confocal imaging (A1RMP PLUS®, Nikon) using an excitation wavelength of 850 nm. Second harmonic generated light from collagen was collected at a channel with a specific band-pass filter of 400–490 nm. A 25×, 1.1-NA water immersion objective (CFI Apo LWD 25XW; Nikon) was used. The image field of view was 512 × 512 μm^2 with a resolution of 0.5 μm. To scan the thickness of the meniscus, stacks of 2D images were recorded in each area, with a time scan of 2 s and an average of two scans per image, every 2 μm from 0 to 200 μm in depth.

The whole stack of 2D images of the tibial and femoral surfaces of the menisci was projected using ImageJ 1.47v (NIH, Bethesda, Maryland, USA) on a single slice. Each pixel of the output image contained the maximum intensity value over all of the images in the stack at the particular pixel location. Moreover, 3D reconstructions of the image stack acquisitions in the deep zone were performed using NIS element Viewer (Nikon Instruments Europe B.V, France) to characterize the organization of the circumferential collagen fibers. The organization of the tie fibers with respect to the tibial and femoral superficial layers was characterized using the following grading system (Levillain et al., 2017): grade 0: the tie fibers emanate from both surfaces; grade 1: the tie fibers are only linked to the tibial or the femoral surface of the meniscus; grade 2: the tie fibers are not linked to any surface; and grade 3: no tie fibers are detected.

1.5. Detection of GAGs

After microscopic imaging, the meniscal samples were fixed in 10% formalin for GAG semi-quantification. They were then embedded in paraffin and 4-μm-thick sections parallel to the indented surface were sliced using a Microm HM 340 E microtome. In each sample, at least two sections were stained with Safranin O-fast green (SOFG), which turns GAGs red.

Histological sections were imaged using an Eclipse TS100 microscope and a DS-FI2 color camera (Nikon instruments). Red coverage of SOFG staining was semi-quantitatively analyzed using ImageJ (Killian et al., 2010). Color images (Fig. 2A) were first converted to Red-Green-Blue stacks and were viewed as grey-scale images under the blue stack (Fig. 2B). Tissue appeared light, and SOFG-positive stained regions appeared dark. Images were analyzed using the threshold function with a black to red ratio of 1:3 (Fig. 2C). The percentage of GAG coverage was then measured for each section and was averaged for each sample.

1.6. Statistical analyses

Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria). The differences in the macroscopic scores, mechanical data, microscopic score, and GAG coverage among the healthy, operated