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

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Article info
Article history:
Accepted 30 April 2017

Keywords:
Meniscus
Osteoarthritis
Viscosupplementation
Viscoelasticity
Extracellular matrix

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. 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.

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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

http://dx.doi.org/10.1016/j.jbiomech.2017.04.034
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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, Gosnè, 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 veterinarian 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/mL; molecular weight: 6 × 106 Da) 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.

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 aluminum support (glue 3; Lociote®). 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 10%. A constant displacement rate of 5 μm/s 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).

\[ f = \frac{E_{\text{st}}}{E_{\text{eq}}} \]

(1)

where \( E_{\text{st}} \) and \( E_{\text{eq}} \) 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 indenter 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 μm² 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 BVY, 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 (Kilian 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
non-treated, and operated treated groups were analyzed using the Kruskal-Wallis test with a level of significance of 0.05, followed by a post hoc Dunn’s test with Bonferroni correction.

2. Results

2.1. Pre-clinical outcome

All animals but one (which was excluded from the study) recovered uneventfully from surgery. At the time of sacrifice, no patellar maltracking or dislocation of the patella were observed in any group. For all analyses, the healthy and operated non-treated groups had a sample size of $n = 6$, while the operated treated group had a sample size of $n = 5$.

2.2. Gross morphological assessment

The macroscopic scores attributed to meniscus and cartilage degradations as well as osteophyte formation are given in Table 2 for the healthy, operated non-treated, and operated treated groups. All of the healthy rabbits displayed normal menisci, rare cartilage alterations, and minute osteophytes. The surface of the menisci was smooth and glistening (Fig. 3A). Operated non-treated rabbits exhibited meniscal lesions with different degrees of severity (Fig. 3B and C), fibrillation or erosion of both tibial and femoral cartilage, as well as osteophyte formation. One of the menisci displayed a bucket handle tear (Fig. 3C) and the lesions were mainly located in the posterior region. The macroscopic score for meniscal degradation was significantly lower ($p = 0.002$) for operated treated than for operated non-treated groups. All of the menisci from the operated treated knees had a minimal score, with a smooth and brilliant surface (Fig. 3D), as for the healthy group. Moreover, tibial and femoral cartilage scores were significantly lower ($p = 0.0004$ and $p = 0.02$, respectively) for the operated treated group than for the operated non-treated group and were very similar to those of the healthy group. On the contrary, the macroscopic scores for osteophyte formation in the tibial plateau and in the trochlea were significantly higher ($p = 0.0007$ and $p = 0.001$, respectively) for the operated treated group than for the healthy group and were very similar to those of the operated non-treated group.

2.3. Viscoelastic properties

The instantaneous modulus, equilibrium modulus, and elastic fraction of the menisci in each region are given in Fig. 4 for healthy, operated non-treated, and operated treated groups. The equilibrium moduli in the anterior region of the menisci from the operated non-treated and operated treated groups were significantly lower ($p = 0.0149$ and $p = 0.0059$, respectively) than that of menisci from the healthy group, with mean values of $0.26 \pm 0.12$ MPa, $0.26 \pm 0.25$ MPa, and $0.60 \pm 0.18$ MPa, respectively. Moreover, the elastic fractions in the posterior region were significantly lower for the operated non-treated ($p = 0.038$) and operated treated ($p = 0.0078$) groups than for the healthy group, with mean values of $0.16 \pm 0.049$, $0.14 \pm 0.038$, and $0.21 \pm 0.027$, respectively. None

Fig. 2. Representative histological images showing GAGs coverage measurement. (A) Color image, (B) grey-scale image viewed under the blue channel, (C) image after applying a threshold of 1:3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
of the mechanical parameters were significantly different between operated non-treated and operated treated groups.

### 2.4. Collagen fiber organization

Menisci from the healthy knees displayed compact collagen bundles made of straight fibers aligned in the circumferential direction (Fig. 5A and D). By contrast, menisci from the operated non-treated (Fig. 5B and E) and operated treated (Fig. 5C and F) knees exhibited less compact bundles, with undulated fibers in both regions.

The microscopic scores for tie fibers organization are given in Table 3 for healthy, operated non-treated, and operated treated knees, in each region. All of the menisci from the healthy group had a null microscopic score in each region. This score significantly increased in the anterior \((p = 0.006)\) and posterior \((p = 0.004)\) regions of the menisci from the operated treated groups, with mean values of 2 and 1.4, respectively. No significant difference was found between the microscopic scores of the menisci from the operated non-treated and operated treated groups, in any region.

### 2.5. GAG coverage

Measurements of GAG coverage are given in Fig. 6 for healthy, operated non-treated and operated treated groups, in each region of the menisci. The anterior region of the menisci from the operated non treated group demonstrated a significant decrease \((p = 0.0074)\) in GAG coverage compared to the healthy group. The GAG coverage in the anterior region of the menisci from the operated treated group was not significantly different from that of the healthy group, nor from that of the operated non-treated group, and showed a great variability of values. GAG coverage was very
Fig. 4. Instantaneous modulus, equilibrium modulus, and elastic fraction of menisci from the healthy, operated non-treated, and operated treated groups, in the anterior and posterior regions. Boxplots show the median and quartiles. * denotes a significant difference between two groups.
low in the posterior region of the menisci, and no significant difference was found between the groups.

3. Discussion

ACL rupture in animals is a common traumatic procedure of OA induction which mimics PTOA in human (Vignon et al., 1987). In particular, ACL rupture in rabbits results in degenerative changes in the menisci which have been investigated morphologically (Boulocher et al., 2008; Fischenich et al., 2015; Hellio Le Graverand et al., 2001; Killian et al., 2010b; Levillain et al., 2017; Smith et al., 2002), histologically Fischenich et al., 2015, 2017; Hellio Le Graverand et al., 2001; Levillain et al., 2015, 2017; López-Franco et al., 2011) and mechanically (Fischenich et al., 2015, 2017; Levillain et al., 2015, 2017; Wheatley et al., 2015) at an early stage of the disease progression in different animal models. To the best of our knowledge, this is the first study examining the effects of a viscosupplementation therapy on the morphological, microstructural, and mechanical properties of the medial menisci using a post-traumatic model of OA. This study showed promising results of crosslinked HA mono injection for the early treatment of OA menisci at the macroscopic scale, but it did not demonstrate a significant effect on their mechanical behavior.

Intra-articular injection of HA in the OA knees greatly improved the surface integrity of the menisci and cartilages in the medial compartment. All menisci from the operated treated group had a normal appearance, with a smooth and brilliant surface, while most of the menisci from the operated non-treated group had morphological evidence of fibrillation or tearing. This finding supports the effect of viscosupplementation in improving the lubrication over the OA knee (Strauss et al., 2009; Moreland, 2003). Inconsistent results were obtained in another study on rabbit menisci, in which sodium hyaluronan was first injected four weeks after ACLT and then weekly injected for five weeks (Sonoda et al., 2000). They did not find any significant difference in the medial meniscal tear frequencies between control operated and HA-injected knees nine weeks after OA induction. This discrepancy may be due to the different HA formulations (crosslinked or not) or to the different times of first injection. Indeed, Hellio Le Graverand et al. (2001) showed that most rabbit medial menisci presented with fibrillation or tears as early as three weeks after ACLT. Taken together, these findings obtained with an ACLT rabbit model emphasized the need for early viscosupplementation therapy to prevent the occurrence of meniscal lesions at the macroscopic scale. However, this statement should be confirmed using other animal models of OA with different treatment regimes.

Surprisingly, biphotonic confocal imaging did not reveal any significant difference in the organization of the collagen fibers at the surface nor in the deep zone of the menisci between operated non-treated and operated treated groups. Both groups displayed disruption of the tie fibers, due to alterations of the superficial layers from which they emanate (Andrews et al., 2014; Rattner et al., 2011). As a consequence, tie fibers could not resist the separation of the circumferential collagen bundles in the deep zone, which were less packed and more undulated compared to those of the healthy group. This result indicates that, while the viscosupplementation therapy seems to improve the lubrication over the knee and significantly decreased the occurrence of macroscopic lesions, it did not prevent tearing of the surface at the microscopic scale, leading to structural modifications in the deep zone.

As a result, no significant effect of the viscosupplementation therapy was found on the viscoelastic properties of the menisci.
the equilibrium modulus in the anterior region and the elastic fraction in the posterior region of the menisci from the operated treated group were significantly lower than those of the healthy group. These results imply that HA injection in the OA knee did not prevent the degradation of the mechanical properties of the menisci in our model. However, it is worth noting that menisci from the operated treated groups showed a greater variability of the instantaneous and equilibrium moduli in the anterior region, with higher maximal values. One possible explanation is that mechanical alterations occurred in the first weeks following OA induction and that HA injection slowed down the decrease in the mechanical properties of the preserved menisci two weeks after ACLT. This hypothesis is in agreement with the study of Fischenich et al. (2017), which showed that the instantaneous and equilibrium moduli of the rabbit menisci were decreased four weeks after OA induction and were then stabilized, indicating that mechanical alterations occurred very early in the progression of the disease. More samples would be necessary to verify this assumption.

Histological analysis suggests a potential beneficial effect of the viscosupplementation therapy on the GAGs content of the menisci. GAGs are essential in healthy menisci to support the compressive loading and facilitate collagen sliding (Vanderploeg et al., 2012). Several studies demonstrated that their content decreased following post-traumatic OA (Adams et al., 1983; Fischenich et al., 2014, 2015, 2017; Hellio Le Graverand et al., 2001; Kwok et al., 2016) and then increased to revert to normal (Adams et al., 1983; Fischenich et al., 2017). In the present study, it was found that the operated non-treated knees exhibited a significant decrease in GAG coverage of the menisci in the anterior region six weeks after ACLT, while no significant difference was found between the healthy and operated treated groups. These results are consistent with those obtained by Hope et al. (1993) and Sonoda et al. (2000) and suggest that HA played a role on the GAG distribution in OA menisci, as observed in OA cartilage (Williams et al., 2003).

In conclusion, this study demonstrated the positive effect of the viscosupplementation therapy in preventing macroscopic lesions and suggested a potential effect of the treatment on the GAG distribution in post-traumatic OA menisci in an animal model. However, no significant effect of the treatment was shown on the disorganization of the collagen fibers at the surface nor in the deep zone, leading to lower mechanical properties than healthy menisci. These microstructural and mechanical changes likely occurred in the first weeks following OA induction and could not be addressed by the treatment. Several studies have demonstrated the importance of the injection timing on the efficacy of the viscosupplementation therapy (Moreland, 2003). Thus, the effect of an earlier HA injection in preventing alterations of the microstructural and mechanical properties of the menisci merits future studies.

Conflict of interest statement
The authors of this paper have no financial or personal relationships with other people or organizations that could inappropriately influence (bias) our work.

Acknowledgements
The authors wish to thank Morad Bensidhoum and the staff from Biomedical Research Centre of Alfort (CRBM), histology unit of VetAgro Sup, and IVTV plateform, for their technical support. We would also like to thank the IVTV ANR-10-EQPX-06-01 for their financial support.

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