Abnormal Mechanical Loading Induces Cartilage Degeneration By Accelerating Meniscus Hypertrophy And Mineralization After Acl Injury In Vivo

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Introduction: Anterior cruciate ligament (ACL) injury is an extremely common injury in the young placing them at high risk for post-traumatic osteoarthritis (PTOA). Furthermore, surgical ACL reconstruction does not appreciably reduce this risk. Although PTOA is common following ACL injury, the molecular mechanisms behind the onset and progression of the disease remain elusive. Increasing evidence suggests that meniscus, synovium and subchondral bone all may play a critical role in the pathogenesis of PTOA. Meniscal degeneration is positively associated with articular cartilage degeneration in PTOA patients, though it is unclear how the meniscus influence cartilage damage. Our hypothesis was that abnormal mechanical loading induces cartilage damage by accelerating the meniscus hypertrophy and mineralization. We studied this hypothesis in vivo PTOA model of guinea pig ACL injury and in vitro cyclic loading on bovine meniscus explants.

Methods: Animals: Six 3-month-old male Hartley guinea pigs received ACL transection (ACLT) on the right knee, while the contralateral ACL-intact knee in the same animal served as a sham control. These animals were sacrificed 10 weeks after surgery (5.5 month-old). Calcein was injected at day 1 and day 5 before the animals were euthanized to detect new bone formation. Histology: The meniscus and proximal tibia plateau of right (ACLT) and left knee (control) were harvested and measured with a ruler. Eight μm sections were collected after the tibia plateaus were embedded in methyl-methacrylate 72.5%, plastoid N 25%, and benzoylperoxide 2.5% without decalcification. Calcification of the meniscus was determined by Fluorescent microscopy. Alizarin Red and Von Kossa staining were used to evaluate the mineralization of the meniscus. The area and intensity of calcification were calculated with HIS-Elements AR software. The severity of cartilage and meniscus damage was determined by the Modified Mankin Score and meniscus grade after the slides were stained using Safranin O/Fast green. Correlation between the area and intensity of meniscus calcification and the severity of cartilage damage were calculated using regression analysis. Immunohistochemistry: The presence of Ihh, MMP-13, IL-1, type X collagens, ANKH, ENPP1, and ALP in meniscus were determined by immunohistochemistry. Explant culture and cyclic impact loading: To detect if abnormal mechanical loading induced meniscus hypertrophy and/or mineralization, the meniscal explants from mature bovine knee were subjected to a range of loading injury by indenting to 25% strain at 0.3Hz for 1h, 2h, 3h respectively. The 1/4 of the
explants were immediately assayed for cell viability after loading and the rest of the samples were cultured for 24h, 48h, 72h respectively. The mRNA and protein levels associated with hypertrophy and/or mineralization were measured with real-time polymerase chain reaction (RT-qPCR) and Western blot, including Ihh, MMP-13, IL-1, Col X, ANKH, ENPP1, and ALP. Conditioned medium was collected for sGAG release assay.

**Results:** In vivo study: The size, area and intensity of the meniscus calcification were significantly increased in the ACLT group compared to the sham control group (Fig.1 and 2). We observed severe cartilage and meniscus damage were presented in the ACLT group compared with the control. The area and intensity of meniscal calcification were positively correlated with articular cartilage damage ($r=0.925 \ p<0.0001$, $r=0.944 \ p<0.0001$) (Fig.3). Immunohistochemistry staining of typical hypertrophy markers Ihh, MMP-13, type X collagen and mineralization markers ANKH, ENPP1, ALP were increased in ACLT animals compared to the control.

Explants loading study: Live/dead staining indicated a single layer of necrotic cell in the superficial zone of the bovine meniscus explants after 25% dynamic compressive strain for 3 h at 0.3Hz. The mRNA and protein levels of MMP-13, ANKH, ENPP1, and ALP were up-regulated in the dynamic loading group compared to the explants w/o loading at 24h, 48h, 72h cultures respectively after 3h loading. The GAG content in the culture medium was increased by 17-19% in the loading group compared with no loading control.

**Discussion:** We are the first to demonstrate that the abnormal loading after ACL injury results in meniscus hypertrophy and calcification, which is correlated with cartilage damage. The meniscus calcification results in the increase of the meniscus stiffness. The increased stiffness of the meniscus between articular cartilages may initiate cartilage damage. ANKH, ENPP1, and ALP are critical genes that regulate the calcium deposition and mineralization. Here, we showed that abnormal cartilage loading up-regulates these genes in vivo and in vitro, which are responsible and promote meniscus calcification. Our findings indicate that the meniscus hypertrophy and calcification caused by the abnormal loading after ACL injury plays a critical role in the development of PTOA.

**Significance:** It is known that the surgical ACL reconstruction does not reduce the risk of PTOA but the pathogenic mechanism remains unclear. Thus, understanding the mechanisms behind the onset and progression of the PTOA is critical. Our findings suggest that the meniscus calcification induced by abnormal loading after ACL injury plays a critical role for PTOA. The results of our study suggest that the suppression of the meniscus calcification could provide chondral protection for the prevention of PTOA.

**Figure 1:** Abnormal loading results in increased meniscus size after ACL injury compared with the sham control.

**Figure 2:** Abnormal loading accelerates the meniscus calcification after ACL injury. Fluorescent microscopy shows more new bone formation in meniscus tissue (A) and slide (B and C). Alizarin Red staining (D) indicates a strong calcification staining in the ACLT animals compared to control. The graphs show area and intensity of the meniscus calcification.

**Figure 3:** The meniscus calcification is correlated with the cartilage damage. Safranin O staining shows the meniscal (A) and cartilage (B) damage. Bar graphs on top right depict the Modified Mankin Score and meniscus grade. Both the area and the intensity of meniscal calcification are positively correlated with the articular cartilage damage (C).
ORC 2015 Annual Meeting
Poster No: 0333