Devitalizing effect of high hydrostatic pressure application on xenogenic graft material

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INTRODUCTION: Lesions of the anterior cruciate ligament and the Achilles tendon are the most common tendon and ligament injuries. Especially for elite athletes, these injuries can be career-ending. Since therapeutic approaches based on autografts may be mechanically more instable to native tissue grafting, it is difficult to fully restore the joint function. This also concerns allografts and xenografts in particular, which require processing steps to eliminate both pathogens and immune-system activating cellular components according to European guidelines. Those decellularization processes often include strong chemical and physical applications, which reduce biomechanical properties of the grafts. A gentle alternative processing method could be high hydrostatic pressure (HHP) which is used in food industry for decontamination. With HHP, devitalization of ligaments and tendons may be possible avoiding aggressive chemical processing and rinsing steps in order to preserve protein integrity and biomechanicsal properties. The aim of this study was to analyze HHP effects on xenogenous graft material, i.e., porcine flexor tendons, regarding types of cell death and protein structures.

METHODS: Five porcine upper flexor tendons supplied were extracted under sterile conditions by the Cells+Tissue Bank (Krems, Austria) and used for the present study. Tendons were cut into 10 x 10 mm pieces with an average thickness of 4 mm. Samples were placed into cryogenic tubes which were filled with 0.9 % saline solution and closed air bubble free. HHP of 150 MPa, 250 MPa, 350 MPa, 450 MPa and 600 MPa were applied for 20 min each at 30 °C. After HHP, the surrounding medium of the samples was changed from NaCl to Dulbecco's Modified Eagle Medium and sample were incubated under standard culture conditions (37 °C, 5 % CO_2) for 2 hours. Afterwards, RNA was isolated and gene expression analysis were performed with regard to *caspase-9*, *caspase-8*, *caspase-3*, *MLKL* and *caspases-1*. Furthermore, a sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine changes in the primary structure of the tendons. Control samples (n = 5), which was not affected by HHP, but apart from this treated in the same way, were also characterized.

RESULTS SECTION: A pressure dependent cell reaction was observed in HHP treated tendinous tissue. In general, the HHP application resulted in an overexpression of cell-death related genes compared to the untreated control. HHP treatment led to an overexpression of *caspase-9*, whereas *caspase-8* could not be detected. The strongest *caspase-9* expression could be detected for samples treated with 250 MPa and 450 MPa. In addition, it was found that an application of 600 MPa resulted also in the expression of *MLKL* which represented 30% of the total cell death. This could also be observed for samples treated with 350 MPa but not as much as for 600 MPa. Analysis regarding protein integrity showed no distinct differences between treated and non-treated groups in SDS-PAGE.

DISCUSSION: Our presented data reveal the devitalizing effect of HHP application on porcine tendinous tissue while preserving the primary structure of proteins. Interestingly, cells responded to HHP primarily with the expression of *caspase-9* which gives hints of an intrinsic apoptotic cell death, whereas *caspase-8*, suggesting an extrinsic apoptotic cells death, could not be detected. *Caspase-1*, indicative for the proinflammatory pyroptosis was only expressed to a certain extent. Therefore, it can be assumed that this cell death does not occur under the applied HHP conditions. However, *MLKL*, a characteristic marker for necroptosis, could be detected with a strong overexpression at HHPs of 350 MPa and 600 MPa. It is known that necroptosis leads to uncontrolled membrane rupture with the release of proinflammatory cytokines, which in turn would increase the probability of graft rejection after transplantation. Considering this, high hydrostatic pressures causing an anti-inflammatory cell death should be chosen for tissue devitalization in order to reduce the risk of rejection of the xenogenic graft material. Based on the presented data, HHP of 250 MPa and 450 MPa should be examined more closely in further studies using allogeneic graft material, as these pressures led mainly to an anti-inflammatory apoptotic reaction. Since only gene expression analyses are shown here, the type of cell death as well as the distribution of cell death should be further investigated e.g., via histology. In addition, protein analysis showed the preservation of the primary protein structure of tendon-specific matrix proteins. Besides scanning electron microscopy, with which the matrix integrity could be further investigated, biomechanical analyses including tensile tests should also be performed to evaluate the biomechanical properties after HHP application.

SIGNIFICANCE/CLINICAL RELEVANCE: HHP treatment could be a gentle alternative processing method for devitalization of allogeneic or xenogeneic tendon grafts. Since HHP triggers various cell reactions that could partly lead to graft reactions after transplantation, HHP which induce primarily anti-inflammatory cell death should be verified in further studies.