Silk-Fibroin/Methacrylated Gellan Gum Hydrogel As An Novel Scaffold For Application In Meniscus Cell-Based Tissue Engineering

H. Pereira[1,2,3,4], J. Silva-Correia[1,2], L.P. Yan[1,2], S.G. Caridade [1,2], A.M. Frias [1,2], A.L. Oliveira[1,2], J.F. Mano[1,2], J.M. Oliveira[1,2], J. Espregueira-Mendes[1,2,3], R.L. Reis[1,2]

[1]3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Guimarães, Portugal;
[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal;
[3]Saúde Atlântica Sports Center – F.C. Porto Stadium; Minho University and Porto University Research Center, Portugal;
[4]Orthopedic Department Centro Hospitalar Póvoa de Varzim – Vila do Conde, Portugal

One sentence summary of your abstract to be included in the final program (400 characters max): *"Silk-Fibroin/Methacrylated Gellan Gum Hydrogel have adequate properties for use in either acellular or cellular approaches for partial and/or total meniscus replacement while enabling control of neovascularization."*

Introduction: Knee meniscus injury is highly prevalent and there is a demand for new cost-effective treatment solutions. Tissue engineering (TE) and regenerative medicine strategies using acellular scaffolds are being used in clinical application for total or partial meniscus replacement [1]. Although this strategy has been considered as a safe and promising approach, progressive volume reduction of the implant and early failure have been described. Advances in the field of meniscus TE are required and greatly depend on increased knowledge of meniscus biology, improvement of biomaterials and cell-based therapies [2]. Advanced scaffolds for meniscus TE should possess adequate mechanics, biodegradability and biocompatibility, and also be able to mimic and preserve the asymmetric vascular network of this complex tissue, i.e. enable controlling the segmental vascularization during the regeneration process. Silk fibroin scaffolds derived from Bombyx mori cocoon have been recognized as a versatile biomaterial for application in meniscus TE [3]. The purpose of this study is to: 1) contribute to the knowledge of meniscus aiming future clinical applications (namely, the aspects dealing with the characterization of cellular phenotypes and density, biomechanics and extracellular matrix composition) and 2) to develop an alternative and viable silk fibroin scaffold possessing adequate properties for either use in acellular or cellular approaches for partial and/or total meniscus replacement, and combine it with the methacrylated gellan gum hydrogel (iGG-MA) hydrogel, which is able to prevent the ingrowth of endothelial cells and blood vessels into the hydrogels [4,5].

Patients & Methods: Morphologically intact menisci were collected from 44 human donors (12 male, 32 female). All menisci (30 lateral and 14 medial) were divided into anterior, middle and posterior segments prior to mechanical, biological or histological characterization. Human meniscus cells (HMC´s) were isolated using an enzymatic standard protocol. HMC´s phenotype was characterized by flow cytometry analysis. Haematoxylin and eosin (H&E), safranin-O and collagen I staining were performed for

segmental characterization of the extracellular matrix. For the evaluation of the viscoelastic properties, dynamic mechanical analysis (DMA) was performed using fresh tissue samples. The three segments of menisci were cut in cylindrical shapes with 4 mm diameter and analyzed at 37ºC in PBS (pH 7.4). The microstructure of freeze-dried meniscus was investigated by micro-computed tomography (micro-CT) analysis. Silk-based scaffolds (10 and 12 wt%) were produced by means of combining salt leaching and freeze-drying methods [3], in order to match human tissue biological and biomechanical features. HMC's were seeded onto the different silk scaffolds at a cell density of 5x104 cells/disc. Then, the cell-laden scaffolds were cultured in static conditions, for times of culturing up to 21 days. After specific times of culturing (from 1 day up to 21 days), HMC´s adhesion, viability and proliferation were investigated by scanning electron microscopy (SEM), calcein-AM assay and DNA quantification tests, respectively. In addition, the mechanical properties of the cell-loaded scaffolds were evaluated by DMA. The HMC's-laden hydrogel/silk scaffolds were produced by encapsulating the HMC's into a 2 wt% iGG-MA hydrogel, followed by impregnation onto the 12 wt% silk scaffold. A chorioallantoic membrane (CAM) assay was used to investigate in vivo the control of segmental vascularization of the acellular and cell-laden hydrogel/silk scaffolds by the effect of iGG-MA hydrogel, until day 14 of embryonic development.

Results & Discussion: The biological characterization of this meniscus tissue, although not yet completely accomplished, has evolved significantly in the last few years. In this work, DMA analysis has shown that medial meniscus has significantly higher stiffness (E' and Tan d) than lateral meniscus. There is also significant regional variation form anterior to posterior menisci segments regarding biomechanical features. Age, gender and bone mass index (BMI) also influences meniscus stiffness. The FACS analysis revealed that cells isolated from the human meniscus are a mixed population of cells, i.e. fibrochondrocyte-like and MSCs (cells are positive for CD105, CD73 and CD90, and lack CD34 and CD45). HMC's maintained their phenotype for 21 days when cultured in tissue culture polystyrene plate (2D). The micro-CT analysis revealed that the human freeze-dried meniscus possessed a mean porosity of 58.0±20.3% and interconectivity of 41.9%±53.7. The mean pore size and trabeculae thickness was 220.7±111.5 µm and 159.7±78.6 µm, respectively. The knowledge arising from the present study allowed us to develop a novel polymeric scaffold made of silk fibroin, which was subsequently characterized without cells and after cell-loading. SEM analysis revealed that the HMC´s adhered to the surface of the scaffolds. The viability assay and DNA quantification showed that HMC´s were viable and proliferated well when cultured onto both silk-10 and silk-12 scaffolds, until 21 days. DMA analysis has shown that the moduli of the acellular scaffolds immersed in culture medium for 14 days were 27.6 ± 7.9 kPa and 61.1 ± 0.4 at 10 Hz, for silk-10 e silk-12, respectively. By its turn, the moduli determined at 10 Hz of the cell-laden scaffolds cultured after 14 days of culturing were 48.2± 19.8 and 140.1 ± 15.6 kPa, for silk-10 and silk-12, respectively. The in vivo study allowed investigating the number of macroscopic blood

vessels converging to the implants. The evaluation of possible inflammation and endothelial cells ingrowths was performed by histological (H&E staining) and immunohistochemical methods (SNA-lectin staining). Results have shown that iGG-MA hydrogel prevented the endothelial cells adhesion and blood vessels infiltration into the HMC's hydrogel/silk scaffolds, after 4 days of implantation, even in the presence of VEGF.

Conclusions: This study showed that the developed silk scaffolds can support the adhesion, proliferation and viability of HMC's, in vitro. Cells increased the biomechanical features of acelular scaffolds. Medial and lateral menisci present diferent biomechanical properties which are also influenced by age, gender and BMI. Moreover, the silk scaffolds combined with the iGG-MA hydrogel enabled controlling the segmental vascularization. This pioneer study has contributed for developing a novel tissue engineering strategy based on combining silk scaffolds with hydrogels and cells, and thus it can possibly mimic the native vasculature architecture during meniscus regeneration.

References

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