Development of a reliable *in vitro* osteoarthritis model responsive to mTORC1-inhibitors via oxidative stress reduction and autophagy promotion

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INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease, which causes pain, swelling, deformation, and in the most severe cases, hypomobility of the joint. Intuitively, these pathologic conditions seriously get in the way for daily movements, and cause invalidating conditions due to the need for supporting assistance[1]. Hyaline cartilage is the more heavily involved joint area. However, the underlying mechanisms responsible of osteoarthritis are complex and interconnected, and interest all joint structures such as subchondral bone, synovium, muscles, and ligaments[2,3]. Recent epidemiologic analyses[4] pointed out the age related nature of osteoarthritis, and highlighted that the OA incidence in 75 years old population is 4 times higher than in 40 years old people for hip osteoarthritis, and 14 times higher for knee osteoarthritis. Moreover, age-related comorbidities with an incidence between 14% and 20% ha been reported[5]. In the last decade, interdisciplinary studies involving regenerative medicine, molecular biology, and pharmaceutical chemistry, are directed to discover new pathways involved in OA to be used as target for innovative pharmacological approaches. However, the challenge is particularly hard due to heterogenous OA-related molecular mechanisms involving a wide plethora of biochemical factors. Very recently, the pathways activated by the interaction of lipopolysaccharide (LPS) with its extracellular receptor (toll like receptor 4, TLR4) in chondrocytes has been strictly correlated with the onset of osteoarthritis. The point of contact was found in the intestinal disorder known as leaky gut (LG), which is one of the most frequent OA comorbidities age-related[6]. The etiology of leaky gut is reportedly connected with a dysregulation of the homeostasis in the intestinal epithelium, which is mainly composed of enterocytes covered by a layer of protective mucus. Here, cells are interconnected each others through tight junctions, a cell-cell connection which reduce to zero intercellular gaps blocking paracellular translocation of substances (Figure 1). LG is caused by the partial or total laxity of tight junctions, which promote the diffusion in the bloodstream of LPS produced by resident microbiota triggering systemic inflammatory responses (SIR) that damage joint cartilages. Our work is included in this complex scenario, and aims to create a stimuli responsive 3D *ex vivo* model to be used as bench test for new molecules or innovative drug delivery systems (DDS).

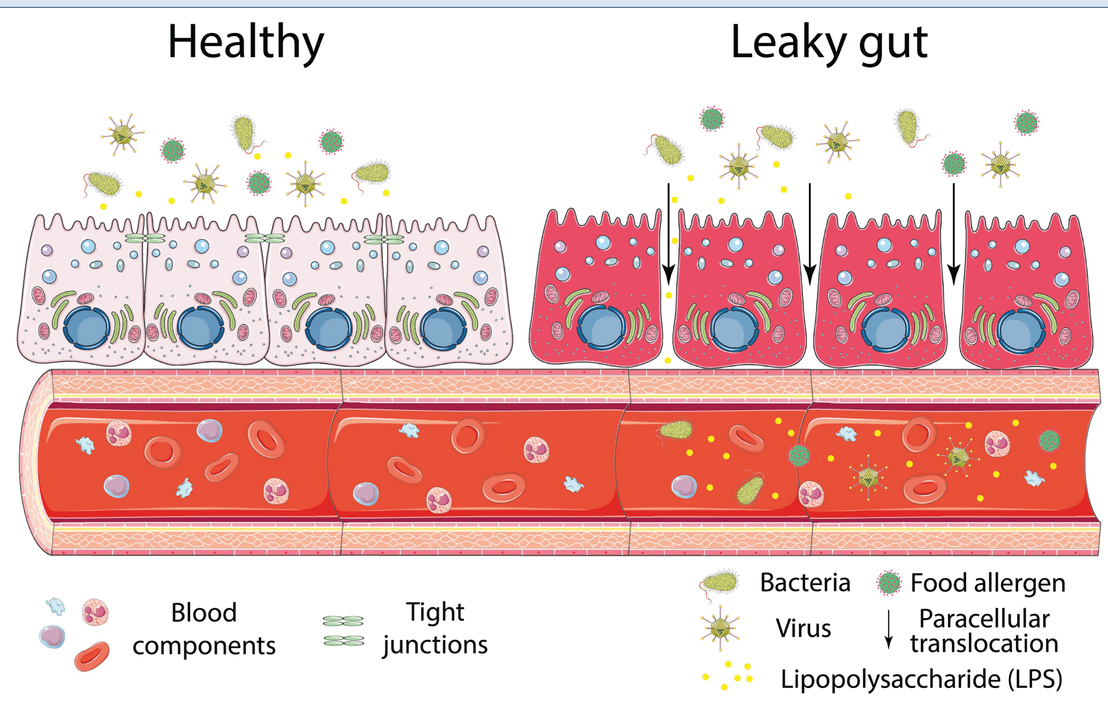


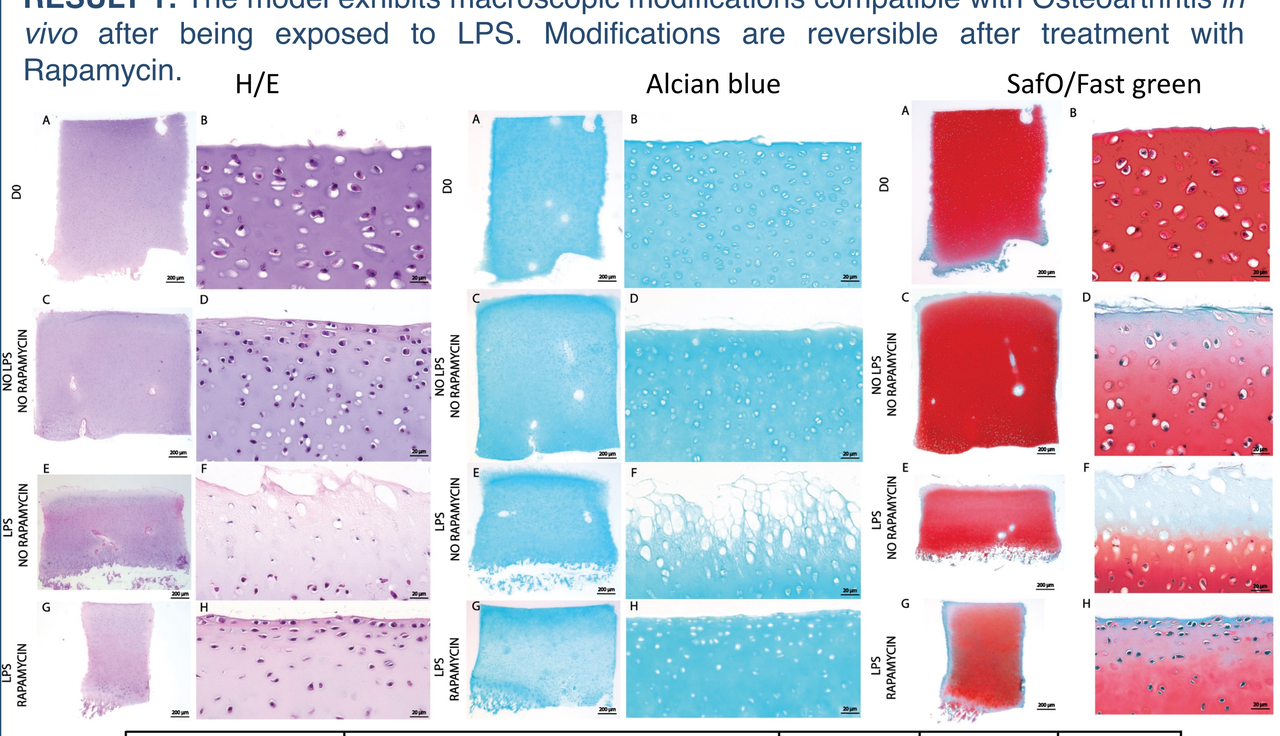
Fig. 1: Schematic representation of the anatomy of a healthy gut and its pathologic modification in a leaky gut condition

MATERIALS AND METHODS

We followed a multimodal approach to obtain a reliable model under two different perspectives. The first one, is to develop a realistic *ex vivo* OA-model reproducing LPS-induced osteoarthritis derived from disorders involving gut-osteoarthritis axis *in vivo*. The second one, is to create a 3D model able to overcome the disadvantages of canonical 2D OA *in vitro* models based on cell cultures or co-cultures, which often result in unrealistic biological responses due to the lack of a 3D structural support. Despite different technologies has been developed to fabricate synthetic 3D models, *ex vivo* explants are the best choice in highly demanding environment as cartilage, which requires an accurate control of cell phenotype. Here, we created an OA-model based on healthy porcine cartilaginous explants harvested from knee joints, chemically stimulated with LPS to induce realistic OA-like conditions. To test the reliability of our model we studied its response after being exposed to a selective inhibitor of the mTORC1 pathway, which is involved in the control of chondrocytes phenotype. Samples were labeled as D0 (untreated), -L-R (without LPS and Rapamycin), +L-R (stimulated with LPS, not treated with Rapamycin), and +L+R (stimulated with LPS and treated with Rapamycin).

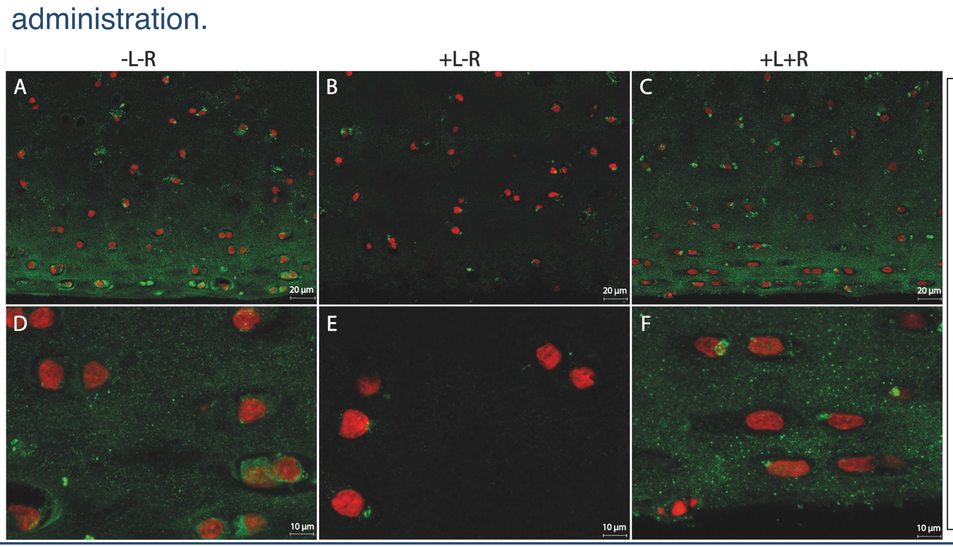
RESULTS AND DISCUSSION

RESULT 1: The model exhibits macroscopic modifications compatible with Osteoarthritis *in vivo* after being exposed to LPS. Modifications are reversible after treatment with Rapamycin.

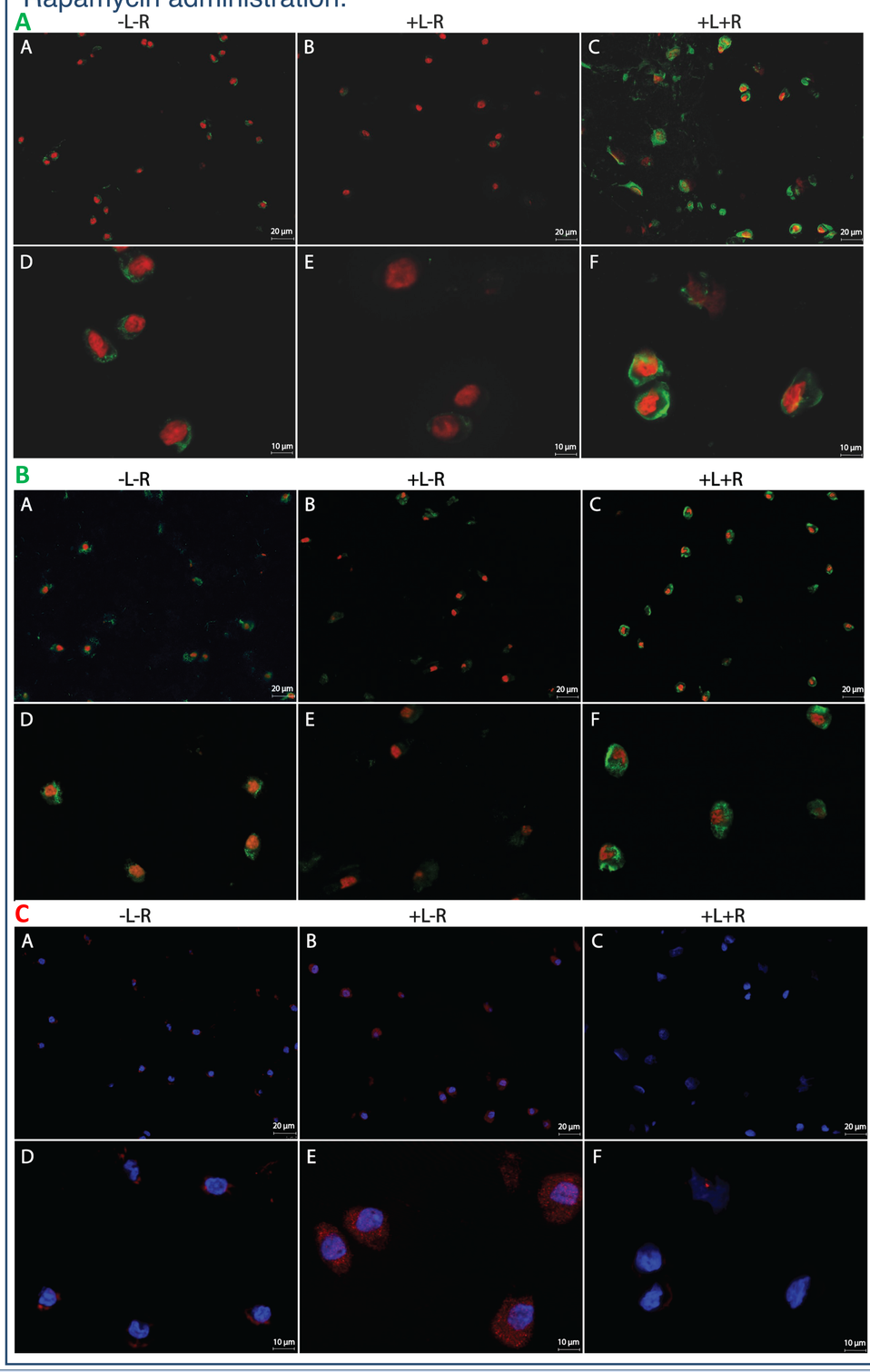


Sample	Morphology	Grade	Stage	Score
D0	Cartilage surface smooth, ordered zones, no chondrons changes	0	0	0
-L-R	Superficial microscopic fissurations, no cartilage oedema, < 10% horizontal involvement, no swelling	1	0	0
+L-R	Superficial fissurations, deep fibrillation, 25-50% horizontal involvement	2	3	6
+L+R	Cartilage surface smooth, ordered zones, no chondrons changes	0	0	0

RESULT 2: The deposition of the hyaline cartilage-specific type II collagen (green) is decreased after pro-inflammatory stimuli. The recovery is complete after Rapamycin administration.



RESULT 3: We successfully induced reversible pathways associated with Osteoarthritis in vivo. Pathways involving autophagy-promoting factors as LC3b (A-green) and ULK1 (B-green) were activated after Rapamycin treatment. Conversely, the pathway involving oxidative stress mediated by ROMO1 enzymatic complex (C-red) was inhibited after Rapamycin administration.



CONCLUSIONS

Our model shows macroscopic and molecular features characteristic of Osteoarthritis *in vivo*. Remarkably, the model is responsive to external stimuli, which is the key point of the overarching hypothesis underlying this work. Overall, the model represents a realistic *ex vivo* platform to be used for advanced in vitro studies supporting the development translational research aimed to develop a pharmacological therapies toward Osteoarthritis

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