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Micelle-to-Gel: Thermosensitive intra-articular hydrogels for osteoarthritis management☆

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ABSTRACT

Osteoarthritis (OA) is a chronic and degenerative joint disease with a rising incidence worldwide. Current therapeutic approaches primarily focus on symptom relief through systemic administration, which raises safety concerns related to side effects and long-term use. In this context, the local administration of natural compounds with anti-inflammatory and anti-arthritic properties, such as β-Lapachone constitutes an interesting alternative. In this work, we prepared and characterized injectable thermosensitive hybrid hydrogels loaded with β-Lapachone. A comprehensive characterization of the hydrogel systems was performed, including micellar diameter, mechanical properties at different temperatures, the ability to control drug release and microstructure. The anti-inflammatory activity of the free drug, as well as that of the blank or loaded hydrogels was then evaluated *ex vivo*, using OA cartilage explants. Additionally, *in vivo* studies were carried out in a rabbit model of OA to assess their clinical potential. The results suggest that the hydrogel systems possess a composite microstructure integrating micelles, together with a temperature-responsive stiffness and the ability to modulate drug release. In addition, β-Lapachone-loaded hydrogels display an interesting immunomodulatory potential *ex vivo*, as they were able to efficiently reduce the secretion of several proinflammatory mediators, such as IL-6, MMP9, MMP13 and CXCL8. Furthermore, the drug-loaded hydrogels were found to improve *in vivo* cartilage and bone histomorphometric markers, such as subchondral bone thickness, as well as early signs of cartilage damage, such as the fibrillation index. Therefore, the developed β-Lapachone-loaded thermosensitive hydrogels constitute a promising alternative for OA management.

1. Introduction

Osteoarthritis (OA) is a chronic and disabling degenerative joint condition affecting over 500 million people worldwide. Its prevalence has significantly increased over the last century due to rising life expectancy and higher rates of obesity [1]. The disease includes complex alterations of the synovial joint including subchondral bone loss, structural defects in hyaline cartilage, increased synovium vascularity, tissue hypertrophy and instability in ligaments or tendons [1]. Current OA management mainly focuses on reducing pain and stiffness while preserving joint function [2,3]. Standard treatments often include oral and topical non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, as well as intraarticular corticosteroids and hyaluronic acid [3,4]. However, these therapeutic approaches raise safety concerns

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related to liver toxicity, cardiovascular, gastrointestinal or renal problems associated with long-term NSAIDs oral use [5]. Furthermore, the effectiveness of intra-articular treatments is often limited by the rapid clearance of the therapeutic molecules, and the efficacy of topical treatments is relatively low [5].

Recently, natural products have gained attention as alternative treatments for OA, demonstrating positive effects in both preclinical and clinical studies. These benefits are related to the ability of certain natural compounds and metabolites to control cell death, inflammation, and tissue anabolism and catabolism [6]. β-Lapachone, a naphtoquinone obtained from the bark of Lapacho trees (*Tabebuia avellanedae*) native to South America, exhibits anti-inflammatory, anti-arthritic and wound-healing properties [7,8]. These therapeutic effects have been linked to the immunomodulatory activity of this compound, which reduces the secretion of pro-inflammatory cytokines and nitric oxide [7]. Despite these promising features, the clinical application of β-Lapachone remains limited by its low aqueous solubility and the risk of side effects, such as haemolytic anaemia, following systemic administration [9,10].

The development of innovative hydrogel-based local injectable formulations constitutes a promising approach to harness the therapeutic potential of natural products. By increasing their local concentration, these formulations can enhance the active compounds retention time at the target site, improve solubility and, minimize systemic side effects [11,12]. Thermosensitive polymers, such as Pluronics, are promising candidates to develop injectable hydrogels, due to their ability to undergo sol-gel transitions at body temperature [13]. These polymers have a PEO-PPO-PEO structure, comprising hydrophilic (polyethylene oxide, PEO) and hydrophobic (polypropylene oxide, PPO) segments, that self-assemble into micellar structures [13]. These micelles can incorporate poorly soluble drugs like β-Lapachone, thereby increasing their aqueous solubility [14]. Upon self-assembly, micelles form hexagonal or cubical ordered phases, generating the thermosensitive hydrogels [13]. Among this family of copolymers, Pluronic® F-127 is the most widely used in drug delivery due to its low toxicity and immunogenicity. However, its fast dissociation in aqueous environments could hinder its ability to achieve sustained drug release profiles [13].

An effective strategy to modulate drug release from pluronic-based hydrogels involves combining them with other polymers, such as chitosan, hyaluronic acid (HA) or alginates [13]. Notably, hyaluronic acid is already used intra-articularly in OA treatments to improve synovial fluid hydration and viscoelasticity, thereby enhancing joint structural integrity [4].

In this sense, hybrid hydrogels combining Pluronic® F-127 and HA, loaded with the natural anti-inflammatory compound β-Lapachone, are hypothesized to offer dual therapeutic benefits for OA treatment. The controlled release of β-Lapachone is expected to provide sustained anti-inflammatory effects, while HA is anticipated to enhance the hydrogels' mechanical properties and stability. This dual functionality could improve joint function and potentially slow the progression of OA.

In our previous work, Artificial Neural Networks were used to rationally design Pluronic® F-127/hyaluronic acid hydrogels capable of loading β-Lapachone. These systems demonstrated *in vitro* anti-inflammatory effects and exhibited rheological properties suitable for use as a viscosupplement [15]. Building on this foundation, this study aims to further characterize β-Lapachone-loaded Pluronic® F-127/hyaluronic acid gels by investigating their micellar characteristics, gel stiffness and hydrogel microstructure to better understand their rheological behavior. Moreover, the release profile of the obtained hydrogels was studied and compared to that of hydrogels containing only Pluronic® F-127. Additionally, for the first time, we will evaluate the clinical potential of these drug-loaded hydrogels in an *in vivo* rabbit model of OA, with the goal of demonstrating their efficacy as a treatment for the disease.

2. Materials and methods

2.1. Materials

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione; C15H14O) was kindly provided by Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE (Recife, Brazil) with a 99.9 % purity, as determined by DSC and HPLC. Pluronic® F-127 (PF127) was purchased from Sigma-Aldrich (St. Louis, USA). Hyaluronic acid (HA) of high molecular weight, 1.54 ± 0.16 MDa (PDI 1.25), and a purity greater than 98 % was obtained and characterized as previously described [15]. Multiplex immunoassay kits were acquired from R&D Systems (USA). DPBS, penicillin/streptomycin, Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin/amphotericin and fetal bovine serum were purchased from Thermo Fisher Scientific (USA). Bupaq and Ketamidor were purchased from VetViva Richter GmbH (Austria). Sededorm was acquired from VetPharma Animal Health SL (Spain). Ventflurane and Syvaquinol were obtained from Virbac SA (France) and Laboratorios Syva SA (Spain), respectively. Loxicom and Dolethal were purchased from Norbrook Laboratories Limited (Ireland) and Vetoquinol Especialidades Veterinarias, SA (Spain), correspondingly. The light curing resin Technovit® 7200-VLC was obtained from Heraeus Kulzer GmbH (Germany) and absorbable sutures Vicryl 4-0 were purchased from Ethicon (USA). Ganadexil was obtained from Industrial Veterinaria SA (Spain).

2.2. Hyaluronic acid production and purification

HA was obtained by fermentation of the producer bacterium *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 grown in a culture medium formulated with fish wastes using a 2 L-bioreactor with continuous control of pH, temperature and agitation, and operating in batch mode [16]. After 18 h of cultivation, the bacterial biomass was precipitated and centrifuged, and the supernatant was chemically treated for the selective isolation of HA. Subsequently, the HA was purified by ultrafiltration using a 300 kDa membrane [17], freeze-dried and vacuum-packaged until use.

2.3. Hydrogels preparation and physicochemical characterization

2.3.1. Hydrogels preparation

Pluronic® F-127/hyaluronic acid hybrid hydrogels were prepared following a protocol previously developed by our group using Artificial Intelligence tools [15]. In brief, hyaluronic acid was initially dissolved in ultrapure water (MilliQ®) at a concentration of 6.37 mg/mL. The solution was cooled, and Pluronic® F-127 was added at a concentration of 15 % (*w/v*). The mixture was then continuously stirred at 500 rpm until complete dissolution. For β-Lapachone-loaded hydrogels, the drug was added to the Pluronic® F-127/hyaluronic acid mixture to achieve a final concentration of 0.16 mg/mL.

2.3.2. Micellar diameter characterization

Pluronic® F-127 solutions (5 % *w/v*) were prepared in ultrapure water and filtered through 0.45 μm filters. Dynamic light scattering (DLS) and zeta potential measurements were performed in quadruplicate both at 25 °C and 37 °C in a Zetasizer Nano ZS (Malvern Instruments, UK), according to a previously described protocol with slight modifications [18]. To assess the effect of hyaluronic acid inclusion as well as to evaluate β-Lapachone-micelle interactions, Pluronic® F-127-hyaluronic acid binary mixtures and Pluronic® F-127-hyaluronic acid-β-Lapachone ternary mixtures were also prepared and characterized. The reduction in the Pluronic concentration compared to the concentration used to prepare the gels, was deliberate to obtain a solution with reduced viscosity, to avoid issues during the measurements, while maintaining a concentration above the CMC (critical micelle concentration). Hyaluronic acid (2.12 mg/mL) and β-Lapachone (0.053 mg/

mL) concentrations were adjusted accordingly to replicate the ternary system behavior in the original hydrogels.

2.3.3. Gel stiffness

The rheological properties of Pluronic® F-127/hyaluronic acid hybrid hydrogels containing β-Lapachone were assessed in a stress-controlled AR1000N rheometer (TA Instruments, USA). Samples were subjected to frequency sweeps with a rotation speed from 0.05 to 50 rad/s under an oscillatory stress of 0.1 Pa. The storage (G′) and the loss modulus (G″) were recorded using these conditions both at 25 °C and 37 °C. Additionally, a temperature ramp from 20 to 40 °C at 2 °C/min rate with the same oscillatory stress at 5 rad/s was applied recording G′ and G″.

2.3.4. Injectability tests

Hydrogel injectability was evaluated following a previously described protocol [19] using a TA XT Plus Texture Analyser (Surrey, UK). In brief, Pluronic® F-127/hyaluronic acid hydrogels were loaded into 1 mL syringes coupled with a needle, ensuring no bubble formation. The syringes were then vertically positioned on a support, with a gap between the texturometer punch and the syringe plunger. Finally, the punch moved downward at a speed of 2 mm/s, pressing the syringe plunger over a distance of 2 cm. Measurements were performed in triplicate at room temperature (22 °C), and injectability was analyzed by measuring the injection force.

2.3.5. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was employed to analyse the microstructure of the Pluronic® F-127/hyaluronic acid hybrid hydrogels. For this purpose, samples were air-dried for at least 24 h at 37 °C and sputter coated with gold. Images were then acquired using a ZEISS FESEM Ultra Plus (Zeiss, Germany) scanning electron microscope.

2.3.6. Drug release studies

To evaluate the capacity of the developed hydrogels to efficiently control β-Lapachone delivery, drug release studies were performed using horizontal Franz diffusion cells. For this purpose, 1.5 mL of either β-Lapachone-loaded Pluronic® F-127 or β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hybrid hydrogels were placed in the donor compartment, together with 1.5 mL of simulated synovial fluid at pH 7.4, prepared following a previously described protocol [20]. On the other hand, the receptor compartment was filled with 3 mL of simulated synovial fluid. Both compartments were separated by a 3500 Da pore size Spectra/Por 3® dialysis membrane (Fisher Scientific, USA). The pore size selected enables free drug diffusion, while preventing the passage of the polymeric components of the hydrogels. At suitable timepoints samples were taken from the receptor chamber and replaced with fresh simulated fluid. Finally, the amount of released drug was determined by UV–visible spectrophotometry at 257 nm using a plate reader (FLUOstar Omega, BMG Labtech, Germany).

2.4. Ex vivo anti-inflammatory activity

Cartilage tissue samples were obtained from OA patients undergoing total knee replacement surgery, after obtaining their informed consent, and with the approval of the Ethics Committee (CEIC Galicia 2015/029). Following surgery, cartilage samples were immersed in a sterile saline solution and washed twice with DPBS supplemented with 1 % penicillin/streptomycin/amphotericin. Subsequently, 6-mm tissue biopsies were obtained using sterile punches and then washed again with supplemented DPBS. The cartilage explants were cultured for 24 h in Dulbecco's modified eagle's medium (DMEM) supplemented with 2 % fetal bovine serum and 1 % penicillin/streptomycin, under standard culture conditions (37 °C and 5 % CO_2), using 24-well plates. The following day, the cell culture media was removed, and the explants were stimulated with tumor necrosis factor alpha (TNF-α) at 25 ng/mL. They were then treated with β-Lapachone at varying concentrations (1 and 2 μM), dexamethasone (2 μM) or the Pluronic®-F127/HA hydrogels, either blank or loaded with β-Lapachone (equivalent final drug concentration 2 μM). After 48 h, cell culture supernatants were collected, and the secretion of pro-inflammatory cytokines and extracellular matrix degradative mediators, such as IL-6, MMP-13, IL-1ra, CXCL8 and MMP-9 were evaluated using multiplex immunoassay kits, following the manufacturer's instructions. The cytokine concentration was determined by measuring the plate fluorescence in a MAGPIX system (Luminex) and employing the corresponding calibration curve. The anti-inflammatory potential of the different treatments was then quantified by calculating the reduction percentage in protein secretion compared to untreated cartilage explants stimulated with TNF-α.

2.5. In vivo therapeutic activity

2.5.1. Experimental animal model

Twenty-four healthy skeletally mature adult female New Zealand White rabbits (Granja San Bernardo, Tulebras, Navarra, Spain), 7 months old and with a mean weight of 4.73 kg were used for the study following the approval of the protocol by the Ethics Committee of the University of Santiago de Compostela (03/18/LU-002). All procedures adhered to Spanish and European Union regulations on animal research and were carried out by personnel trained in laboratory animal science. The rabbits were housed individually at the Animal Experimentation Service Facility of the University of Santiago de Compostela (Lugo, Spain), with free access to food and water, environmental enrichment, and controlled conditions of temperature, ventilation, and light cycle. Throughout the study, the animals were monitored daily to assess their health status and promptly detect any signs of discomfort.

2.5.2. Osteoarthritis induction

After a three-week acclimatization period, osteoarthritis (OA) was induced in one knee of each rabbit through anterior cruciate ligament transection (ACLT) and partial medial meniscectomy. The contralateral knee served as a healthy control and was left untreated. The side for OA induction was randomly selected, with half of the animals undergoing surgery on the left knee and the other half on the right knee to minimize bias. The surgical wounds were then closed in two layers using 4-0 absorbable Vicryl sutures.

For the surgical procedure, animals were premedicated with a combination of medetomidine (50 mg/kg i.m. Sededorm 1 mg/mL) and ketamine (25 mg/kg i.m., Ketamidor 100 mg/mL), along with buprenorphine 0.03 mg/kg (Bupaq 0.3 mg/mL i.m.). Anesthesia was then maintained with isoflurane administered *via* facemask (Inspiratory Fraction ISO 2.5-4 %, Vetflurane 1000 mg/g). All animals received antibiotic prophylaxis with an initial dose of enrofloxacin (5 mg/kg i.m., Syvaquinol 25 mg/mL) and were given enrofloxacin in drinking water (1 mL/L; Ganadexil 10 %) for 21 days. Pain relief was managed with an initial dose of meloxicam (0.2 mg/kg i.m., Loxicom 5 mg/mL), followed by oral meloxicam (0.1 mg/kg) once daily for two days (Loxicom 1.5 mg/mL).

After surgery, once the animals had fully recovered from anesthesia, they were returned to individual cages, allowed to resume normal activity without joint immobilization, and monitored daily to assess any changes in their health status.

2.5.3. Intra-articular administration

Treatments began 3 weeks after the OA induction surgery. For administration, animals were anesthetized using the same regimen as during the surgery. The skin was disinfected with a combination of ethanol and povidone, and the systems were injected under sterile conditions using a 23-gauge needle. Injections were administered once a week for a total of 4 weeks.

The animals were divided into three groups (Fig. 1), each consisting of eight animals: one group was treated with sterile saline solution

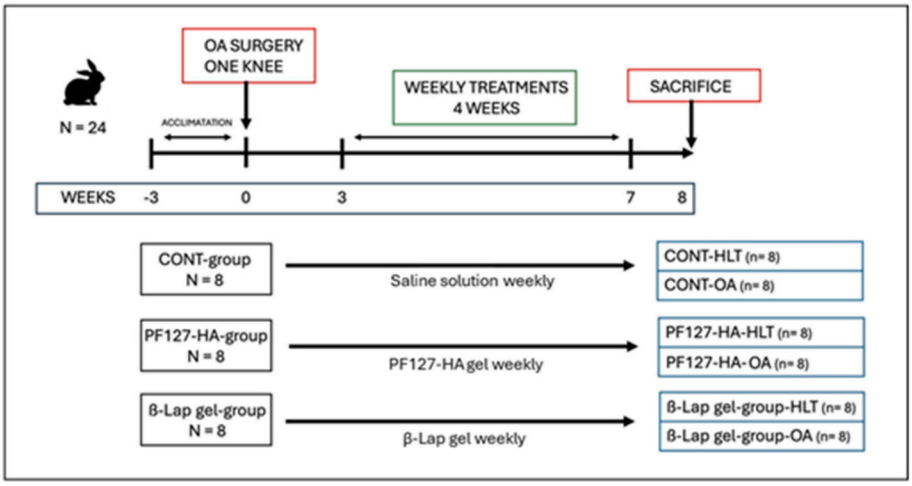


Fig. 1. Diagram illustrating the experimental design and treatment groups. CONT group: animals treated with placebo (sterile saline solution); PF127-HA-group: animals treated with Pluronic® F-127/hyaluronic acid hybrid hydrogels without β-Lapachone; β-LAP gel-group: animals treated with β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hydrogels. Animals were administered once a week for 4 weeks, after which they were sacrificed for sample collection.

(CONT-group), another received the Pluronic® F-127/hyaluronic acid hybrid hydrogels without β-Lapachone (PF127-HA group), and the third group was treated with β-Lapachone-loaded Pluronic® F-127/hyaluronic hydrogel (β-Lap gel group). As detailed in Section 2.5.2, the contralateral knee of all animals was used as a healthy control (HLT).

2.5.4. Necropsy and sample preparation

Animals were euthanized 8 weeks after the initial surgery using an overdose of sodium pentobarbital (Dolethal 100 mg/kg) preceded by sedation with medetomidine and ketamine at the same dosages used during surgery. Following euthanasia, the entire knee joints were dissected and fixed in 10 % formalin solution for histological analysis.

After fixation, specimens were processed for undecalcified thin section histology as described by Donath [21] and stained using the Lévai-Laczkó method [22], a combination of methylene blue, azure II and safranin [23], that enables evaluation of the presence of mineral in the tissues [24]. In addition, the undecalcified plastic embedding was selected following OARSI recommendations [25]. Briefly, samples were dehydrated and embedded in a light-curing resin (Technovit® 7200-VLC). Once embedded, the distal femur was sectioned in the middle and polished using a grinding machine (EXAKT Apparatebau, Germany) to achieve approximately a 40 μm thickness.

2.5.5. Microscopic evaluation of undecalcified sections

A masked examiner performed the histological analysis using pre-established morphometric parameters and employed PC-based image analysis software, including CellSens 1.5 (Olympus Corporation, Japan) and Image-Pro Premier 4.0 (Media Cybernetics, USA) [26]. The morphometric parameters used for the cartilage evaluation were previously published by our group and are detailed in Fig. 2 [27].

Subchondral cortical bone thickness (SB.Th) was defined as the mean distance between the cartilage and the subchondral cortical bone boundary. Mean cartilage thickness (Cg.Th) is further divided into non-calcified cartilage thickness (nCg.Th) and calcified cartilage thickness (cCg.Th), using the tidemark as a reference.

The fibrillation index (FI), which measures the superficial undulations of the cartilage, is defined as the difference between the length of the upper margin of the cartilage (line 1) and the width of the same

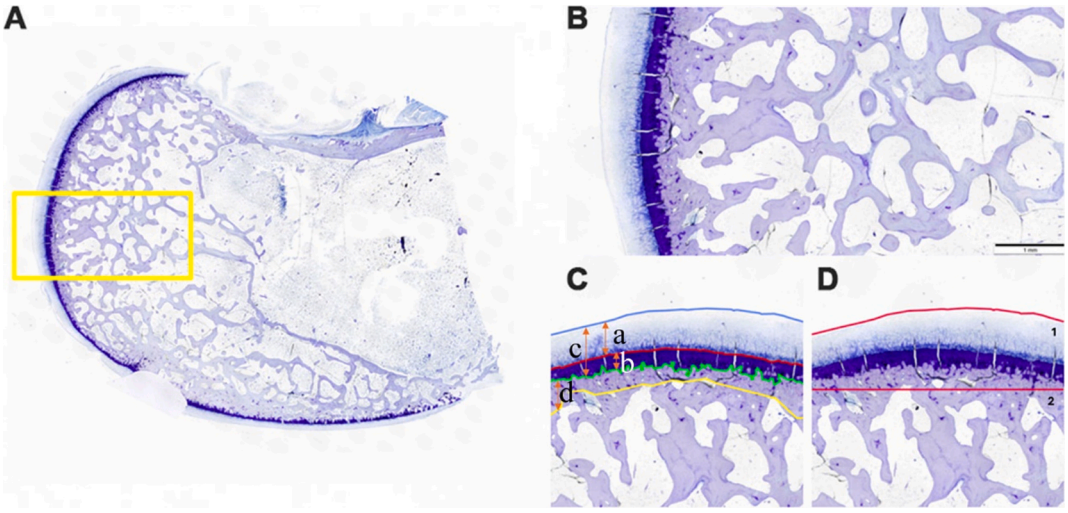


Fig. 2. A) Representative image of the femoral condyle from a control knee. The yellow rectangle highlights the section used for measurement, which is also depicted in image B. C) Measurements of cartilage and subchondral bone thickness: a) nCg.Th (non-calcified cartilage thickness); b) cCg.Th (calcified cartilage thickness); c) Cg.Th (mean cartilage thickness) and d) SB.Th (Subchondral cortical bone thickness). D) Representative image of fibrillation index measurement: line 1 indicates cartilage surface and line 2 the width of the FI measurement area. Scalebar: 1 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measurement area (line 2).

For all the samples, only the mid surface of the condyle was used.

2.6. Statistical analysis

Experimental values were presented as means and standard deviations. Statistical analyses were performed using GraphPad Prism 8 (Informer Technologies Inc., USA). ANOVA followed by Dunnett's *post hoc* test, was applied to identify significant differences between the treatment groups and the control group. Pairwise comparisons between two groups were performed using Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant in both analyses.

3. Results and discussion

3.1. Hydrogels physicochemical characterization

The β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hydrogels under evaluation were previously optimized in terms of composition using Artificial Intelligence tools to enable efficient drug incorporation while maintaining appropriate viscosupplementation capacity [15]. The high molecular weight hyaluronic acid (HA) used to prepare the hydrogels, obtained through bacterial fermentation using fish-based products [16], offers several advantages, including the avoidance of concerns related to the transmission of infectious diseases and potential religious or cultural objections associated with the use of mammalian-derived products [28]. Moreover, the thermoresponsive properties of the hydrogels will allow easy injection and increased joint residence time. Additionally, the viscosupplementation capacity of the systems will improve joint function while their micellar structure enhances the solubilization of poorly soluble drugs. Therefore, the developed systems show several advantages over current OA treatments and are promising drug delivery systems for intra-articular administration.

Building on this foundation, the present study represents a step forward on the clinical translation of the developed hydrogels and provides a thorough characterization of the hydrogels in terms of micellar properties, hydrogel microstructure, temperature-responsive stiffness, drug release in biologically relevant environments, and *ex vivo* anti-inflammatory potential in human cartilage explants. Additionally, the therapeutic potential of the β-Lapachone-loaded hydrogels was evaluated in an osteoarthritis (OA) rabbit model.

3.1.1. Micellar diameter characterization

Poloxamers, such as Pluronic® F-127, can be used to develop thermosensitive micelle-based hydrogels that enhance the solubility of poorly soluble drugs like β-Lapachone by incorporating them into the hydrophobic micelles core [15].

Micelle formation can be disrupted or altered by interactions between poloxamers and other formulation components, such as hyaluronic acid (HA). Given the critical role of micelle formation in drug solubilization and, consequently, in the performance of hydrogels as drug delivery systems, dynamic light scattering (DLS) was used to characterize the micellar hydrodynamics of Pluronic® F-127 solutions before and after the incorporation of hyaluronic acid and β-Lapachone. All analyses were conducted at 25 °C and 37 °C to evaluate micelle properties at both room and body temperatures. At room temperature, Pluronic® F-127 systems showed a bimodal size distribution with a predominant micellar population at 52 ± 4 nm and a smaller population of 5 nm, likely associated with the presence of unimers in solution (Fig. 3.A, blue color) [18]. Interestingly, at 37 °C, Pluronic® F-127 systems displayed increased homogeneity, showing a unimodal and reduced micellar size of 24 ± 2 nm (Fig. 3.A, orange color).

This reduction in particle size and size distribution is consistent with previous studies and can be associated with the dehydration of the PPO chains within the micellar core, resulting in more compact micelles [18,29]. Additionally, this dehydration with temperature is believed to enhance micelle formation and decreases the critical micelle concentration (CMC), which might explain the absence of unimers at physiological temperature [30].

The inclusion of hyaluronic acid in the systems led to a slight increase in the micellar dimensions at both temperatures. Specifically, the micelles exhibited sized of 67 ± 7 nm at 25 °C and 31 ± 4 nm at 37 °C (Fig. 3.B). This increase in micelle size may be attributed to the high molecular weight of hyaluronic acid (MW = 1.4–1.5 MDa), which is likely to be incorporated into the hydrophilic shell of the micelles [18]. Similarly, the addition of β-Lapachone to the Pluronic® F-127/hyaluronic acid hybrid systems, led to micelles of 69 ± 6 nm and 33 ± 5 nm at 25 °C and 37 °C, respectively (Fig. 3.C). These findings suggest that

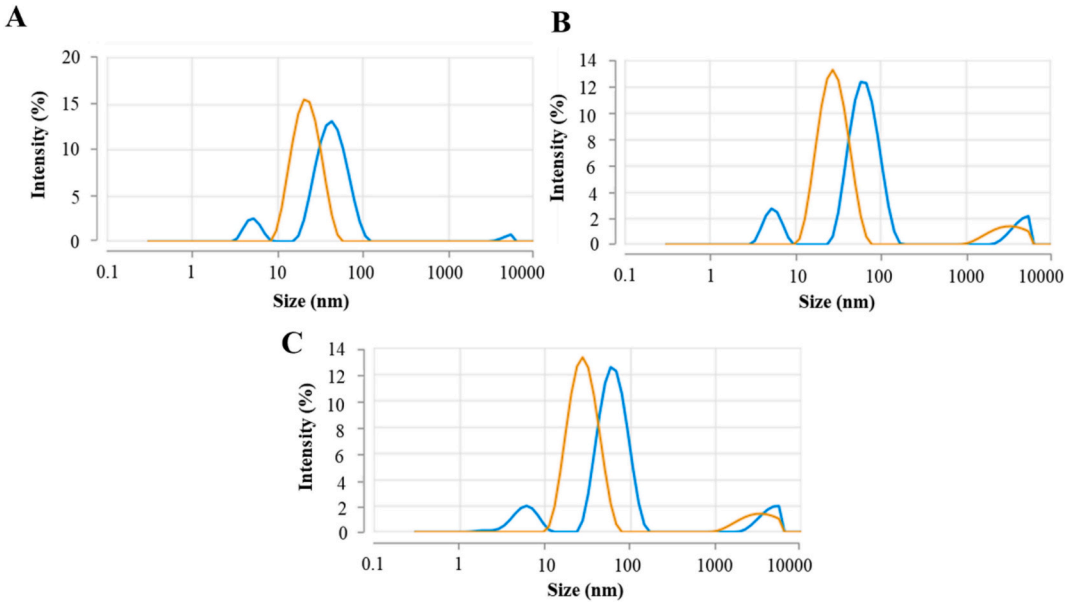


Fig. 3. DLS analysis of micellar systems: A) Pluronic® F-127, B) Pluronic® F-127/hyaluronic acid, and C) Pluronic® F-127/hyaluronic acid/β-Lapachone. Measurements were performed at 25 °C (blue) and 37 °C (orange). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the drug does not significantly disrupt the micellar organization.

In addition to characterizing micellar hydrodynamics, the surface charge of the micelles was evaluated. Pluronic® F-127 systems exhibited a zeta potential of −2 mV, both at 25 °C and 37 °C. However, the incorporation of the hyaluronic acid significantly increased the negative surface charge, with zeta potential values of −21 ± 1 mV at 25 °C and −18 ± 1 mV at 37 °C. This enhanced negativity can be attributed to the presence of ionized carboxylic groups of hyaluronic acid on the micelle surface, consistent with the incorporation of HA into the hydrophilic shell of PF127 micelles [31]. Furthermore, the inclusion of β-Lapachone in these systems resulted in zeta potential values of −22 ± 5 mV at 25 °C and −17 ± 1 mV at 37 °C, which are closely comparable to those observed for the PF127/hyaluronic acid micelles.

These results confirm the successful integration of hyaluronic acid into the Pluronic® F-127 micellar systems.

3.1.2. Gel stiffness

The rheological properties of the β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hybrid hydrogels were analyzed through frequency sweep tests conducted at 25 °C and 37 °C. Frequency sweeps are a well-established rheological method that provide insights into the cohesiveness and mechanical strength of fluids and gels [32].

As depicted in Fig. 4.A, both storage (G′) and loss (G″) moduli exhibit frequency-dependent variations. At 25 °C and low frequencies, the G″ values exceed G′ values, indicating that the material initially behaves as a viscous fluid [33]. At higher frequencies, G′ surpasses G″, suggesting a transition to solid-like behavior. The gelation point is identified at the crossover of G′ and G″ curves [32].

In contrast, Fig. 4.B shows that at 37 °C, G′ values consistently exceeds G″ across all frequencies tested, indicating that the hydrogel exhibits a solid-like structure [34]. Unlike the behavior observed at 25 °C results, no crossover between G′ and G″ occurred at 37 °C, suggesting the formation of a more entangled fibrous network [34]. This observation is further supported by the increased stiffness of the hydrogels at 37 °C, as evidenced by the higher storage modulus values (>300 Pa).

The results suggest temperature-responsive behavior, with gel cohesiveness and stiffness increasing with temperature. To confirm these findings, the storage and loss modulus of the Pluronic® F-127/hyaluronic acid hydrogels was also determined at temperatures ranging from 20 to 40 °C. The data show a gelation temperature of 28 °C (Fig. 4.C). These thermoresponsive properties agree with the micellar behavior previously observed by DLS and are particularly advantageous for injectable hydrogel formulations, as they remain in a liquid state at room temperature while behave as a gel at physiological body temperature (37 °C) [35].

3.1.3. Injectability tests

Injectable hydrogels are versatile biomaterials that can enhance the OA management by delivering therapeutic agents directly to the site of action, through a minimally invasive administration [36]. To confirm that the β-Lapachone-loaded Pluronic® F-127/hyaluronic hydrogels have suitable mechanical properties for their intra-articular injection, injectability was evaluated at room temperature (22 °C). Injectability is defined as the ability of an injectable material to pass through a needle prior to administration [37]. The results obtained showed that a force of 1.3 ± 0.3 N was required to inject the drug-loaded formulations. According to previous studies, this force is compatible with manual injection [38].

3.1.4. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was used to investigate the microstructure of Pluronic® F-127/hyaluronic acid hydrogels and examine the assembly of their components at the microscale. As shown in Fig. 5, the hydrogels in their solid-state exhibit micellar structures with sizes ranging from approximately 20–80 nm to 200–300 nm. The dimensions of the smaller micelles align with the micellar sized

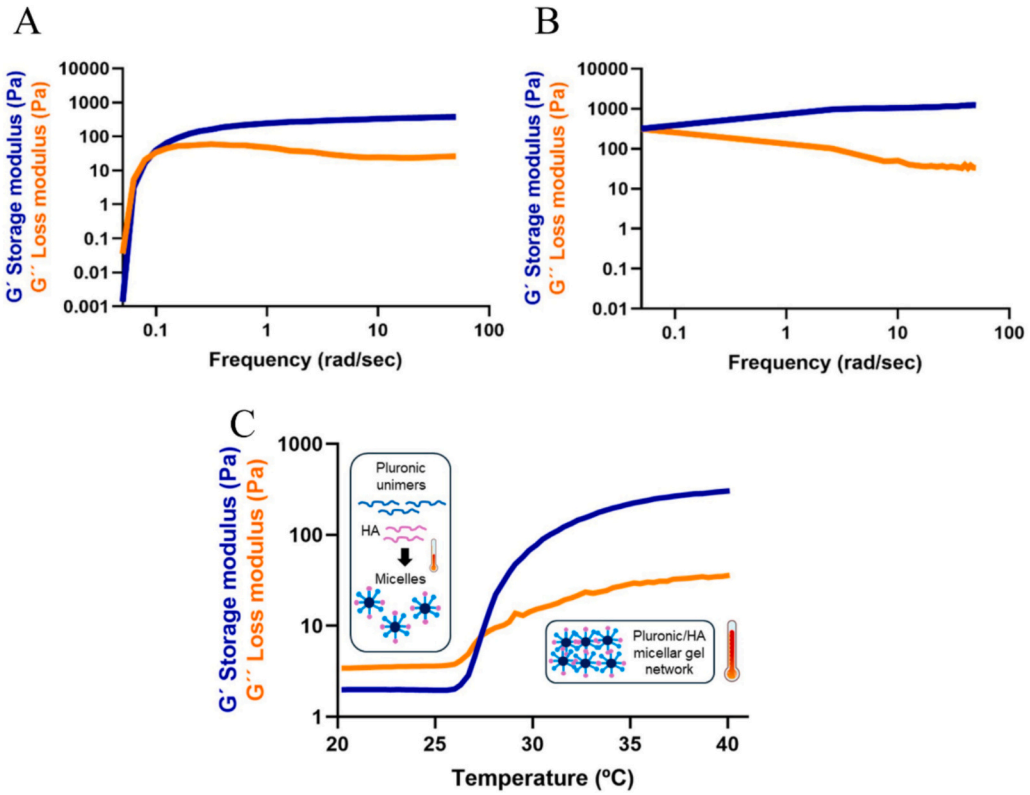


Fig. 4. Variation of storage modulus (G′) and loss modulus (G″) with frequency at A) 25 °C and B) 37 °C. C) Variation of storage modulus (G′) and loss modulus (G″) with temperature and graphical diagram of Pluronic® F-127/hyaluronic acid micellar arrangements below and above gelation temperature.

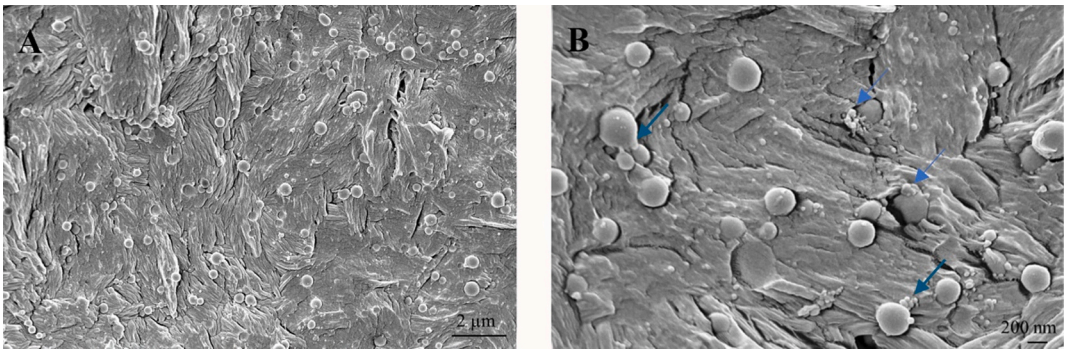


Fig. 5. Scanning electron microscopy (SEM) images of Pluronic® F-127/hyaluronic acid hybrid hydrogels at two magnifications: A) 15,000× and B) 50,000×. Blue arrows indicate micelle aggregation phenomena. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

previously measured by DLS. The larger micelles, highlighted in Fig. 5.B, indicate micelle aggregation phenomena, which are consistent with the known gelation mechanism of Pluronic® F-127 [39].

3.1.5. Drug release studies

Drug release studies were performed in simulated synovial fluid to assess the capacity of the Pluronic® F-127/hyaluronic acid hybrid hydrogels to efficiently control β-Lapachone release under physiological conditions. Moreover, drug release from β-Lapachone-loaded Pluronic® F-127 hydrogels was also evaluated as a control. As shown in Fig. 6, both hydrogels exhibited an initial fast release, followed by a more sustained release profile, fitting a Korsmeyer-Peppas model. Furthermore, based on the n values obtained, which were found to be 0.48 and 0.53 for the Pluronic® F-127 and Pluronic® F-127/hyaluronic acid hydrogels, respectively, drug release follows a diffusion-controlled mechanism [40]. On the other hand, the high similarity between the drug release profiles obtained for both hydrogel systems suggests that β-Lapachone release is primarily controlled by the polymeric Pluronic® F-127 micelles.

3.2. Ex vivo anti-inflammatory activity of Pluronic F-127®/hyaluronic acid hydrogels

The anti-inflammatory activity of β-Lapachone and dexamethasone solutions, along with β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hydrogels (β-Lap gel) and blank hydrogels (PF127-HA) was assessed *ex vivo* using TNF-α-treated OA cartilage explants. TNF-α is a known pro-inflammatory cytokine that increases cartilage degradation by promoting the release of matrix metalloproteinases (MMPs) and inhibiting chondrogenesis through the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway [41]. This cytokine can hinder the natural healing processes of chondral and osteochondral defects following cartilage repair [41]. Interestingly, β-Lapachone has been reported to counteract inflammation by inhibiting NFκB activation [42].

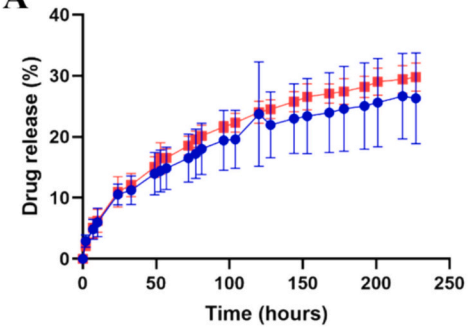
As illustrated in Fig. 7, both free β-Lapachone and drug-loaded hydrogels led to a significant reduction in the secretion of the pro-inflammatory cytokine IL-6, which plays a critical role in OA pathophysiology [43]. This reduction in cytokine secretion, of approximately 99 %, was comparable to that achieved with dexamethasone, a potent anti-inflammatory agent [44], confirming the anti-inflammatory potential of β-Lapachone.

Additionally, treatments with free β-Lapachone, β-Lapachone-loaded hydrogels, and blank hydrogels resulted in a substantial decrease in the secretion of matrix metalloproteinases MMP9 (around 95 %) and MMP13 (88–96 %). This is particularly significant as MMP13 is a key collagenase secreted by chondrocytes involved in OA progression [15], while MMP9 degrades the hyaline articular cartilage [45]. Statistically significant differences ($p < 0.05$) were observed only between dexamethasone and β-Lapachone 2 µM groups for MMP9 secretion.

The reduction in metalloprotease secretion observed with blank hydrogels might be attributed to the anti-inflammatory properties of hyaluronic acid. This finding is in agreement with previous studies, which have reported decreased MMP13 expression following hyaluronic acid treatment, in osteochondral cylinders from OA patients stimulated with both IL-1β and mechanical load [46].

Furthermore, although there was notable variability between replicates, treatment of cartilage explants with either β-Lapachone 1 µM, blank hydrogels or drug-loaded gels led to a reduction in the range of 25–65 % in CXCL8 secretion, a chemokine that enhances pro-inflammatory cytokine secretion by mononuclear cells [47]. Conversely, a decrease in IL-1 receptor antagonist (IL-1ra) secretion,

A



B

Kinetic model	R^2 PF-127	R^2 PF-127/HA
Zero order	0.8933	0.8981
First order	0.6934	0.662
Higuchi	0.9826	0.9772
Korsmeyer-Peppas	0.9927	0.9913

Fig. 6. A) Drug release profiles of β-Lapachone-loaded Pluronic® F-127 (blue color) and Pluronic® F-127/hyaluronic acid hybrid hydrogels (red color). B) Correlation coefficient value (R^2) obtained for the different drug release kinetic models. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

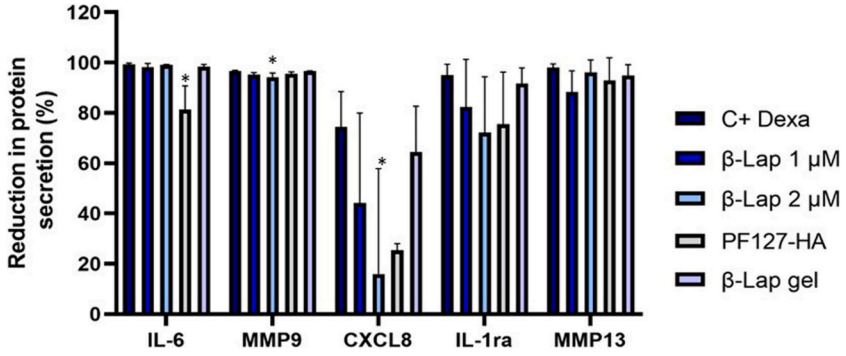


Fig. 7. Reduction in cytokine (IL-6), matrix metalloprotease (MMP9 and MMP13), chemokine (CXCL8), and receptor antagonist (IL-1ra) secretion by TNF-α-treated OA cartilage explants cultured for 48 h with the different treatments: dexamethasone (positive control), β-Lapachone 1 μM, β-Lapachone 2 μM, blank Pluronic® F-127/hyaluronic acid hydrogels (PF127-HA) or Pluronic® F-127/hyaluronic acid hydrogels containing β-Lapachone (β-Lap gel). *Denotes statistically significant differences compared to the positive control (dexamethasone) ($p < 0.05$).

ranging from 72 to 92 %, was observed across all treatments. It is relevant to note that IL-1ra is an inhibitor that blocks the action of the pro-inflammatory cytokines IL-1α and IL-1β, thus exerting an anti-inflammatory effect [48]. Interestingly, glucocorticoids such as cortisol and dexamethasone have been reported to suppress both IL-1 and IL-1ra expression as part of their mechanism of action [49]. These findings suggest that β-Lapachone and hyaluronic acid treatments might similarly influence these pathways.

In conclusion, both the β-Lapachone solutions and Pluronic® F-127/hyaluronic acid hydrogels, particularly the β-Lapachone-loaded formulations, exhibit an anti-inflammatory potential *ex vivo* comparable to that of the glucocorticoid dexamethasone, which is extensively used in treating inflammatory conditions.

3.3. *In vivo therapeutic potential of Pluronic® F-127/hyaluronic acid hydrogels*

3.3.1. *Clinical findings*

All OA induction surgeries were completed without complications. However, one rabbit in the β-Lap gel-treated group died during anesthesia for the second intra-articular injection and was excluded from the study. Another rabbit in the PF127-HA group died at the end of the study due to diarrhea but was not excluded as it had completed all the treatments. The remaining animals successfully tolerated the intra-articular treatments.

3.3.2. *Evaluation of cartilage and subchondral bone thickness*

During OA progression, joint structures such as cartilage and subchondral bone experience an imbalance between anabolic and catabolic processes [50]. In this context, changes in cartilage and subchondral bone take place, contributing to the disease severity [50]. OA often includes increased cartilage hypertrophy, together with enhanced porosity and subchondral bone thinning [50–52]. To assess these pathological changes, histomorphometric analysis was conducted on the cartilage and subchondral bone in the femoral condyles. Fig. 8 shows the thickness of non-calcified, calcified, and total cartilage together with the subchondral bone thickness, for healthy animals (CONT HLT) and animals treated with either saline solution (control group; CONT OA), blank Pluronic® F-127/hyaluronic acid hydrogels (PF127-HA) or β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hydrogels (β-LAP gel).

In the CONT OA group, a mild tendency towards cartilage thickening and hypertrophy was generally observed. The observed increase in cartilage thickness is characteristic of the early stages of the disease, and has been associated with a swelling phenomenon [53]. Notably, animals treated with blank Pluronic® F-127/hyaluronic acid hydrogels (PF127-HA) exhibited a statistically significant decrease in non-calcified cartilage thickness (nCg.Th) compared to the CONT OA ($p = 0.0016$).

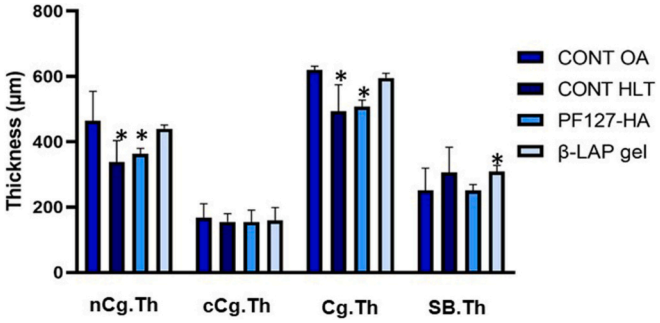


Fig. 8. Results for cartilage and subchondral bone thickness. nCg.Th: Non-calcified cartilage thickness; cCg.Th: Calcified cartilage thickness; Cg.Th: Total cartilage thickness; SB.Th: Subchondral bone thickness. Data are presented as mean ± standard deviation. *Indicates statistically significant differences compared to the CONT OA group ($p < 0.05$).

Additionally, total cartilage thickness (Cg.Th) was significantly reduced in both the β-Lapachone-loaded hydrogel group (β-Lap gel, $p = 0.008$) and the blank hydrogel group (PF127-HA, $p = 0.0001$) when compared to the CONT OA.

These results suggest that hyaluronic acid incorporated into the formulations may lead to a protective effect on cartilage, helping to prevent tissue hypertrophy. This therapeutic effect could be attributed to the various roles of hyaluronic acid at the joint level, including its chondroprotective, anti-inflammatory and antioxidative properties. Additionally, the ability of hyaluronic to enhance cartilage lubrication and prevent extracellular matrix (ECM) degradation also likely contributes to its protective effects [54]. Furthermore, the chondroprotective effect observed is in agreement with several works in the field, that describe a retardation in cartilage degeneration and OA progression after intra-articular administration of high molecular weight crosslinked hyaluronic acid in an OA rabbit disease model [55,56].

Conversely, in the CONT OA group, a trend towards subchondral bone thinning was observed following OA induction. Notably, a statistically significant increase in subchondral bone thickness was found in the group treated with β-Lapachone-containing hydrogels (β-Lap gel, $p = 0.0213$) compared to the saline solution (CONT OA). In contrast, the blank Pluronic® F-127/hyaluronic acid hydrogels treatment did not appear to prevent bone degeneration, suggesting that the therapeutic effect on bone may be specifically linked to β-Lapachone administration. This outcome could be attributed to the drug's ability to inhibit the NFκB pathway [42]. The activation of NFκB, a signaling pathway involved in OA pathogenesis, is known to induce the expression of osteoclastogenic factors, such as IL-1β, which trigger subchondral bone resorption [57,58]. Interestingly, the intraarticular administration of a conjugate of

hyaluronic acid and salmon calcitonin, a well-known anti-resorptive drug, in an analogue OA rabbit model, has been reported to ameliorate subchondral bone morphology [59]. Authors associate the obtained positive outcomes with the ability of the covalent system to prevent calcitonin clearance from the joint cavity [59]. We hypothesize the developed Pluronic® F-127/hyaluronic acid system could also modulate β-Lapachone's release and residence time, allowing to take full advantage of its anticatabolic effect at the bone level.

In summary, osteoarthritic joints are characterized by increased cartilage thickness and decreased subchondral bone thickness compared to healthy knees, characteristic of early stage OA [51,53]. Furthermore, the presence of hyaluronic acid in the formulations appears to protect cartilage, while the inclusion of β-Lapachone was found to be crucial for enhancing other histomorphometric markers, such as subchondral bone thickness.

3.3.3. *Fibrillation index*

In addition to the previously mentioned histomorphometric parameters, early signs of cartilage degeneration that do not involve the entire tissue thickness, such as flaps, fibrillation or blistering should be assessed [60]. The fibrillation index (FI) is a commonly used metric to evaluate the surface characteristics of cartilage, defined as the root mean square of variations in the cartilage surface profile compared to an ideally smooth surface after optimal debridement [60]. The FI was measured for saline solution-treated OA control group (CONT OA), the blank Pluronic® F-127/hyaluronic acid hydrogels treated animals (PF127-HA) and β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hydrogels treated animals (β-Lap gel), to investigate the effects of the different treatments on cartilage surface features (Fig. 9).

The fibrillation index results demonstrated a significant reduction in superficial fissures and undulations in the cartilage of animals treated with β-Lapachone-loaded hydrogels (β-Lap gel, $p = 0.0005$) compared to the saline solution-treated control group (CONT OA). In contrast, there were no significant differences in the fibrillation index between the CONT OA group and the group treated with blank hydrogels (PF127-HA). This suggests that the presence of β-Lapachone in the hydrogel formulation is crucial to improve the surface of the femoral cartilage.

Representative histological images for each tested condition, used to assess the histomorphometric parameters shown in Figs. 8 and 9, are presented in Fig. 10. These images enable the visual evaluation of cartilage degeneration differences between experimental groups. Animals treated with β-Lapachone-loaded formulations (β-Lap gel), Fig. 10F, exhibited a markedly improved cartilage surface appearance compared to both the saline solution group (CONT OA, Fig. 10D) and the group treated with blank hydrogels (PF127-HA, Fig. 10E), featuring a smoother surface with reduced fibrillation like that of the healthy contralateral controls (Fig. 10A–C). These observations align with previous studies highlighting the immunomodulatory and anti-arthritic properties of β-Lapachone, pointing out its potential in preventing and treating various inflammatory diseases [7,61]. Furthermore, previous studies suggest the intra-articular administration of high molecular

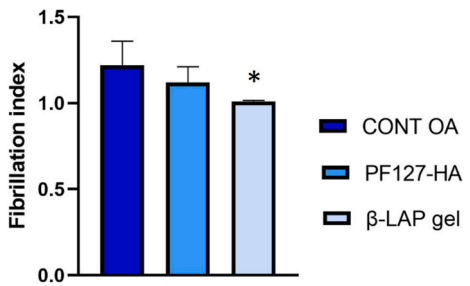


Fig. 9. FI measurements for cartilage surface characteristics across the three study groups. Data are presented as mean ± standard deviation. *Denotes statistically significant differences compared to the CONT OA group ($p < 0.05$).

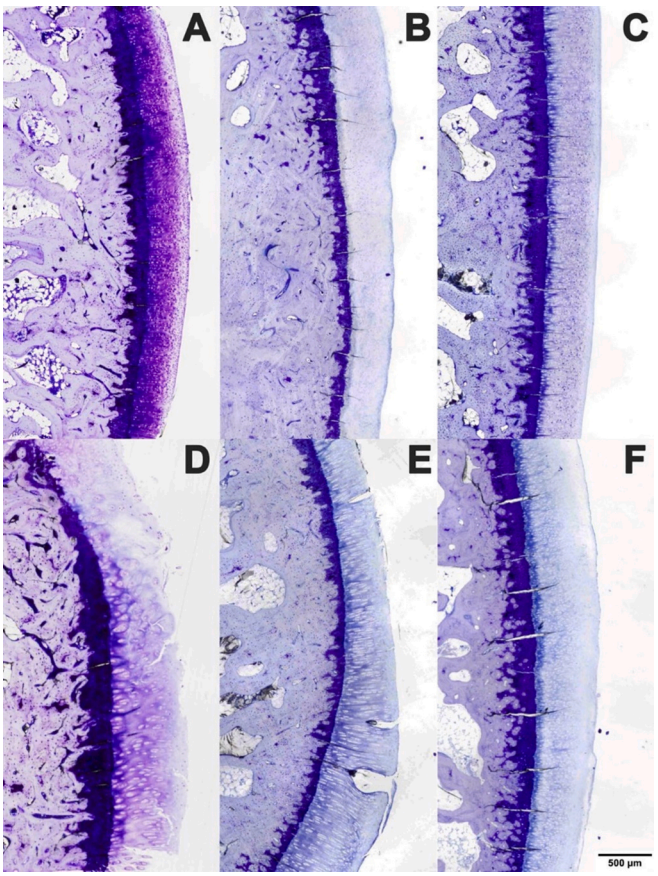


Fig. 10. Histological comparison of samples from the different treatment groups. A: CONT-HLT (healthy contralateral knee of rabbits treated with saline solution); B: PF127-HA-HLT (healthy contralateral knee of rabbits treated with blank hydrogels); C: β-Lap gel-HLT (healthy contralateral knee of rabbits treated with β-Lapachone-loaded hydrogels); D: CONT-OA (osteoarthritic knee, saline solution-treated); E: PF127-HA (osteoarthritic knee treated with blank hydrogels); F: β-Lap gel (osteoarthritic knee treated with β-Lapachone-loaded hydrogels). Staining: Levai-Lazckó. Magnification: 4×.

weight hyaluronic acid, like the one present in the formulations, exerts a chondroprotective effect by triggering a reduction in the severity of cartilage degeneration in OA rabbit models [55,56]. This might explain the improved cartilage appearance in osteoarthritic knees of blank hydrogels treated mice (Fig. 10E) in comparison with the saline solution-treated control (Fig. 10D). Therefore, a synergistic effect between the immunomodulatory activity of β-Lapachone and the chondroprotective action of hyaluronic acid could be responsible for the significant reduction in early cartilage damage parameters for rabbits treated with the β-Lapachone-loaded formulations.

Despite the promising preclinical findings derived from this study, it has some minor limitations. First, saline solution injections used in the control group might slightly affect synovial fluid viscosity and, therefore, joint lubrication. This concern has been previously raised by other authors, that did not find any significant differences in the rheological properties of synovial fluid after saline injection [55]. However, we have not performed *in vivo* evaluations. On the other hand, another limitation of the present study is the inability to evaluate the therapeutic potential of free β-Lapachone *in vivo*, due to the reduced solubility of the drug in aqueous media.

4. Conclusions

Pluronic® F-127/hyaluronic acid hydrogels, loaded with the natural product β-Lapachone were successfully obtained through a procedure

previously optimized using Artificial Intelligence tools that enabled the obtention of hydrogels with reproducible characteristics. Hydrogel formulations showed a composite microstructure integrating micelles, together with a temperature-responsive stiffness. The treatment of TNF-α stimulated OA cartilage explants with the drug, before and after its inclusion within the hydrogel systems, and with the blank hydrogels, led to a decrease in several pro-inflammatory mediators, producing a similar effect to that obtained with dexamethasone, widely used to treat inflammatory processes. Furthermore, after the intra-articular administration of the drug-loaded hydrogels in an *in vivo* rabbit model of OA, formulations were able to attenuate subchondral bone loss, cartilage hypertrophy and generation of cartilage superficial fissures and undulations. Therefore, the developed β-Lapachone-loaded Pluronic® F-127/hyaluronic acid hybrid hydrogels constitute a promising candidate for OA management.

CRediT authorship contribution statement

Helena Rouco: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Maria Permuy:** Writing – original draft, Methodology, Investigation. **Fernando Muñoz:** Writing – review & editing, Methodology, Investigation. **José Antonio Vázquez:** Writing – review & editing, Methodology, Investigation, Conceptualization. **José R. Caeiro:** Writing – original draft, Methodology. **Mariana Landin:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Patricia Diaz-Rodriguez:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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