Materials Science & Engineering C 128 (2021) 112254

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Targeting joint inflammation for osteoarthritis management through stimulus-sensitive hyaluronic acid based intra-articular hydrogels

Patricia Diaz-Rodriguez [a,b,*], Cibrán Mariño [a], Jose Antonio Vázquez [c], Jose Ramon Caeiro-Rey [d], Mariana Landin [a]

[a] R+D Pharma Group (GI-1645) Department of Pharmacology, Pharmacy and Pharmaceutical Technology, School of Pharmacy, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[b] Drug Delivery Systems Group, Department of Chemical Engineering and Pharmaceutical Technology, School of Pharmacy, Universidad de La Laguna, La Laguna, Spain
[c] Group of Recycling and Valorisation of Waste Materials (REVAL), Marine Research Institute (IIM-CSIC), Vigo, Spain
[d] Department of Orthopaedic Surgery and Traumatology, Complexo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Spain

ARTICLE INFO

Keywords:
Hyaluronic acid
Intra-articular hydrogels
Thermo-sensitive systems
Anti-inflammatory activity
Osteoarthritis

ABSTRACT

Numerous therapeutic strategies have been developed for osteoarthritis (OA) management, including intra-articular (IA) injections. The ideal IA formulation should control cartilage degradation and restore synovial fluid viscosity. To this end, we propose to combine thermo-sensitive polymers (poloxamers) with hyaluronic acid (HA) to develop suitable beta-lapachone (βLap) loaded IA formulations. The development of IA formulations with these components entails several difficulties: low βLap solubility, unknown βLap therapeutic dose and the bonded commitment of easy administration and viscosupplementation. An optimized formulation was designed using artificial intelligence tools based on the experimental results of a wide variety of hydrogels and its therapeutic capacity was evaluated on an *ex vivo* OA model. The formulation presented excellent rheological properties and significantly decreased the secretion of degradative (MMP13) and pro-inflammatory (CXCL8) molecules. Therefore, the developed formulation is a promising candidate for OA treatment restoring the synovial fluid rheological properties while decreasing inflammation and cartilage degradation.

1. Introduction

Osteoarthritis (OA) is a degenerative musculoskeletal disease involving the full-thickness of the osteochondral unit. It is a highly prevalent disease affecting over 250 million people worldwide representing a huge socioeconomic burden. Moreover, the disease numbers keep increasing due to the ageing of the population [1,2]. Big efforts have been made to find strategies able to avoid or control OA progression such as local administration of stem cells, autologous blood derived cell concentrates, autologous chondrocyte implantation, TNF inhibitors, growth factors and cell derived exosomes. However, these approaches have shown limited clinical success and variable outcomes, restricted by the limited self-repair potential of hyaline cartilage and relegating current OA treatments to those mainly devoted to alleviate the disease symptoms [2–7].

Quite recently, the crucial role of inflammation on OA progression has been pointed out. In fact, the level of some pro-inflammatory cytokines on the synovial fluid, such as IL-6, serves as a diagnostic tool of joint damage and can promote disease progression [8,9]. These cytokines, secreted by activated synovium and cartilage stablish a chronic joint inflammation and a catabolic/anabolic imbalance leading to further cartilage degradation [10–12]. Despite the huge efforts made to counterbalance this altered environment, there is no therapeutic molecule nowadays able to revert this pathological state making necessary to explore novel strategies for OA management [5].

New strategies to avoid disease progression should be aimed at modulating the above mentioned imbalance of catabolic and anabolic factors stablished on the disease. Beta-lapachone (βLap) is a natural naphtoquinone identified *in vitro* as a highly active molecule for the treatment of several types of cancer [13]. Beta-lapachone is "activable" by the enzyme NAD(*P*)H dehydrogenase [quinone] 1 (NQO1) leading to a futile cycle in which the molecule gets converted to its unstable hydroquinone form which, therefore, is oxidized to the semiquinone form and further oxidized to the initial quinone form [14]. This cycle causes a

* Corresponding author at: R+D Pharma Group (GI-1645) Department of Pharmacology, Pharmacy and Pharmaceutical Technology, School of Pharmacy, Universidade de Santiago de Compostela, Santiago de Compostela, Spain.
E-mail address: patricia.diaz.rodriguez@usc.es (P. Diaz-Rodriguez).

https://doi.org/10.1016/j.msec.2021.112254
Received 4 March 2021; Received in revised form 19 May 2021; Accepted 8 June 2021
Available online 16 June 2021

dramatic increase in NAD^+ levels and a subsequent ATP loss modifying cell metabolism. More recently, βLap has been pointed out as an anti-inflammatory and wound healing promoter molecule improving oxidative stress, renal dysfunction and skeletal muscle function in renal injury, skeletal muscle atrophy and neuroinflammatory disease models [15–18]. The controversial responses to βLap treatment in healthy *versus* cancer cells can be attributed to the increased NQO1 expression levels in numerous tumours [14,19]. On the other hand, in non-tumoral cells, βLap induce heme oxygenase-1 (HO1) expression and AMP-activated protein kinase activation exerting cytoprotective effects [20,21]. In fact, βLap has been already proposed as an alternative treatment for multiple sclerosis, rheumatoid arthritis and osteoarthritis due to promising *in vitro* results [22,23].

Intra-articular (IA) drug administration provides several advantages over systemic treatments such as lower side effects, reduced cost, decreased systemic exposure and increased local concentration [5]. The administration of therapeutic molecules (mainly glucocorticoids) though the intra-articular route is already well-stablished for OA management. However, several limitations as a fast drug clearance from the joint due to the lymphatic drainage and/or the short half-life of the therapeutic molecules limit their efficacy. Moreover, there is a controversy on while currently in use intra-articular treatments offer clinically relevant benefits for OA management [24].

The quantitative composition of the synovial fluid is modified during OA, characterized by an increase in volume and a decrease in viscosity. This change in viscosity is associated with a hyaluronic acid (HA) degradation and concentration reduction on the synovial fluid [25]. The high HA concentration is essential for the synovial fluid normal function and lubricating properties, a decrease in concentration causes progressive joint degeneration and limit patient mobility [26]. These changes in synovial fluid properties could be compensated by the intra-articular injection of HA for viscosupplementation purposes. In this sense, the administration of HA through intra-articular route is already included in most of the OA management clinical guidelines [27].

The main goal of the present work is to develop βLap loaded intra-articular formulations able to increase the drug residence time on the joint and simultaneously serve as viscosupplementation systems. Despite the βLap therapeutic capacity, its low solubility limits the clinical use and makes necessary the use of different excipients. Micelle-forming block copolymers as some poloxamers (Poly(ethylene oxide)-Poly(propylene oxide)- Poly(ethylene oxide)) allow for developing thermo-sensitive hydrogels in which hydrophobic molecules are incorporated in the micelles core increasing its solubility [28–30]. Taking advantage of these properties we propose to combine different poloxamers with naturally derived HA to obtain thermo-sensitive hydrogels for local delivery of βLap. Injectable hydrogels present several advantages for intra-articular administration [27] but their development requires the understanding of complex polymeric interactions. To obtain optimized hydrogel formulations with the required properties in terms of thermosensitivity and drug solubility we took advantage of Artificial Intelligence tools. Optimized formulations were physiochemically evaluated *in vitro* and their therapeutic activity was evaluated on an *ex vivo* OA model.

2. Materials and methods

2.1. Materials

β-Lapachone (batch L503; 3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione; C15H14O3; Mw 242.3) was kindly provided by Laboratorio Farmacêutico do Estado de Pernambuco, LAFEPE with a 99.9% purity estimated by DSC and HPLC. Pluronic F123® (PF123) was acquired from BASF and Pluronic F127® (PF127) was purchased from Sigma–Aldrich.

2.2. Production and purification of microbial hyaluronic acid

Hyaluronic acid (HA) was produced by fermentation of the bacterium *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 on low-cost medium based on marine peptones according to the procedure described in [31]. Briefly, 18 h-cultures of *S. zooepidemicus* were run on a 2 L-biorreactor (with a working volume of 1.8 L) at 37 °C, 500 rpm of agitation, without aeration, and pH controlled with sterile solutions of NaOH and HCl (both 5 M) at 6.7. The nutritive broth was composed by glucose, mineral salts and peptones obtained from small-spotted catshark (*Scyliorhinus canicula*) viscera as source of organic nitrogen. At the end of the fermentation, bacterial biomasses were precipitated after mixing SDS with post-incubated medium for 10 min and its subsequent centrifugation at 13000 *g* for 30 min. HA was isolated from the correspondent supernatant by a combination of chemical treatment and membrane purification [32,33].

Thus, this supernatant was treated with a hydroalcoholic-alkaline-saline solution (0.5 M NaOH, 1.02 volumes of EtOH and 2.5 g/L of NaCl) for 1–2 h for the selective precipitation of HA at 4 °C. The impure HA recovered after centrifugation (13000 *g*/30 min/4 °C) was redissolved in water, neutralized with 6 M HCl and centrifuged again (12000 *g*/30 min/4 °C). Then, HA clarified supernatant was purified by ultrafiltration using a membrane of 300 kDa MWCO (spiral polyethersulfone, 0.56 m^2, Prep/Scale-TFF, Millipore Corporation, USA) employing a first step of concentration (by 2-folds) and a subsequent stage of diafiltration, applying 6 diavolumes of distilled water for the elimination of low proteins and peptides. The final HA purity in relation to proteins was higher than 98%. HA molecular weight (Mw) was determined as previously described by gel permeation chromatography [34] indicating a biopolymer size of 1.54 ± 0.16 MDa (PDI 1.25).

2.3. Preparation and physicochemical characterization of hydrogels

2.3.1. Preparation of hydrogels

Poloxamers (PF127 or PF123) were first dissolved at variable concentrations in ultrapure water (MilliQ) using ice-cold water and stored overnight at 4 °C. Isolated hyaluronic acid was incorporated into poloxamer solutions (PF127 or PF123) at the proportions required to achieve the desired concentrations showed in Supplementary Table 1. Solutions were continuously stirred (500 rpm) until complete dissolution.

2.3.2. Drug solubility assessments

An excess of βLap was added to the polymeric solutions and systems were sonicated for 15 min and kept under magnetic stirring for 7 days protected from light. After this period, solutions were filtered through 0.45 μm Nylon filters (GE Healthcare Europe GmBH) to remove not solubilized βLap. Filtered systems were accurately diluted with a 50:50 ethanol:water solution and the drug concentration was determined by spectrophotometry at 258 nm (Agilent Technologies 8453) using an adequate calibration curve.

2.3.3. Rheological properties

The rheological properties of the systems were evaluated, using a stress-controlled rheometer (AR1000N Rheometer, TA Instruments). A temperature ramp from 15 to 40 °C at 2 °C/min with an oscillatory stress of 0.1 Pa at 5 rad/s was carried out recording the storage and elastic moduli. In addition, samples were subjected to a frequency sweep with a rotation speed from 0.5 to 50 rad/s at 25 °C and 37 °C. Through the rheological characterization, the complex viscosity at 25 °C and 37 °C and gelling temperature were obtained.

2.4. Artificial intelligence tools

The experimental databases generated were analyzed using two artificial intelligence tools: FormRules® v3.31 and INForm® v4.11

(Intelligensys Ltd., 2008). FormRules® is a software that combines two AI technologies, artificial neural networks and fuzzy logic. It allows the database modelling and the generation of "IF… THEN" rules to generate knowledge about the effect of the different process variables on the characteristics of the drug delivery systems.

On the other hand, INForm® combines artificial neural networks with genetic algorithms, which allows to carry out an optimization procedure, that is, to select the most appropriate combination of variables capable of obtaining a delivery system with the desired characteristics.

The modelling was carried out using the conditions indicated in Table 1 employing the entire data set to obtain the model using neurofuzzy logic (FormRules®) and with the data split into two groups; one for network training (training data = 40 conditions) and one for error evaluation (test data = 5 conditions) when modelled using INForm®.

The predictability of the models was evaluated using the determination coefficient R^2 (Eq. (1)) and the analysis of variance (ANOVA) which allows to estimate the predictability and accuracy of the models, respectively.

$$R^2 = 1 - \frac{\sum \left(y_i - \widehat{y}_i\right)^2}{\sum \left(y_i - \overline{y}_i\right)^2} \quad (1)$$

where y_i is the experimental value, $\widehat{y}_i$is the predicted value and $\overline{y}_i$ is the predicted average value.

The higher the value of R^2, the better the predictability of the model is. Values between 70 and 99.9% are indicative of good predictability [35].

2.5. Drug delivery profiles

The evaluation of the βLap release profile from the systems was carried out using vertical Franz diffusion cells. Drug-containing formulations (3 mL) were placed in the donor compartment and 6 mL of phosphate buffer pH 7.4 were used in the receptor compartment. Both compartments were separated by a 7000 Da pore size membrane (Medicell International Ltd). This pore diameter allows the drug to diffuse, while prevents the passage of the polymeric components of the gels. At pre-set time points media samples were removed from the receptor chamber and replaced by fresh media. The amount of the released drug was determined by UV–Visible spectrophotometry as described above.

Table 1
Parameters used to model the experimental databases.

FormRules® v3.31	INForm® v4.11	
Ridge regression factor 1 e^{-6}	Momentum	0.80
Number of set densities 2	Learning rate	0.70
Set densities 2 and 3	Target iteractions	1000
Statistical fitness criteria: structural risk	Target MS error	0.0001
Minimisation (structural risk minimization)	Random seed	10,000
C1 > 0.680; C2 = 4.80	Smart stop	Enabled
Maximum inputs per submodel 4	Network structure	
Maximum nodes per input 15	Number of inputs	2
Adapt nodes TRUE	Number of hidden layers	1
	Hidden layer	1
	Number of nodes	2
	Transfer type function	Asymmetric sigmoid
	Output transfer type	Linear
	Back propagation type	RPROP

2.6. Biocompatibility of extracted hyaluronic acid

The biocompatibility of poloxamers and hyaluronic acid has been already extensively reported [36,37]. However, to ensure the hyaluronic acid produced in this work is not toxic, cell viability was evaluated for cells treated with our HA. Murine fibroblasts BALB/3 T3 clone A31 (ATCC® CCL-163™) were cultured in DMEM/F12 HAM medium (Sigma) supplemented with 10% foetal bovine serum (Hyclone) and 1% penicillin/streptomycin (ThermoScientific). Cells were seeded on 96-well plates at 25,000 cells/well and allowed to attach for 24 h. On the next day cells were incubated with hyaluronic acid at variable concentrations (25–200 μg/mL) for 24 h. Cell viability was assessed by means of a WST-1 assay (Roche) following manufacturer's instructions. Untreated cells served as 100% cell viability.

2.7. Evaluation of βLap anti-inflammatory activity

The potential anti-inflammatory effect of βLap was initially evaluated on LPS-stimulated macrophages. Murine macrophages Raw 264.7 (ATCC® CRL-2278) were cultured in DMEM/F12 HAM medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (ThermoScientific). Cells were cultured in 75 cm^2 vented flasks at 37 °C with 5% CO_2 and 90% relative humidity. Cell culture medium was changed every 2 days and cells were passaged when reaching 70% confluency. Macrophages were seeded in 24-well plates at a density of 100,000 cells per well, allowed to attach for 4 h and afterwards, stimulated with lipopolysaccharide (LPS) (Sigma) at 100 ng/mL. After 24 h, stimulation media was removed. Cell monolayers were washed with DPBS and treated with variable concentrations of βLap (1–4.5 μM) or control for 24 h, media was then collected, and cell cytotoxicity evaluated by a commercial LDH quantification kit (Roche). Positive control was obtained by lysing the cells according to manufacturer's instructions. The cytocompatibility of blank and βLap-loaded optimized formulations was also evaluated. Anti-inflammatory activity was analyzed by quantifying the secretion of the pro-inflammatory molecules prostaglandin E_2 (PGE_2) and tumor necrosis alpha (TNF-α). PGE_2 concentration was evaluated by a competitive EIA following manufacturer's instructions (Ann Arbour) and TNF-α through a commercial ELISA (Sigma-Aldrich). Values are expressed as percentage of cytotoxicity with respect to positive control or percentage of decreased PGE_2 and TNF-α secretion with respect to LPS-stimulated untreated cells and their corresponding standard deviations.

βLap anti-inflammatory activity was also evaluated on LPS stimulated OA cartilage explants. Tissues were obtained from OA patients undergoing total knee replacement after patients consent according to Ethics Committee approval (CEIC Galicia 2015/029). Immediately after surgery, samples were placed on sterile saline solution. Cartilage was washed twice with DPBS supplemented with 1% penicillin/streptomycin amphotericin (Gibco) and 6-mm tissue biopsies were obtained using sterile punches. Samples were washed again with supplemented DPBS and placed on 24-well plates containing 0.5 mL of Dulbecco's modified eagle's medium (DMEM) supplemented with 2% fetal bovine serum and 1% penicillin/streptomycin. Cartilage explants were allowed to settle for 48 h. Afterwards, media was removed and explants were treated with LPS at 100 ng/mL with or without βLap at variable concentrations (0–3 μM) or dexamethasone (2 μM) for 48 h. Then, cell culture supernatants were collected to evaluate PGE_2 secretion as previously described.

2.8. Therapeutic activity evaluation of optimized formulations

To mimic the disease environment, an *ex vivo* OA model was stablished by co-culturing human cartilage and synovium explants. Tissues were obtained as previously described and cartilage explants treated as above mentioned. Synovium was washed twice with supplemented DPBS and any excess of fat tissue was removed. Samples were washed

again and placed on supplemented media. Cartilage and synovium samples were allowed to settle for 24 h using standard culture conditions (37 °C and 5% CO_2) and then combined using cell culture inserts (8 μm pore size; Corning). Synovium was placed on the upper compartment while cartilage was placed on the bottom. This co-culture was acclimated for 48 h and then media was removed and co-cultures were treated for additional 48 h with βLap (2 μM) or βLap loaded optimized formulation with equivalent drug concentration. Dexamethasone (2 μM), clinically used to treat OA, was selected as control. Unstimulated co-cultures and mono cultures were also analyzed. After 48 h, media supernatant was collected and used to assess the secretion of pro-inflammatory and ECM degradative mediators. The secretion of IL-13, IL-6, IL-8, MMP13 and TNF-α was studied using multiplex immunoassay kits (R&D Systems) according to the manufacturer's instructions. Samples were diluted 1:1 in assay buffer and loaded into a 96-well plate, after which magnetic bead suspensions, detection antibodies, and streptavidin-phycoerythrin were added to the samples. The plate was then loaded into a MAGPIX system (Luminex) and analyte concentrations were obtained using the correspondent calibration curve. Co-culture samples not treated with any of the therapeutic molecules, cartilage explants treated with LPS (100 ng/mL) for 48 h and cartilage explants without any treatment served as controls to evaluate the generated OA-like environment.

2.9. Statistical analysis

Experimental values are represented as mean ± standard deviation. Experiments were run in quadruplicate unless otherwise stated. One-way ANOVA following HSD Tukey's *post hoc* (IBM SPSS Statistics 26) was performed to detect statistical differences between treatments with *p*-values lower than 0.05 considered as statistically significant.

3. Results and discussion

3.1. Design of optimized formulations

The design of intra-articular formulations aims to obtain systems with suitable performance in terms of easy administration, long residence time in the diseased joint and effective therapeutic molecule release. Thermosensitive hydrogels are ideal systems as drug carriers for intra-articular administration as their administration by injection is minimally invasive, and they can gel at body temperature, giving rise to controlled release drug delivery systems that maximize drug local effects [38]. However, most of the anti-inflammatory drugs currently used for OA treatment, also βLap, are characterized by low aqueous solubility, entailing a challenge for their inclusion in hydrophilic polymeric networks. Poloxamers are able to generate themosensitive micelle-based hydrogels that meet the requirements for intra-articular administration of these drugs, increasing their solubility by their incorporation into the hydrophobic micelles core.

Viscosupplementation is a widely used strategy in OA management with good clinical outcomes. To this end, hyaluronic acid is mainly the polymer used owing to its major role in joint lubrication.

In an attempt to combine the advantages of local drug delivery platforms (thermosensitive micelle-based hydrogels) with viscosupplementation (hyaluronic acid), binary systems (poloxamer+HA) were developed. The design of suitable systems for OA treatment requires a deep knowledge of both polymer-polymer interactions and critical parameters conditioning the developed systems properties. Innovative technologies as artificial intelligence tools can speed up the challenging goal of formulation optimization.

In this work, the performance of PF123 + HA and PF127 + HA binary systems at variable polymer concentrations was evaluated in terms of drug solubility enhancement and rheological behaviour (Supplementary Table 1).

The range of HA concentrations (0–15 mg/mL) was selected based on the marketed HA formulations for viscosupplementation (8–15 mg/mL). Three formulation parameters (model outputs) were selected as crucial to assess system performance, solubilized βLap, viscosity at 25 °C (η_{25}) and viscosity at 37 °C (η_{37}). The first factor is important to ensure adequate drug loading while hydrogel viscosities are used to evaluate hydrogel injectability (η_{25}) and viscosupplementation effect (η_{37}). Table 2 shows the FormRules® results, including the main inputs that explain the variability of each property (output) together with the parameters that guarantee the quality of the models. FormRules® successfully modelled the three parameters studied with R^2 values over 70%, indicative of good predictabilities. Moreover, the ANOVA computed f values were higher than the f critical values in all cases indicative of not statistically significant differences between experimental and predicted values ($p < 0.01$). Therefore, the three models presented excellent predictability and accuracy and can be used to explain the composition effect on the systems performance.

According to the models, βLap loading capacity is determined by the interaction between the type of poloxamer and its concentration. As depicted on the 3D plot of this output (Fig. 1A and B) and the "IF-THEN" rules (Supplementary Table 2), a higher drug solubility was observed for hydrogels containing PF123. This polymer has a greater hydrophobic/hydrophilic (PPO/PEO) ratio and lower critical micelle concentrations (CMC) than PF127. These characteristics conferee PF123 a higher solubilization efficiency for medium hydrophobic drugs when compared to PF127 despite its lower molecular weight [39]. This behaviour is confirmed by our experimental data for the highly hydrophobic drug βLap.

The effect of the composition on hydrogel rheological properties was clearly dependent on the temperature (Fig. 1C–F). At 25 °C the systems viscosity was controlled by the concentration of hyaluronic acid while at 37 °C this feature was affected by the type of poloxamer used and its concentration. This performance is explained by the sol to gel thermosensitive transition attribute of poloxamer solutions. These polymers behave as solutions under the transition temperature and as semi-solid systems above this temperature due to the ordered packing of micelles [40]. Despite PF123 presents lower CMC, its low molecular weight hinders the sol to gel transition of the hydrogels at the concentrations used on this work. Therefore, only hydrogels prepared with PF127 at concentrations higher than 10% were able to undergo this shift and presented a clear increase in viscosity as shown on the 3D plot (Fig. 1D–F). On the other hand, the variations in viscosity at 25 °C were mainly explained by the incorporation of HA to the formulation. An increase in hyaluronic acid concentration above 9 mg/mL led to a concentration dependent enhancement in system viscosity reaching the maximum value at the highest HA concentration, 15 mg/mL (Fig. 1C–D).

Once the influence of the hydrogel's composition on the above mentioned parameters was stablished, the next step is to select an optimized composition able to fulfil the requirements of IA administration for viscosupplementation and drug delivery. PF127 was selected as the thermosensitive polymer to be used based on the above mentioned rheological characteristics. The following attributes were selected as desirable $\eta_{25} < 2$ Pa.s, and solubilized βLap >100 μg/mL and

Table 2
Inputs selected by FormRules® to explain the outputs variability and parameters to assess the quality of the generated models; R^2 (predictability) and ANOVA (accuracy) (d.f. stands for degrees of freedom).

Outputs	Inputs from FormRules®	R^2	d.f.	Calculated *f-ratio*	*f* critical for ($p < 0.01$)
Solubilized βLap	PF × [PF]	85.61	8 and 26	13.39	3.29
η_{25}	[HA]	89.07	3 and 41	103.29	4.30
η_{37}	PF × [PF]	72.93	6 and 35	15.71	3.37

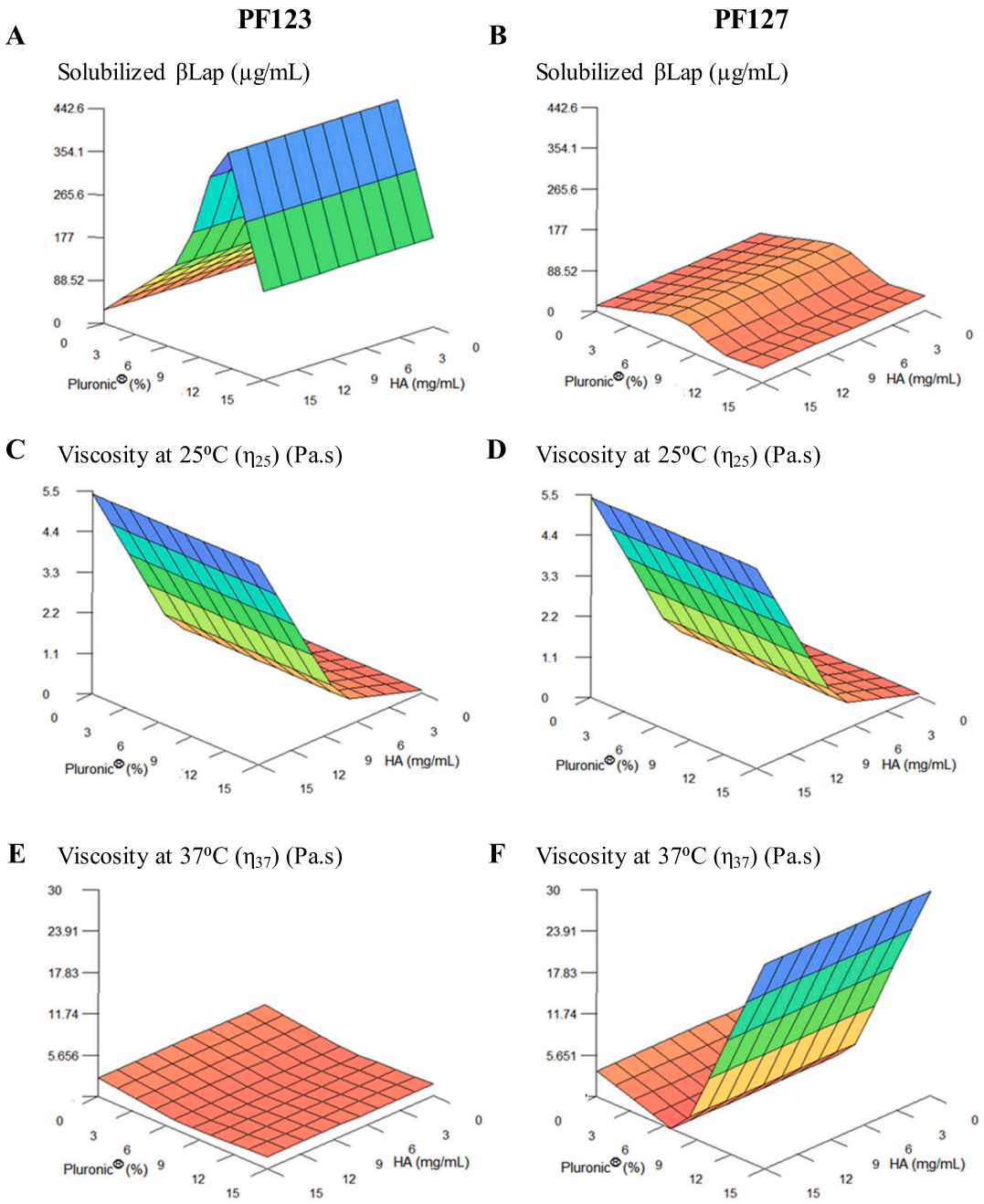

Fig. 1. 3D plots of the obtained models for hydrogels prepared with PF123 (A, C, E) or PF127 (B, D, F) of the analyzed parameters; drug solubility (A&B), viscosity at 25 °C (B&D) and viscosity at 37 °C (E&F).

the optimization was performed using the software INForm®. The composition selected by the model through genetic algorithms was 15% PF127 and 6.37 mg/mL HA. Theoretically, according to the model, this composition should give systems with η_{25} = 1.24 Pa.s and solubilized βLap = 83.97 μg/mL.

3.2. Performance of optimized system: physicochemical properties

Optimized formulations containing PF127 and HA were prepared and characterized as previously described. Hyaluronic acid solutions at the same proportion as in the optimized formulation (6.37 mg/mL) were also prepared and characterized in terms of rheological properties to serve as control. The experimental characteristics of the optimal formulation (solubilized βLap = 85.25 ± 20.64 μg/mL; η_{25} = 1.11 ± 0.01 Pa.s) were remarkably close to those predicted by the model, confirming its validity. βLap loaded optimized systems presented the characteristic orange colour of the drug (Supplementary Fig. 1). The combination of PF127 with the hyaluronic acid produced on this work (1.54×10^6 Da) provided a positive effect on the viscoelastic properties of the obtained systems. Formulations exhibited at 37 °C a non-newtonian shear thinning behaviour, similar to that reported for human synovial fluid (Fig. 2A) [41]. The values of elastic (G') and viscous (G") moduli at variable frequencies showed a viscous behaviour over the entire range of frequencies for HA alone with a viscous modulus higher than the elastic modulus (Fig. 2B). On the other hand, when PF127 is added, a gel behaviour is observed, with the elastic modulus being higher than the viscous modulus throughout the range of frequencies. The sol to gel transition of the biphasic system is clearly observed on Fig. 2C behaving as a viscous solution at low temperatures and as a gel after a temperature threshold of 26.45 ± 1.17 °C. This

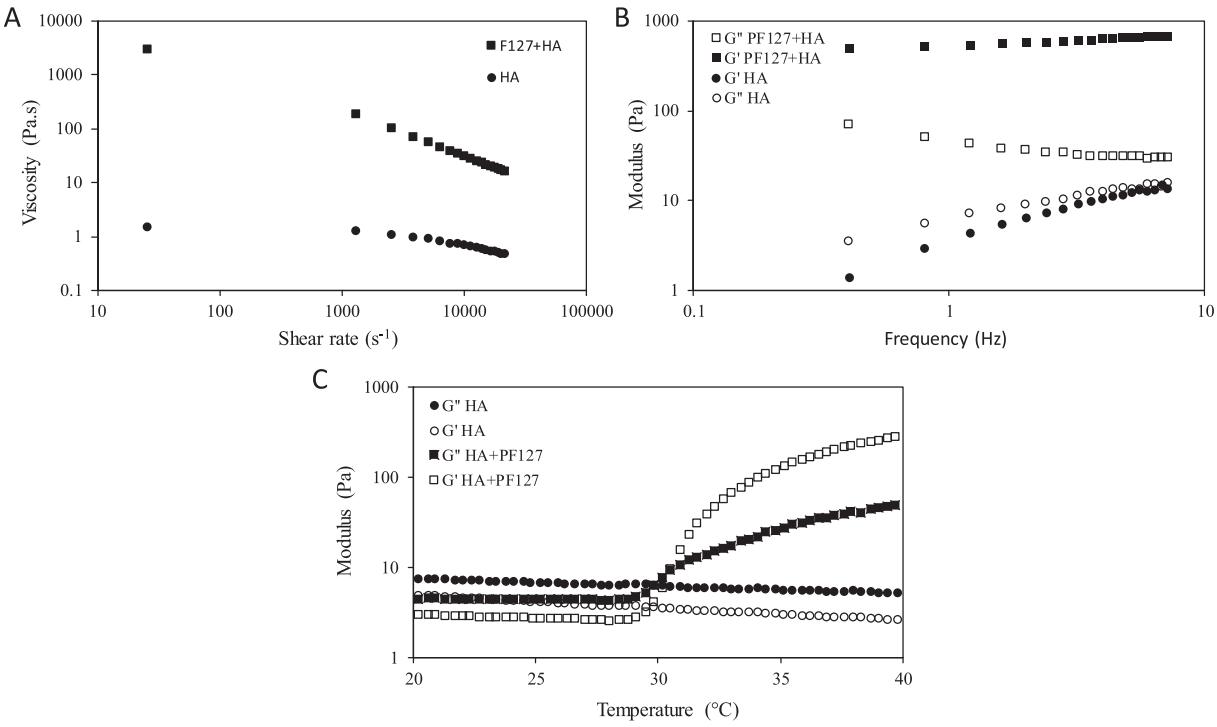

Fig. 2. Rheological properties of the optimized formulation (HA + PF127) and hyaluronic acid solution at equivalent concentration.

property allows for an easy administration at room temperature while acting as a good viscosuppplementator after injection (37 °C). In fact, PF127 + HA formulation showed a viscosity at 37 °C and low shear rate (179.3 ± 41.9 Pa.s) superior to the already commercialized solutions using HA of low molecular weight, presenting excellent viscosupplementation capacity [41].

3.3. Drug delivery profiles of optimized systems

Optimized binary system showed a linear drug release profile (Fig. 3), fitting a zero-order release kinetic, characteristic of depot systems. The obtained release constant was 0.45 μgβLap/h (r^2 = 0.9996) ensuring the βLap sustained release. IA depot systems have been reported to significantly increase therapeutic molecules half-life in the affected joint endorsing sustained drug distribution and allowing to use lower drug doses and longer dosing intervals [42].

3.4. Biocompatibility of extracted hyaluronic acid

To ensure the extracted hyaluronic acid did not promote undesirable effects on cells, fibroblast monolayers were treated with variable concentrations of HA (25–200 μg/mL). Fig. 4 shows the cell metabolic activity after 24 h of treatment. No cytotoxic effect was observed for any of the concentrations tested with values similar to those of non-treated cells (Control -) in agreement with the already well-known high biocompatibility of hyaluronic acid [37].

3.5. Evaluation of βLap anti-inflammatory activity

Recent experiments have suggested the anti-inflammatory capacity of βLap. However, its anti-inflammatory effects on chondrocytes has not been elucidated yet. In this work, we have evaluated the anti-inflammatory capacity of βLap on both LPS-stimulated macrophages and OA cartilage explants to select an appropriated βLap therapeutic concentration. The experimental data (Fig. 5A–B) showed a dose-

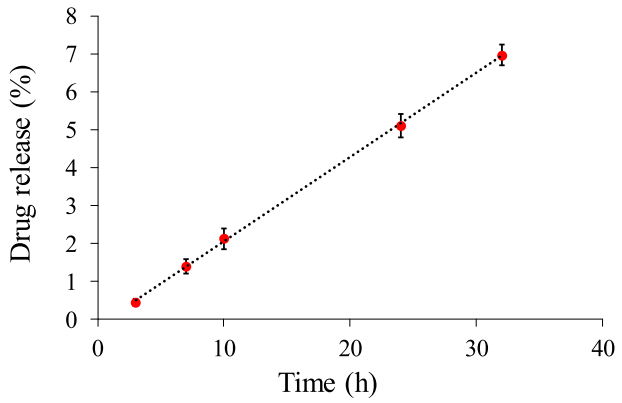

Fig. 3. Drug release profiles of optimized systems in PBS pH 7.4.

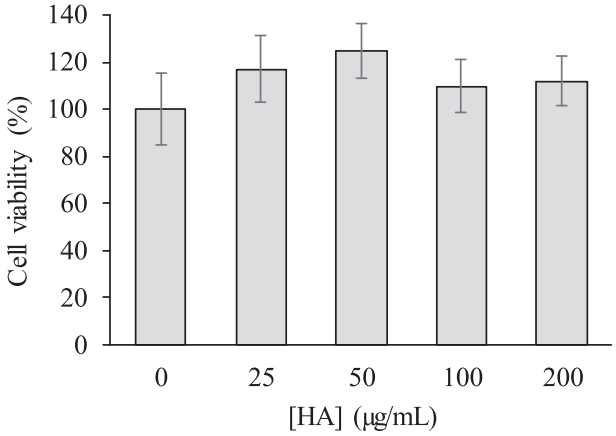

Fig. 4. Fibroblast metabolic activity after 24 h of treatment with HA solutions.

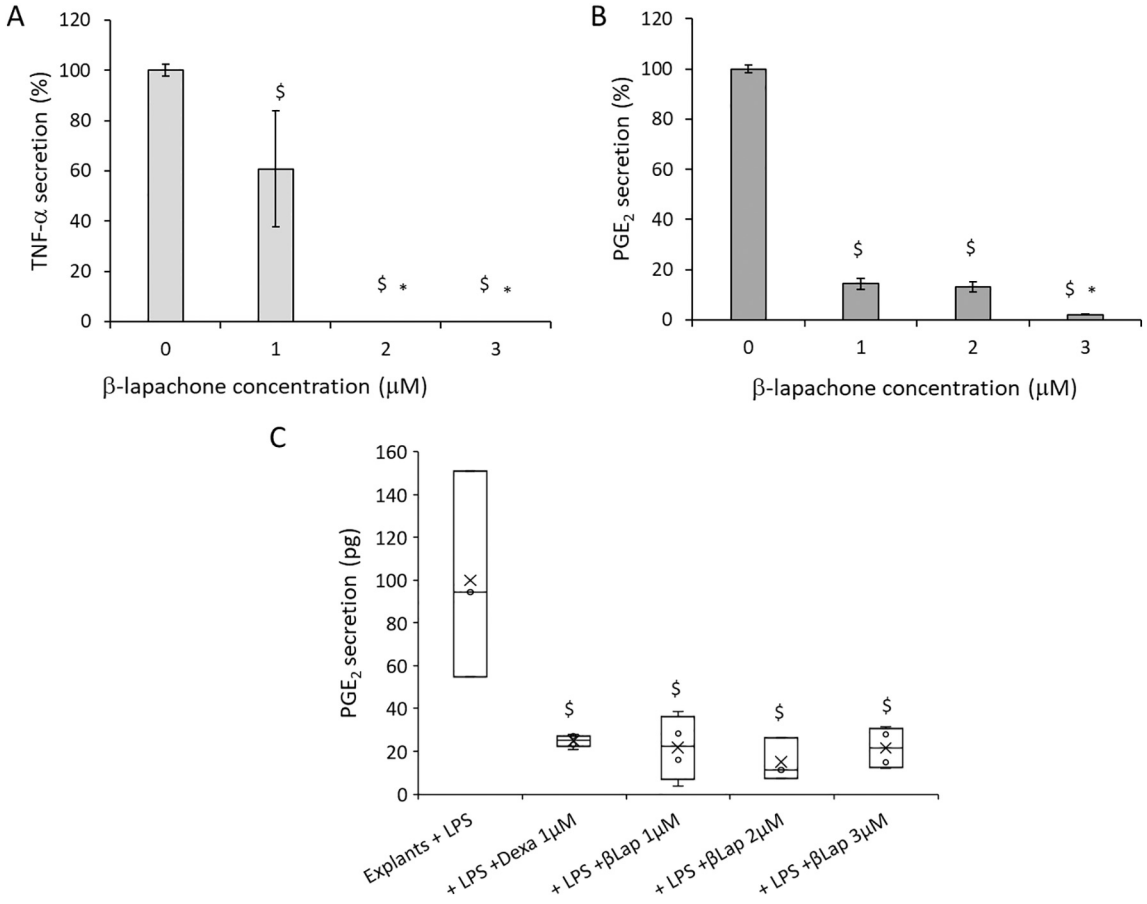

Fig. 5. A) TNF-α secretion and B) PGE_2 secretion of LPS-stimulated macrophages after 24 h treatment with variable βLap concentration. C) PGE_2 secretion of LPS-stimulated OA cartilage explants after 72 h treatment with Dexamethasone 1 μM or variable βLap concentrations. $ denotes statistical significance ($p < 0.05$) when compared to stimulated cells or explants without treatment. * denotes statistical significance ($p < 0.05$) when compared to 1 μM βLap.

dependent decrease in TNF-α and PGE_2 secretion when pro-inflammatory macrophages were treated with βLap solutions. The dose-dependent decrease in TNF-α secretion after βLap treatment has been previously reported on LPS stimulated microglia [18]. Moreover, in agreement with studies using other cell types the treatment with βLap at concentrations over 3 μM led to cell death (Supplementary Fig. 2) [43]. Also, no cytotoxic effects were observed for blank and βLap loaded optimized formulations (Supplementary Fig. 2).

Interestingly, stimulated explants did not show a dose-dependent response to βLap. The addition of βLap significantly decreased the secretion of PGE_2, but no differences were noted between the concentrations tested (Fig. 5C). This decrease in the pro-inflammatory mediator secretion was similar to that observed for Dexamethasone control reaching levels close to non-stimulated explants (data not shown).

The anti-inflammatory effects exerted by βLap have been associated to the inhibition of NF-κB activation. TLRs are sensors of the innate immune system recognizing pathogens and triggering the host defense. The activation of the transcription factor NF-κB is the end step of toll-like receptors (TLRs) signalling pathways and controls the expression of a wide variety of genes encoding pro-inflammatory molecules. Therefore, βLap could modulate cytokine gene expression by suppressing TLR signalling pathways [44–46]. Other authors have pointed out the ability of βLap to not only inhibit NF-κB activation but also inhibit the phosphorylation of several types of mitogen-activated protein kinases (MAPKs) and AKT, promoting an anti-inflammatory effect involving several pathways [18,47].

3.6. Therapeutic activity of optimized formulations in a OA model

Based on the βLap activity (comparable to Dexamethasone), the systems release profile and the reported synovial fluid volume, optimized formulations were loaded with βLap at 70 μM (16 μg/mL). The activity of these systems was evaluated on the developed *ex vivo* OA model. To elucidate whether this co-culture efficiently led to an OA mimetic environment we evaluated the secretion of several cytokines and matrix degradative enzymes (Fig. 6A).

TLRs play an important role on cartilage inflammation during OA progression. Their activation by danger-associated molecules leads to the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 [48]. Moreover, LPS is a well-known TLR4 agonist. In this way, the stimulation of cartilage explants with LPS significantly increased the secretion of all the analyzed cytokines (TNF-α, IL-6, CXCL8 and IL-13) (Fig. 6A). On the other hand, the co-culture of cartilage explants with synovial tissue also led to increased levels of TNF-α, IL-6, CXCL8 and IL-13 on the cell culture supernatant when compared to unstimulated explants similarly to LPS stimulation except for IL-6. Interestingly, the co-culture set-up also allowed for a statistically significant increase in the secretion of MMP13, a key degradative enzyme during OA progression. These findings suggest the developed synovium/cartilage co-culture achieved a more OA mimetic environment. These results are in agreement with other authors [49] who have reported increases in MMP-3 and IL-6 at long times in co-cultures of osteochondral explants and synovial membrane with respect to osteochondral explants cultured alone.

MMP13 is a matrix degradative enzyme secreted by chondrocytes, implicated in collagen and proteoglycan degradation. MMP13 is considered the most important collagen degradative enzyme in the

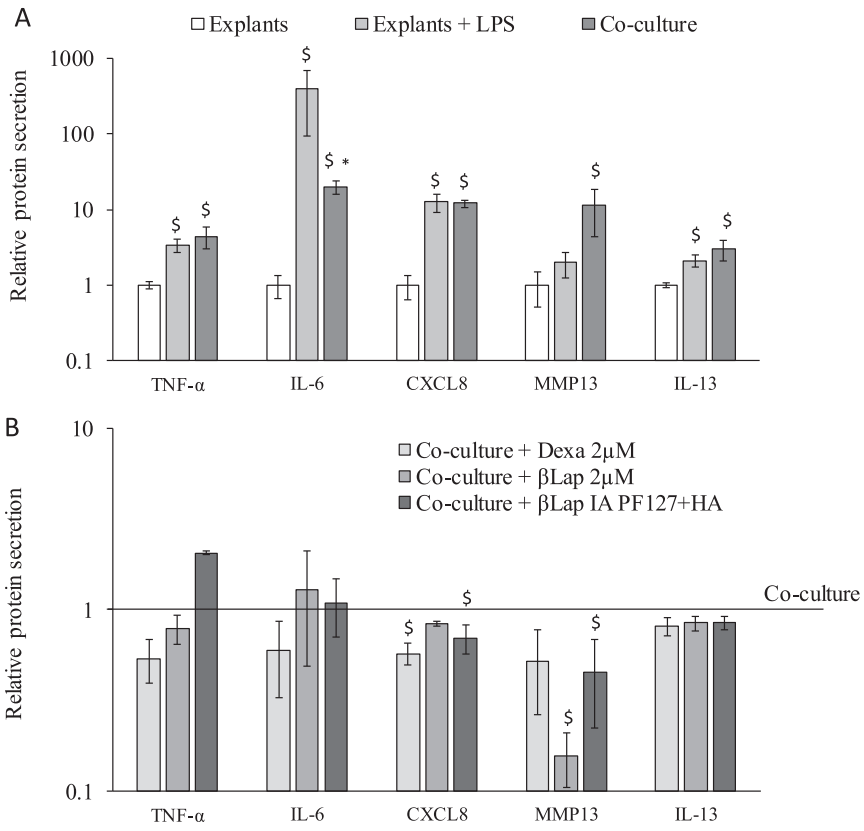

Fig. 6. A) Cytokines (TNF-α, IL-6, CXCL8 and IL-13) and matrix metalloproteases s (MMP13) secretion of OA cartilage explants cultured for 48 h without treatment (explants), treated with LPS at 100 ng/mL (Explants + LPS) or co-cultured with patient matched synovial tissue (Co-culture). B) Cytokines and MMPs secretion of OA cartilage and synovial tissue co-cultured for 48 h and treated with Dexamethasone (control drug), βLap or βLap loaded optimized formulation. $ denotes statistical significance ($p < 0.05$) when compared to explants without treatment (6A) or untreated co-culture (6B). * denotes statistical significance ($p < 0.05$) when compared to Explants treated with LPS.

cartilage and one of the main drivers and inductors of the OA degradative process. In fact, different strategies to control OA are being focused on inhibiting MMP13 [50]. Interestingly, βLap alone or loaded in the optimized formulation was able to significantly decrease the secretion of MMP13 (Fig. 6B). The capacity of βLap to decrease MMP expression has been already described in interleukin-1 stimulated primary chondrocytes [22]. Moreover, the treatment with the optimized formulations was able to significantly decrease the secretion of CXCL8 to a similar extend as Dexamethasone. Despite co-cultured explants treated with βLap alone showed lower TNF-α and MMP-13 secretion than those treated with the βLap loaded optimized formulation the differences between both treatments were not statistically significant (p-values >0.67).

The results suggest the combination of HA and βLap could lead not only to an increase in the synovial fluid viscosity but also to a decrease in the degradation rate of both, injected and patient's HA, due to the anti-inflammatory action of βLap that would control the pro-inflammatory environment of the joint [42]. Therefore, the developed formulation could increase the half-life in the affected joint of both βLap and injected HA characterized by a short half-life of about 13 h [51].

4. Conclusions

The use of artificial intelligence tools has allowed to stablish the suitable composition of Pluronic+HA hydrogels for IA. The optimized formulation postulated as adequate for the treatment of OA was injectable and achieved adequate viscosupplementation while ensured βLap controlled release.

The treatment of M1 macrophages and LPS-stimulated cartilage explants with βLap shows the drug ability to reset the inflammatory environment towards a less pro-inflammatory profile. The use of an *ex vivo* synovium/cartilage co-culture set up allowed for a more OA-like environment, suitable to test the developed formulations.

The optimized formulation is a promising candidate for the treatment of OA, as it can restore the rheological properties of synovial fluid improving joint function while decreasing inflammation and the established cartilage degradative environment.

CRediT authorship contribution statement

Patricia Diaz-Rodriguez; Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing - original draft; Writing - review & editing.

Cibrán Mariño; Investigation; Methodology;

Jose Antonio Vázquez; Methodology; Resources; Funding acquisition; Writing - review & editing.

Jose Ramon Caeiro-Rey; Resources; Funding acquisition; Writing - review & editing.

Mariana Landin; Conceptualization; Formal analysis; Software; Supervision; Validation; Visualization; Funding acquisition; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support was received from Interreg V-A POCTEP (0245_IBEROS_1_E) EU (FEDER). Research groups are also supported by Xunta de Galicia (Competitive Reference Groups, ED431C 2020/17-FEDER) and (Grupos de Potencial Crecimiento, IN607B 2018/19-FEDER).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2021.112254.

References

[1] M. Kloppenburg, F. Berenbaum, Osteoarthritis and Cartilage, 2020.
[2] N.R. Fuggle, C. Cooper, R.O.C. Oreffo, A.J. Price, J.F. Kaux, E. Maheu, M. Cutolo, G. Honvo, P.G. Conaghan, F. Berenbaum, J. Branco, M.L. Brandi, B. Cortet, N. Veronese, A.A. Kurth, R. Matijevic, R. Roth, J.P. Pelletier, J. Martel-Pelletier, M. Vlaskovska, T. Thomas, W.F. Lems, N. Al-Daghri, O. Bruyere, R. Rizzoli, J. A. Kanis, J.Y. Reginster, Aging Clinical and Experimental Research, 2020.
[3] L. Kou, S. Xiao, R. Sun, S. Bao, Q. Yao, R. Chen, Drug Deliv. 26 (2019) 870–885.
[4] K.L. Caldwell, J. Wang, Osteoarthr. Cartil. 23 (2015) 351–362.
[5] I.A. Jones, R. Togashi, M.L. Wilson, N. Heckmann, C.T. Vangsness Jr., Nat. Rev. Rheumatol. 15 (2019) 77–90.
[6] L.C. Garbin, C.S. Olver, J. equine Vet. Sci. 86 (2020), 102820.
[7] P. Diaz-Rodriguez, J. Erndt-Marino, H. Chen, J.F. Diaz-Quiroz, S. Samavedi, M. S. Hahn, Regen. Eng. Transl. Med. 5 (2019) 297–307.
[8] N.A. Sandler, M.J. Buckley, J.E. Cillo, T.W. Braun, J. Oral Maxillofac. Surg., 56 (1998) 534–543; discussion 543–534.
[9] R. Liu-Bryan, R. Terkeltaub, Nat. Rev. Rheumatol. 11 (2015) 35–44.
[10] S. Glyn-Jones, A.J. Palmer, R. Agricola, A.J. Price, T.L. Vincent, H. Weinans, A. J. Carr, Lancet (Lond. Engl.) 386 (2015) 376–387.
[11] C.Y. Wenham, P.G. Conaghan, Therapeutic advances in musculoskeletal disease 2 (2010) 349–359.
[12] A. Mathiessen, P.G. Conaghan, Arthritis Res. Ther. 19 (2017) 18.
[13] A.B. Pardee, Y.Z. Li, C.J. Li, Current cancer drug targets 2 (2002) 227–242.
[14] X. Ma, Z.R. Moore, G. Huang, X. Huang, D.A. Boothman, J. Gao, J. Drug Target. 23 (2015) 672–680.
[15] S. Park, M.-G. Shin, J.-R. Kim, S.-Y. Park, Exp. Gerontol. 126 (2019), 110711.
[16] G.T. Gang, J.H. Hwang, Y.H. Kim, J.R. Noh, K.S. Kim, J.Y. Jeong, D.E. Choi, K. W. Lee, J.Y. Jung, M. Shong, C.H. Lee, Free Radic. Biol. Med. 67 (2014) 139–149.
[17] J.S. Park, Y.Y. Lee, J. Kim, H. Seo, H.S. Kim, Free Radic. Biol. Med. 97 (2016) 168–178.
[18] E.-J. Lee, H.-M. Ko, Y.-H. Jeong, E.-M. Park, H.-S. Kim, J. Neuroinflammation 12 (2015) 133.
[19] D.W. Kim, J.-Y. Cho, Int. J. Mol. Sci. 19 (2018) 3813.
[20] S.J. Byun, Y. Son, H.O. Pae, Eur. Rev. Med. Pharmacol. Sci. 18 (2014) 949–958.
[21] S.J. Byun, Y. Son, B. Hwan Cho, H.T. Chung, H.O. Pae, J. Clin. Biochem. Nutr. 52 (2013) 106–111.
[22] J.K. Jackson, T. Higo, W.L. Hunter, H.M. Burt, Inflammation research : official journal of the European histamine research society [et al.] 57 (2008) 126–134.
[23] J. Xu, G. Wagoner, J.C. Douglas, P.D. Drew, J. Neuroimmunol. 254 (2013) 46–54.
[24] A.J. Kompel, F.W. Roemer, A.M. Murakami, L.E. Diaz, M.D. Crema, A. Guermazi, Radiology 293 (2019) 656–663.
[25] N. Gerwin, C. Hops, A. Lucke, Adv. Drug Deliv. Rev. 58 (2006) 226–242.
[26] A. Migliore, M. Granata, Clin. Interv. Aging 3 (2008) 365–369.
[27] R.H. Koh, Y. Jin, J. Kim, N.S. Hwang, Cells 9 (2020).
[28] S. Seoane, P. Diaz-Rodriguez, J. Sendon-Lago, R. Gallego, R. Perez-Fernandez, M. Landin, Eur. J. Pharm. Biopharm. 84 (2013) 497–504.
[29] P. Diaz-Rodriguez, M. Landin, Int. J. Pharm. 433 (2012) 112–118.
[30] M. Rahimi, G. Charmi, K. Matyjaszewski, X. Banquy, J. Pietrasik, Acta Biomater. 123 (2021) 31–50.
[31] J.A. Vázquez, L. Pastrana, C. Piñeiro, J.A. Teixeira, R.I. Pérez-Martín, I.R. Amado, Mar. Drugs 13 (2015) 6537–6549.
[32] M.A. Murado, M.I. Montemayor, M.L. Cabo, J.A. Vázquez, M.P. González, Food Bioprod. Process. 90 (2012) 491–498.
[33] J.A. Vazquez, M. Blanco, J. Fraguas, L. Pastrana, R. Perez-Martin, Food Chem. 198 (2016) 28–35.
[34] J.A. Vazquez, J. Fraguas, R. Novoa-Carballal, R.L. Reis, R.I. Perez-Martin, J. Valcarcel, Carbohydr. Polym. 210 (2019) 302–313.
[35] L. Garcia-del Rio, P. Diaz-Rodriguez, M. Landin, Mater. Sci. Eng. C, 106 (2020), 110252.
[36] W. Zhang, Y. Shi, Y. Chen, J. Ye, X. Sha, X. Fang, Biomaterials 32 (2011) 2894–2906.
[37] K. Ghosh, in: R.L. Reis, N.M. Neves, J.F. Mano, M.E. Gomes, A.P. Marques, H. S. Azevedo (Eds.), 28 - Biocompatibility of Hyaluronic Acid: From Cell Recognition to Therapeutic Applications, Natural-Based Polymers for Biomedical Applications, Woodhead Publishing, 2008, pp. 716–737.
[38] Z. He, B. Wang, C. Hu, J. Zhao, Colloids Surf. B: Biointerfaces 154 (2017) 33–39.
[39] A.M. Bodratti, P. Alexandridis, J. Funct. Biomater. 9 (2018) 11.
[40] G. Dumortier, J.L. Grossiord, F. Agnely, J.C. Chaumeil, Pharm. Res. 23 (2006) 2709–2728.
[41] P. Bhuanantanondh, D. Grecov, E. Kwok, J. Med. Biol. Eng. 32 (2012) 12–16.
[42] M.L. Kang, G.-I. Im, Expert Opin. Drug Deliv. 11 (2014) 269–282.
[43] H.N. Kung, M.J. Yang, C.F. Chang, Y.P. Chau, K.S. Lu, Am. J. Physiol. Cell Physiol. 295 (2008) C931–C943.
[44] S.K. Manna, Y.P. Gad, A. Mukhopadhyay, B.B. Aggarwal, Biochem. Pharmacol. 57 (1999) 763–774.
[45] H.P. Tzeng, F.M. Ho, K.F. Chao, M.L. Kuo, S.Y. Lin-Shiau, S.H. Liu, Am. J. Respir. Crit. Care Med. 168 (2003) 85–91.
[46] J. Xu, G. Wagoner, J.C. Douglas, P.D. Drew, J. Neuroimmunol. 254 (2013) 46–54.
[47] N. Mokarizadeh, P. Karimi, H. Kazemzadeh, N. Fathi Maroufi, S. Sadigh-Eteghad, S. Nikanfar, N. Rashtchizadeh, Int. Immunopharmacol. 87 (2020), 106810.
[48] G. Barreto, B. Senturk, L. Colombo, O. Brück, P. Neidenbach, G. Salzmann, M. Zenobi-Wong, M. Rottmar, Osteoarthr. Cartil. 28 (2020) 92–101.
[49] E. Haltmayer, I. Ribitsch, S. Gabner, J. Rosser, S. Gueltekin, J. Peham, U. Giese, M. Dolezal, M. Egerbacher, F. Jenner, PLoS One 14 (2019), e0214709.
[50] E.E. Mehana, A.F. Khafaga, S.S. El-Blehi, Life Sci. 234 (2019), 116786.
[51] A.S. Hanafy, S.O. El-Ganainy, Int. J. Pharm. 573 (2020) 118859.