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SELECTION OF AN EFFICIENT CHONDROITIN SULFATE-LIPOSOME DELIVERY SYSTEM AS CHONDROPROTECTIVE AGENT FOR LOCAL INFLAMMATION TREATMENT

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ABSTRACT. We aimed to select a biocompatible concentration of chondroitin sulfate loaded in liposomes (L-CS) in a culture of human chondrocytes for its possible use in intra-articular treatment of inflammatory and degenerative disorders. L-CS effect on cell viability and morphology and its anti-inflammatory effect was investigated in cell culture experimental models. Our data demonstrated that 250-500 µg/ml CS from L-CS formulation was the optimal range concentration for *in vitro* experiments. L-CS was more efficient than free CS in preventing cell morphology changes and inhibiting gelatinases secretion in the culture medium of inflamed cells. In conclusion, CS loaded in liposomes could be efficient an chondroprotective agent for intra-articular treatment of osteoarthritis.

Keywords: chondroitin sulfate, liposomes, chondrocytes, cytotoxicity, metalloproteinases

INTRODUCTION

Articular cartilage is a nonvascularized connective tissue that contains specialized cells, chondrocytes, within a highly structured extracellular matrix, composed primarily of type II collagen (COL) fibers and proteoglycans (PG) that account for the tensile strength and load-bearing capacity of the joint (Dijkgraaf *et al.*, 1995). The predominant PG from articular cartilage, aggrecan, consists of a molecule of hyaluronic acid with covalently attached side chains of glycosaminoglycans (GAG), mainly chondroitin sulfate (CS) and keratan sulfate. CS consists of a long, unbranched, polysaccharidic chain composed of repetitive disaccharidic units of N-acetylgalactosamine and glucuronic acid (Lee *et al.*, 2010). Due to its numerous sulfate groups with negative electric charges, CS ensures the PG with swelling and water retention properties, contributing to cartilage characteristic property of compressive strength.

In normal tissue, CS was able to quickly and specifically interact, as linear anionic polyelectrolyte, with proteins on the cell surface or from the extracellular matrix and with enzymes to regulate various cellular activities (Mufamadi *et al.*, 2011). CS action has been attributed to its pivotal role in increasing PG synthesis, decreasing collagenase synthesis and maintaining synovial fluid viscosity. CS also prevented cartilage damage by blocking activity of elastase and hyaluronidase. *In vitro* studies demonstrated that CS had both anabolic and anticatabolic effects on articular cartilage (Fioravanti & Collodel, 2006).

In arthritic cartilage, CS had a beneficial effect on metabolic imbalance, at each of the 3 levels of joint: cartilage, synovial membrane and subchondral bone. There are several evidences that CS may play an important role in preventing and treatment of inflammation and joint destruction in osteoarthritis (OA) (Morelli *et al.*, 2003). Previous *in vitro* studies found three CS action mechanisms in OA treatment through stimulation of chondrocytes to increase the production of extracellular matrix components, supression of inflammatory mediators and inhibition of cartilage degradation (Zhang & Huang, 2012). *In vivo* reports have showed CS anti-inflammatory activity and its role in reducing several parameters of synovitis, including cell infiltration, fibrosis and proliferation of synovial lining cells (Zhang & Huang, 2012). A double-blind controlled study showed that a dose of 2x400 mg CS per day, for 6 months, was well tolerated and efficacious in treating OA patients (Uebelhart *et al.*, 2006). In randomized trials, CS administration showed clinic efficacy as chondroprotective agent in reduction and disappearance of signs and symptoms associated with OA: pain, joint motion, function (Miller & Clegg, 2011). No serious side effects have been reported for CS.

Oral CS bioavailability assessed in healthy human volunteers showed a peak concentration of maximum 22% between 2 and 6 h after administration (Volpi, 2002). To improve CS therapeutic properties, local intra-articular administration of CS at the diseased site is now preferred (Evans *et al.*, 2014). It was reported that biologic active substances entrapped in liposomes (L) and administered intra-articularly in inflamed joints

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provide several advantages, such as targeting the phagocytic cells from synovium, suppressing pro-inflammatory cytokines and reducing adverse systemic effects (Gerwin *et al.*, 2006). CS loaded in L could represent a superior mode of drug administration compared to conventional forms due to high drug intracellular uptake, enhanced biodistribution and controlled release. Moreover, L could prolong CS retention time at the injured site, due to drug protection and cell internalization, improving its anti-inflammatory potential in OA and other local inflammatory diseases (Butoescu *et al.*, 2009). Previous studies demonstrated that administration of a drug in a liposomal formulation improved its anti-inflammatory efficacy compared to its free form (Van den Hoven *et al.*, 2011).

The present study aimed to develop an efficient lipid carrier of CS (L-CS) with improved anti-inflammatory effect for local treatment of affected joints. Its *in vitro* cytotoxicity threshold was assessed in a human chondrocyte cell culture. L-CS ability as chondroprotective agent was investigated by cell morphology observations and metalloproteinases (MMP) secretion analysis using an *in vitro* experimental model of IL-1β inflamed cells.

MATERIALS AND METHODS

Materials

CS-A sodium salt from bovine trachea, L-α-phosphatidylcholine Type XVI-E from egg yolk (PC), dioleoyl-phosphatidyl-ethanolamine (DOPE), stearylamine (SA), cholesterol (Chol), Triton X-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), L-glutamine and all other chemicals and solvents of analytical grade were purchased from Sigma-Aldrich Chemicals (Germany). Sephadex G-25 column was purchased from Sigma-Aldrich Chemicals (Germany). Sterile Syringe Filter Units with 0.2 µm pores were purchased from from Merck Millipore (Germany). A primary culture of human chondrocytes HC (402-05a) was purchased from ECACC (Sigma-Aldrich, Germany). Blyscan kit was purchased from Biocolor Ltd. (UK). LDH cytotoxicity assay kit was purchased from Cayman Chemical (USA).

Preparation of CS liposome-based carrier

Positively-charged L were prepared in the form of multilamellar vesicles (MLV) using the thin film hydration method (Mozafari, 2005). Briefly, a lipid mixture of PC:DOPE:Chol:SA, in 4:2:3:1 molar ratio, was dissolved in a mixture of chloroform:methanol (95:5, v/v) and, then, evaporated in an IKA RV 10 rotary evaporator (Ika-Works, Inc., Germany), at 150 rpm, 40 °C, for 30 min, under reduced pressure. The thin, dry lipid film was hydrated in phosphate-buffered saline (PBS), pH 7.4, containing or not 10 mg/ml CS by vigorous shaking and incubation at room temperature, for 5 h, to allow complete swelling of the lipid film. In order to reduce the vesicles size and to obtain small unilamellar vesicles (SUV) of L-based CS (L-CS) and empty L, respectively, the suspension was sonicated under inert atmosphere of N_2, first in a bath-type sonicator (Grant, UK), at 37 °C, for 2x30 min, followed by a probe-type sonicator (Bandelin Sonopuls, Germany), at 70% amplitude, for 3x10 s. The suspension was incubated at 37 °C, for 10 min, then rapidly cooled in an ice bath and centrifuged at 10,000 rpm, at 4 °C, for 5 min. Before use in cell culture experiments, L-CS and L were sterile filtered through 0.2 µm filters and were stored at 4 °C until used.

Encapsulation efficiency

Free CS was removed from L-CS suspension by size exclusion chromatography using a Sephadex G-25 column. The amount of CS loaded in L was determined using Blyscan GAG kit, after solubilization of L-CS in 0.1% Triton X-100, as previously described (French *et al.*, 2004). The optical density (OD) was measured at a wavelength of 656 nm in a Sunrise microplate reader (Tecan, Austria). CS concentration was determined on the calibration curve in the range 0-5 µg GAG. The encapsulation efficiency (EE) was calculated as percentage of loaded CS from total CS using the following equation:

$$EE\ (\%) = \text{loaded CS} / \text{total CS} \times 100\ \% \quad (1)$$

The results were expressed as mean of three independent determinations ± standard deviation (SD).

Cytotoxicity tests

The cytotoxicity of L-CS was evaluated according to the International standard SR EN ISO 10993-5 using the direct contact method in human chondrocyte cell culture (HC 402-05a), as previously described (Craciunescu *et al.*, 2013). Briefly, cells were seeded in T75 flasks with DMEM containing 10% FCS and 1% antibiotic mixture and incubated at 37 °C, in humidified 5% CO_2 atmosphere. Confluent cells were trypsinized, seeded in 24-well culture plates, at a density of $5x10^4$ cells/well and cultivated in DMEM, in 5% CO_2 atmosphere, at 37 °C, to allow cell adhesion overnight. Then, the cells were treated with different concentrations of CS (250, 500, 1000 µg/ml) loaded in L and the plate was incubated at 37 °C, for 24 h and 48 h, respectively. After incubation, cells were analyzed for cell viability by MTT assay, membrane integrity by LDH assay and cell morphology by DAPI staining. Cells cultured in standard culture medium were the negative control, considered 100% viable and cells cultured with 50 µM hydrogen peroxide were the positive control. Free CS (500 µg/ml) and empty L (700 µg/ml) were also tested, in the same conditions, as controls.

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MTT assay

Cell viability was assessed using the MTT assay (Mossman, 1983). Briefly, at the end of incubation, the culture media were harvested and the cells were washed and incubated with MTT solution at 37 °C, for 3h. The formazan crystals were dissolved in isopropanol and the optical density (OD) was read at 570 nm using a Tecan microplate reader (Austria). All experiments were performed in triplicate, and the results were expressed as percent from the negative control (100%).

LDH assay

The culture media collected from the MTT experiment were assayed for LDH activity, according to the kit manufacturer's instructions (Cayman Chemical). Briefly, an aliquot of 100 µl of culture supernatant was incubated with 100 µl mixed reaction solutions, with gentle shaking, at room temperature, for 30 min. OD was measured at 490 nm using a 96-well plate reader (Sunrise Tecan, Austria). Data were expressed as mean of three determinations ± standard deviation (SD) and the results were reported to control cells considered 1 in arbitrary units (a.u.).

Cell morphology

After 48h of incubation, the cells were fixed with 4% p-formaldehyde in PBS and DAPI stained. Examination of stained cells was performed at an Axiostar Plus microscope equiped with camera and soft (Zeiss, Germany).

In vitro model of inflammation

An *in vitro* model of inflamed cells was developed in human chondrocyte cell culture using the pro-inflammatory cytokine interleukin-1β (IL-1β), as previously described (Aida *et al.*, 2005). Briefly, cell suspension was seeded in 24-well culture plates at a density of $1x10^5$ cells/well and incubated in a humidified 5% CO_2 atmosphere, at 37 ^oC, to allow cell adhesion for 18 h. IL-1β (50 ng/mL) was added in the culture medium and the cells were cultivated for 24 h. Then, the culture medium was replaced with DMEM containing L-CS, to give 250 and 500 µg/ml final concentration CS, and the plate was incubated in standard conditions, for 48h. After incubation, cells were processed for morphology observation by Hematoxylin-Eosin (H&E) staining and the culture medium was analyzed for MMP type 2 and 9 secretion. Free CS (500 µg/ml) and empty L (700 µg/ml) were tested as controls, in the same conditions.

H&E staining

Cells were fixed in 4% p-formaldehyde in PBS and embeded in paraffin. Sections of 6 µm were stained with H&E and observed at a Zeiss Axiostar Plus microscope (Carl Zeiss, Germany).

MMP by gelatin zymography

Culture media harvested after 48 h of L-CS treatment was kept at -20 °C until analysis. The MMP activity was assessed by gelatin-zymography, as previously described (Moldovan *et al.*, 2002). Gelatin type A from pig skin was added to the standard acrylamide mixture to reach a final concentration of 1 mg/ml. Samples having the same protein concentration (2.5 µg protein/well) were mixed with sample buffer and were migrated in 7.5% SDS-polyacrylamide gel, for 2 h. Then, the gel was washed and incubated in Tris-HCl buffer, pH 7.6, containing Triton X-100 and stained with 0.1% Coomassie Brilliant Blue R-250. After 1 h, the gel was unstained and photographed. MMP's activity was visualized as non-colored bands on the blue background of the gel. Samples were co-migrated with a molecular weight marker (10-150 kDa). Variation in MMP activity was assayed using a gel-densitometer (Vilber Lourmat, France) and Gel Analyzer 2010a software.

Statistics

Data were expressed as mean value ± SD for three independent samples (n=3). Statistical analysis of the data was performed using the one-tailed paired Student's *t*-test, on each pair of interest. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

L-CS population obtained in our lab was characterized, as previously showed (Craciunescu *et al.*, 2013) and presented a mean diameter of 250.2 nm and the vesicle's surface charge of -9.44 mV. CS encapsulation efficiency was high (68.7%) due to the interaction of CS negative charges with the cationic lipid SA from the lipid bilayer.

Determination of optimal L-CS concentration in HC cell culture

The optimal dose of CS loaded in L for cell culture studies was established in a primary HC cell line by assessment of cell viability, membrane integrity and fluorescence microscopy observations of cell morphology. Two quantitative assays (MTT, LDH) and one qualitative assay (DAPI staining) were used together to provide complementary information on cytotoxicity threshold of CS conditioned as liposomal formulation.

MTT assay evaluates the activity of mitochondrial dehydrogenases reflecting the cell metabolism and viability. In our study, HC cell viability was assessed after cultivation with several concentrations of L-CS added in the culture medium, to give a final concentration in the range of 250-1000 µg/ml CS. In fig. 1, the variation of cell viability values is presented after 24 h and 48 h of cultivation with L-CS.

The data showed that only 250 and 500 µg/ml L-CS concentrations in the culture medium induced a value of cell viability comparable with the control cells (100%), after 48 h of cultivation. Examining the records, the values for both L-CS concentrations were

significantly higher ($p<0.05$) compared to free CS-treated group and L-treated group. At higher concentration of L-CS (1000 µg/ml) added in the culture medium, the cell viability percent decreased below 70%, after 48 h of cultivation, indicating a mild cytotoxic sample. The cell viability percent for the hydrogen peroxide-treated cell group (63 %) was significantly decreased ($p>0.05$) compared to the control cells, after 48 h of cultivation.

These results suggested that CS loaded in L was highly cytocompatible and stimulated the cellular activity of chondrocytes, in concentrations up to 500 µg/ml. Even if free CS showed to be a safe therapeutic agent in HC cell culture, L-CS systems exhibited a better control of cell metabolic activity. This observation is an advantage next to the other roles provided by liposomal delivery, such as rapid cellular uptake, controlled release at the inflamed site, reported by other teams (Mufamadi *et al.*, 2011). Our previous studies showed that concentrations of 500 µg/ml CS entrapped in unilamelar lipid vesicles didn't induce cytotoxicity in human dermal fibroblasts (Trif *et al.*, 2008) and L929 fibroblasts and the cells maintained their normal morphology (Craciunescu *et al.*, 2013).

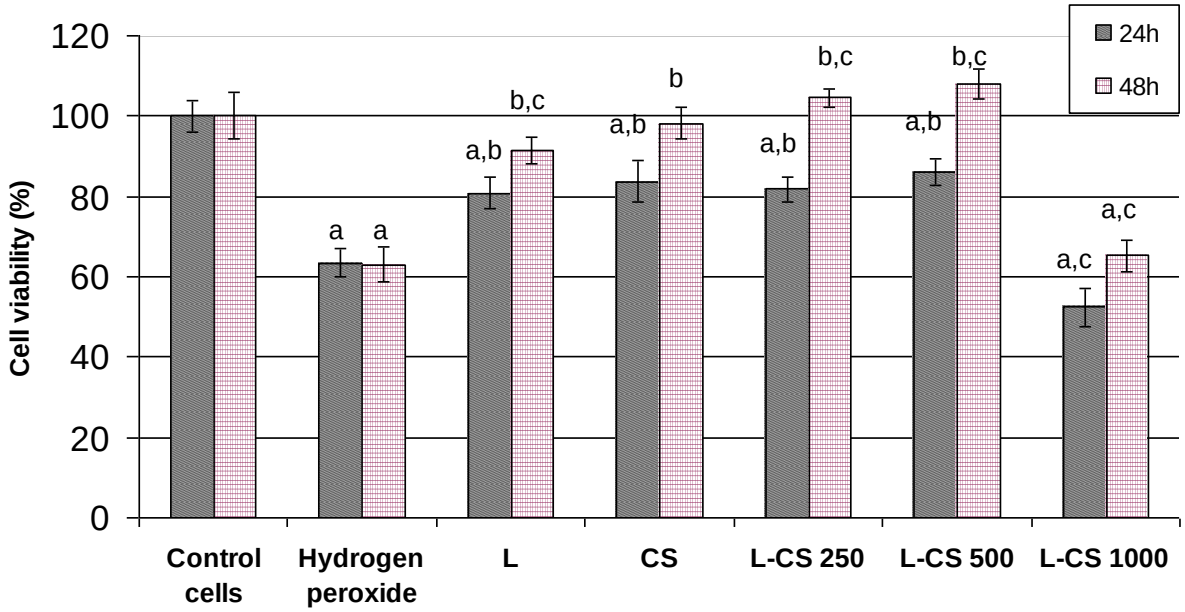

Fig. 1 The metabolic activity of HC cells cultivated in the presence of different concentrations of L-CS (250, 500 and 1000 µg/ml), empty L (700 µg/ml) and free CS (500 µg/ml), for 24 h and 48 h, assessed by MTT assay. The results are expressed as percentage from control cells (100 %). Error bars represent mean ± SD for n=3. One-tailed paired *t*-test analysis showed significant differences ($p<0.05$) compared to control cells (a), H_2O_2 control (b) and CS (c).

LDH assay evaluates the activity and the quantity of enzyme secreted in the culture medium due to cell membrane damage, compared to the control cells. LDH measurements were performed in the media harvested from MTT experiment in order to correlate the results. The data are presented in fig. 2.

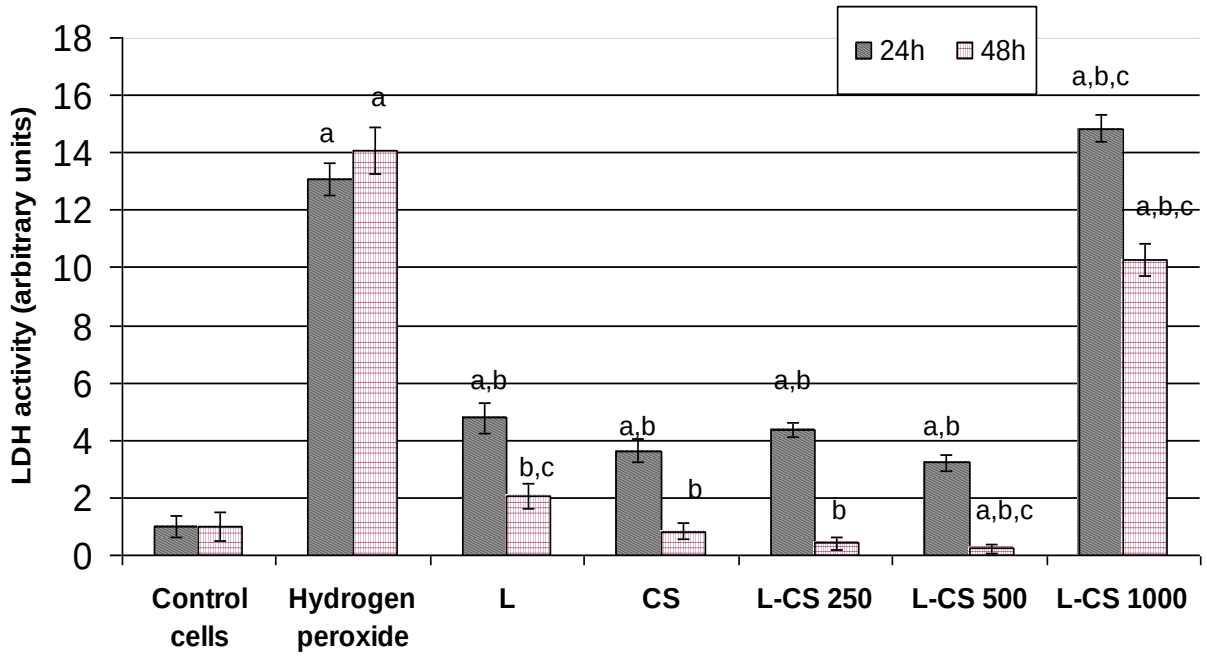

Fig. 2 The LDH activity values in the culture media of HC incubated with different concentrations of L-CS (250, 500 and 1000 µg/ml), empty L (700 µg/ml) and free CS (500 µg/ml), for 24 h and 48 h, respectively. The results were reported to control cells considered 1 a.u. Error bars represent mean ± SD for n=3. Significant differences ($p<0.05$) were observed in comparison to control cells (a), H_2O_2 control (b) and CS (c).

The results showed that cell treatment with 250 and 500 µg/ml concentrations of CS loaded in L didn't affect cell membrane integrity and resulted in no significant differences ($p>0.05$) in LDH activity values compared to the control cells. The LDH value for cells treated with 500 µg/ml L-CS was significantly lower ($p<0.05$) than for free CS-treated group. A significant increase ($p<0.05$) in LDH activity was registered for 1000 µg/ml L-CS. These data correlated with those obtained by MTT assay.

DAPI is a fluorophore that binds the regions rich in A-T bonds from double chain DNA after penetration of intact cell membrane of live cells, to give information about cell population viability. The morphology and density of HC cultivated with different concentrations of CS loaed in L (250, 500 and 1000 µg/ml) were observed by DAPI staining (fig. 3). Micrographs showed that treated cells presented a normal phenotype, similar to the control cells, without signs of apoptosis, after 48 h of cultivation. The density of cells cultured with 250 and 500 µg/ml L-CS was higher than that of cells cultivated with 1000 µg/ml L-CS. The cells had a slightly decreased density after 48 h of cultivation with free CS and empty L, respectively.

Cell morphology analysis confirmed the quantitative results and indicated that L-CS didn't exert cytotoxic effects in HC culture. We selected the doses of 250 and 500 µg/ml L-CS as biocompatible concentrations for further tests of *in vitro* induced inflammation in HC cell culture.

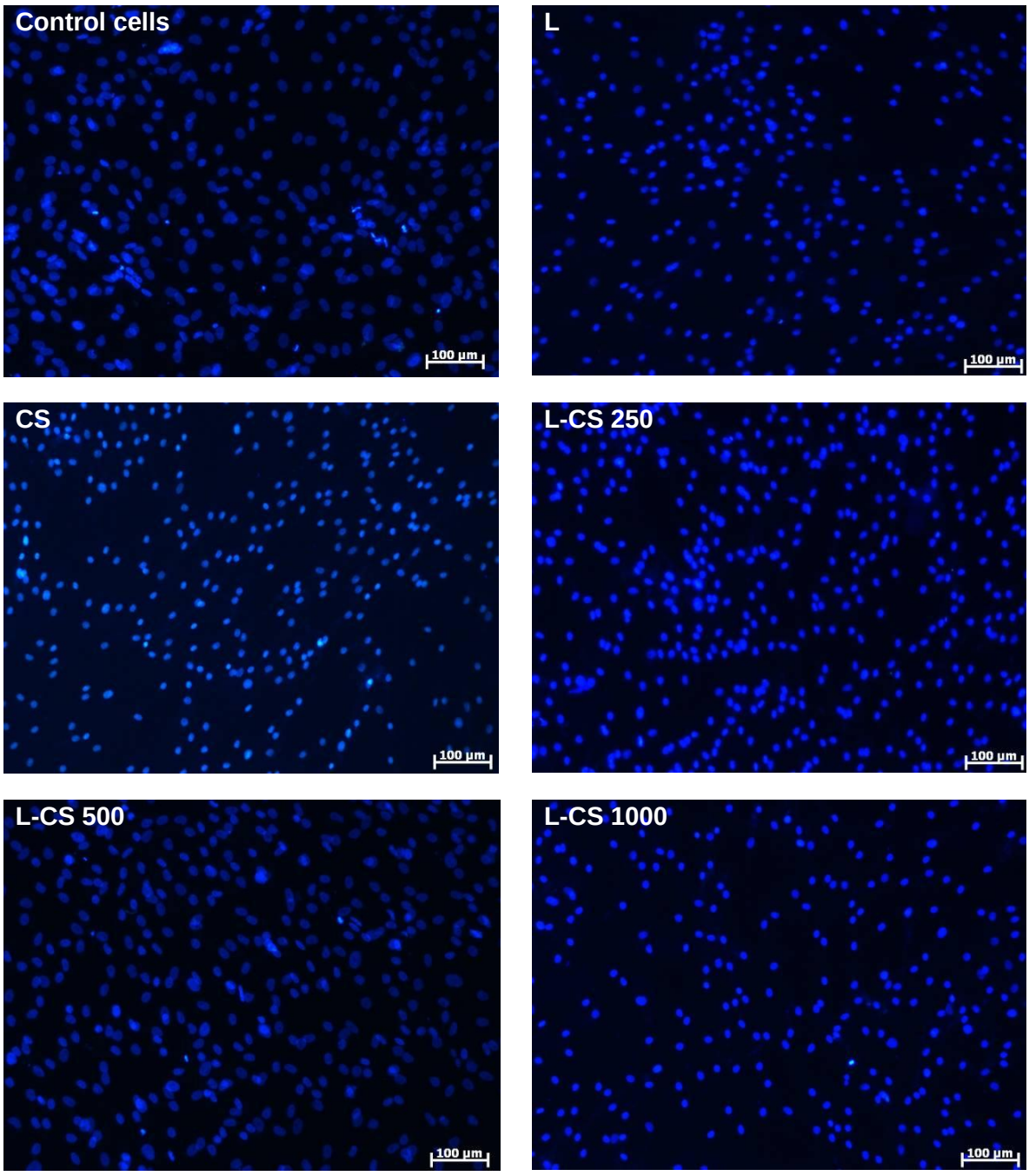

Fig. 3 Morphology and density of HC cells cultivated in the presence of empty L, free CS and different concentrations of L-CS (250, 500 and 1000 µg/ml), for 48 h. (DAPI staining)

Anti-inflammatory activity of CS loaded in L

The anti-inflammatory property of CS loaded in lipid vesicles was tested using an *in vitro* experimental model of HC inflamed with IL-1β and treated with cytocompatible concentrations of L-CS for 48 h. Their effect was comparatively assessed to free CS and empty L treatment by cell morphology observations using light microscopy and analysis of MMP-2/MMP-9 gelatinases secreted in the culture medium using gelatin-zymography assay.

The light micrographs of inflamed cells after 48 h treatment with L-CS samples are presented in fig. 4. Compared to IL-1β-inflamed cells, the cells treated with 250 and 500 µg/ml L-CS had a normal morphology and a density close to 80%, indicating their an anti-inflammatory activity. Treatment with free CS and empty L, respectively, for 48 h induced a restored, chondrocyte-type morphology, but the cell density was near to 50%.

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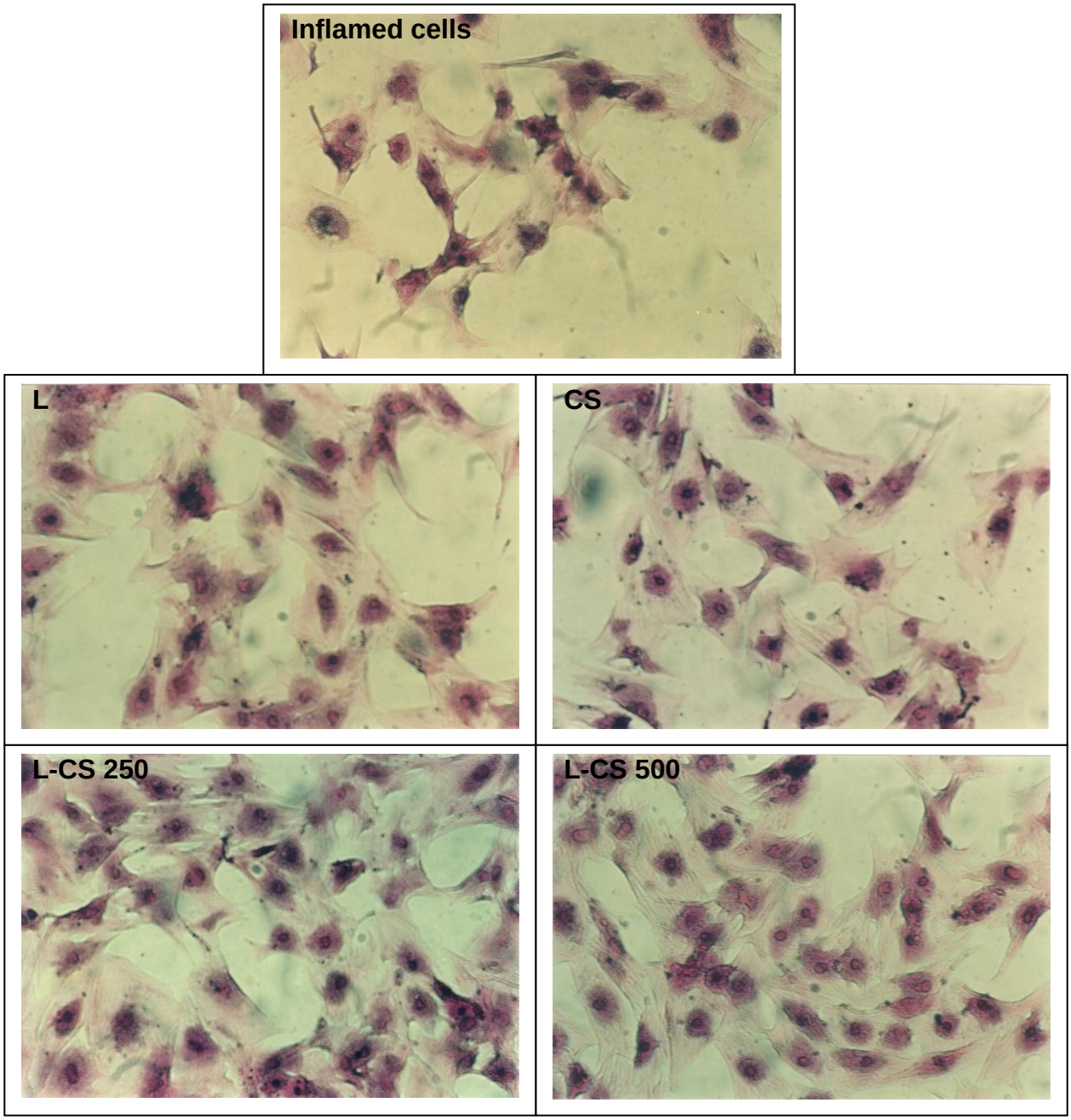

Fig. 4 Light micrographs showing the morphology of inflamed HC cells treated with different concentrations of L-CS (250 and 500 µg/ml), empty L (700 µg/ml) and free CS (500 µg/ml), for 48 h. (H&E staining, x100)

MMP are involved in both development processes and pathological conditions, such as OA, wound healing, acute and chronic cardiovascular diseases (Lim *et al.*, 2010). In our study, the expression of MMP-2 and MMP-9 gelatinases in the culture medium of inflamed cells treated with L-CS was analyzed by gelatin-zymography. The results showed that, in inflamed cells, MMP-2 was detected as a dublet represented by a prominent band corresponding to the latent form, pro-MMP-2, with a higher molecular weight (72 kDa) and a faint band with a higher electrophoretic mobility (62 kDa), corresponding to the active form of MMP-2 (fig. 5). MMP-9 was also detected as a dublet, formed by pro-MMP-9 band, at 92 kDa and the active MMP-9 band, at 82 kDa. In inflamed cells treated with CS, gelatinases were expressed in a similar pattern as in control, excepting active MMP-9 band that was diminished. In L-CS treated cells, faint bands corresponding to active MMP-2 and MMP-9 were detected.

These results highlighted that the treatment of inflamed cells with CS loaded in liposomes resulted in a higher MMP inhibition than CS treatment. Previous studies showed that the variation of active gelatinases was considered beneficial for tissue healing and improvement of procollagen synthesis (Fonseca *et al.*, 2010). All our findings obtained in the experimental model of IL-1β-inflamed HC culture suggested that L-CS had a better anti-inflammatory activity than free CS and could have a therapeutic activity to prevent inflammation damage of chondrocyte cells.

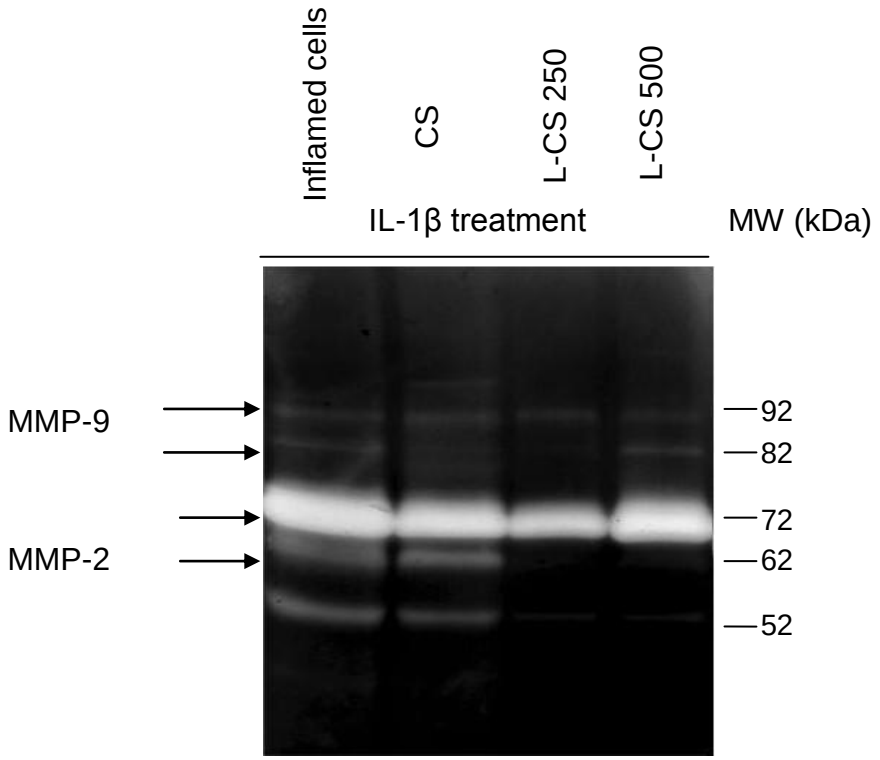

Fig. 5 Effect of L-CS treatment on MMP-2 (72/62 kDa) and MMP-9 (92/82 kDa) secretion in HC inflamed cells, analyzed by gelatin-zymography

CONCLUSIONS

Our study selected optimal concentrations of CS loaded in liposomes formulation (L-CS 250, L-CS 500) that were biocompatible and stimulated cell proliferation in HC culture. They were tested for anti-inflammatory activity in a model of IL-1β-inflamed HC cells showing their ability to prevent cell morphological changes and inhibition of gelatinases secretion. We conclude that L-CS represent a promising therapeutic agent that could be further tested for its impact in OA treatment by intra-articular local administration.

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