



Research paper

Potential therapeutic effect of curcumin loaded hyalurosomes against inflammatory and oxidative processes involved in the pathogenesis of rheumatoid arthritis: The use of fibroblast-like synovial cells cultured in synovial fluid



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ABSTRACT

In the present work curcumin loaded hyalurosomes were proposed as innovative systems for the treatment of rheumatoid arthritis. Vesicles were prepared using a one-step and environmentally friendly method. Aiming at finding the most suitable formulation in terms of size, surface charge and stability on storage, an extensive pre-formulation study was performed using different type and amount of phospholipids. Curcumin loaded vesicles prepared with 180 mg/ml of Phospholipon 90G (P90G) and immobilized with sodium hyaluronate (2 mg/ml) were selected because of their small size (189 nm), homogeneous dispersion (PI 0.24), negative charge (−35 mV), suitable ability to incorporate high amount of curcumin (E% ~88%) and great stability on storage. The *in vitro* study using fibroblast-like synovial cells cultured in synovial fluid, demonstrated the ability of these vesicles to downregulate the production of anti-apoptotic proteins IAP1 and IAP2 and stimulate the production of IL-10, while the production of IL-6 and IL-15 and reactive oxygen species was reduced, confirming their suitability in counteracting pathogenesis of rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis is a complex and autoimmune disease characterized by chronic inflammatory synovitis and progressive joint destruction, which cause severe disability and mortality. Several studies suggest that the pathogenesis of rheumatoid arthritis is mainly controlled by fibroblast-like synovial cells. These apoptosis-resistant cells are responsible for the synovium hyperplasia and the vascular pannus formation [1,2]. Furthermore, they play crucial roles in both joint damage and propagation of inflammation [3,4] as they proliferate in a non-controlled manner and the formed hyperplastic tissue (pannus) is

responsible for cartilage degradation and bone erosion [5]. Numerous studies suggest the involvement of oxidative stress in pathogenesis and degeneration of rheumatoid arthritis [6,7], because of the imbalance between oxidants and antioxidants in favour of the former, leading to the interruption of redox signalling and control, along with molecular damage. In particular, reactive oxygen species (ROS) may initiate a variety of oxidative reactions involved in the pathophysiology of rheumatoid arthritis through a self-perpetuation cycle of inflammation and destruction. ROS may be formed in the inflamed joint of patients either by chondrocytes, by activated macrophages in the synovial membrane, or by activated neutrophils in the synovial cavity [8].

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Currently, synthetic drugs such as analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs and corticosteroids are used for the treatment of this pathology. Unfortunately, the treatment with NSAIDs and corticosteroids is characterized by cumulative adverse effects; many patients cannot tolerate these treatments, and some individuals continue to have active disease during therapeutic treatment. Thus, effective alternative therapies or co-adjuvants, based on natural and safe active ingredients and characterized by reduced side effects, are essential to treat from moderate to severe rheumatoid arthritis and improve the patient compliance. Among all, curcumin, a naturally occurring yellow pigment derived from the rhizome of *Curcuma longa*, has been demonstrated to be safe and possess anti-oxidant, anti-inflammatory, anti-proliferative and anti-arthritis properties [9,10]. It seems to downregulate various pro-inflammatory cytokines such as tumour necrosis factor (TNF- α), interleukins (IL-1, IL-2, IL-6, IL-8, IL-12) and chemokines, most likely through inactivation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). A number of studies have shown that curcumin can inhibit cell proliferation and induce apoptosis in a variety of cancer cells [11–13]. In contrast, several reports indicate that curcumin can protect cells from apoptosis in various disease models including hepatic and testicular injury and Alzheimer's disease [14–16]. Despite its promising properties, the therapeutic use of curcumin is limited by its high lipophilicity (and consequent poor aqueous solubility) and high first-pass metabolism that lead to a poor oral bioavailability [17,18]. To overcome these problems different approaches, mainly related to the use of drug delivery systems, have been proposed. In particular, phospholipid vesicles or liposomes have attracted increasing attention as local carriers capable of facilitating the overcoming of biological barrier and controlling the release at the action site. Aiming at improving the local delivery of biological molecules, the basic formulation of liposomes has been improved by the addition of other functional components, (i.e. cholesterol, co-solvents, penetration enhancers, polymers or dextrans), as their addition was able to positively affect the stability and performances of vesicles [18–23]. In previous works sodium hyaluronate immobilized vesicles so called hyalurosomes were prepared and tested as carrier for different natural molecules or extracts. Their peculiar structure favoured the incorporation and retention of payloads and improved the carrier performances *in vitro* and *in vivo* [18,24–27].

In this work, curcumin loaded hyalurosomes were formulated as carrier for the local treatment of rheumatoid arthritis. In order to get the best formulation with the most suitable physicochemical features and properties, a pre-formulation study has been carried out as a function of the amount and type of phospholipid. The most promising formulation has been chosen to evaluate its anti-oxidative and anti-inflammatory effectiveness in fibroblast-like synovial cells cultured in synovial fluid.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine with different purity degree (Phospholipon® 90G, P90G); Lipoid® S75, S75) and hydrogenated soy phosphatidylcholine, Phospholipon® 90H (P90H) were purchased from AVG S.r.l. (Garbagnate Milanese, Italy). Soy lecithin (SL) was purchased from Galeno (Prato, Italy). Sodium hyaluronate (HY), low molecular weight (200–400 kDa) was purchased from DSM Nutritional Products AG Branch Pentapharm (Aesch, Switzerland). Except where otherwise indicated, all other reagents of analytical grade were purchased from Sigma Aldrich (Milan, Italy).

2.2. Sample preparation

Hyalurosomes were prepared by using different amounts and type of

phospholipids (P90G, S75, P90H or SL). In a typical preparation, 20 mg of sodium hyaluronate and 100 mg of curcumin were dispersed in distilled water (10 ml) and stirred for 5 h at $\sim 25^\circ\text{C}$ to achieve a final polymer dispersion of 0.2% w/v. Subsequently, the curcumin/polymer dispersion (10 ml) was added to 0.9, 1.8 or 3.6 g of each phospholipid (P90G, S75, P90H or SL) and left hydrating overnight; the dispersions were then sonicated (5 s on and 2 s off, 50 cycles; 13 mm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, United Kingdom).

Samples (1 ml) were purified from the non-incorporated curcumin by dialysis against water (2.5 l) using dialysis tubing (Spectra/Por® membranes, 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) at room temperature for 4 h, replacing the water every hour. The used water (10 l total) was able to theoretically remove 10 mg of curcumin contained in 1 ml of dispersion.

2.3. Vesicle characterization

For cryogenic electron transmission microscopy (cryo-TEM), a thin aqueous film was formed on a glow-discharged holey carbon grid and vitrified by plunging into ethane, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous films were transferred to a Tecnai F20 TEM (FEI Company) and the samples were observed in a low dose mode, at 200 kV and at a temperature around $\sim -172^\circ\text{C}$.

The average diameter and polydispersity index (a measure of the size distribution width), were determined by Photon Correlation Spectroscopy, using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, United Kingdom). Samples were backscattered by a helium-neon laser (633 nm) at an angle of 173° and a constant temperature of 25°C . Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility [28]. Samples were diluted with water (1:100 dilution) and immediately analysed.

Entrapment efficiency (EE), expressed as the percentage of curcumin in the vesicles versus that initially used, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with methanol (1/1000), using a SunFire C18 column (3.5 mm, 4.6×150 mm) and an Alliance 2690 chromatograph (Waters, Milano, Italy) equipped with a photodiode array detector and a computer integrating apparatus (Empower™ 3). The mobile phase was a mixture of acetonitrile, water and acetic acid (95:4.84:0.16, v/v), delivered at a flow rate of 0.7 ml/min. Curcumin content was quantified by measuring $A_{424\text{ nm}}$ against a standard calibration curve. Graphs were plotted according to linear regression analysis, which gave a correlation coefficient value (R) of 0.999.

2.4. Stability studies

Vesicle stability was assessed by monitoring their average size and zeta potential over 60 days at room temperature ($25 \pm 2^\circ\text{C}$) [29]. Curcumin retention within the vesicles during storage was evaluated by measuring the entrapment efficiency.

2.5. Antioxidant activity of samples

The antioxidant activity of curcumin loaded vesicles was evaluated by measuring their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH). Each sample (40 μl) was added to a DPPH methanolic solution (2 ml, 40 $\mu\text{g/ml}$) and stored at room temperature for 30 min in the dark. The absorbance was measured at 517 nm against blank. All the experiments were performed in triplicate. The percent antioxidant activity was calculated according to the following formula: antioxidant activity (%) = $[(A_{517\text{ DPPH}} - A_{517\text{ sample}})/A_{517\text{ DPPH}}] \times 100$ [30].

2.6. Synovial fluid samples and extraction of fibroblast-like synoviocytes

Synovial fluid samples and fibroblast-like synovial cells were used. The study was approved by the Hospital Ethics Committee of the Gaetano Pini Institute of Milan (Italy). All patients signed informed consent to take part in the study. All samples of liquid and synovial membrane were taken from the waste materials during arthrocentesis or surgery. The Ethics Committee at the University of Milan approved the whole study (27th March 2012). Synovial fluid was directly aspirated from the joints of patients, collected into heparinized tubes and centrifuged at $1000 \times g$ for 10 min. The acellular portion was stored at -80°C until the use. Samples collected from 10 patients were used to reduce the variability between the different fluids, which were used at a final dilution of 1:8 in culture medium. Synovial tissue was obtained from patients with rheumatoid arthritis ($n = 22$) during joint synovectomies. The tissues were digested with collagenase in Dulbecco's modified Eagle Medium (DMEM, Euroclone, Pero, Italy) for 2 h at 37°C . Dissociated cells were then centrifuged at $1000 \times g$, suspended in DMEM supplemented with 10% opticlone serum (Thermo Scientific, USA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Euroclone, Pero, Italy), and plated. Cells were cultured overnight, non-adherent cells were removed, while adherent cells were cultivated in DMEM supplemented with 10% foetal calf serum at 37°C in 5% CO_2 . The purity of the cells was tested by flow-cytometric analysis using phycoerythrin-conjugated anti-CD14 (Pharmingen, San Diego, CA, USA), fluorescein isothiocyanate phycoerythrin-conjugated anti-CD3, anti-CD19 and anti-Thy-1 (CD90) monoclonal antibodies (R&D Systems Minneapolis, MN). A FACS Calibur flow cytometer (488ex/620em, Becton Dickinson, San Jose, CA, USA) was used for the analysis. At passage 3, the cells were homogeneous from the morphological point of view and exhibited the characteristic properties (typical bipolar configuration under inverse microscopy) of rheumatoid arthritis-derived fibroblast-like synovial cells. Most cells ($> 98\%$) expressed the surface marker for fibroblasts (Thy-1) and were negative for the expression of antigens CD3, CD19, and CD14. Before each experiment, rheumatoid arthritis-derived fibroblast-like synovial cells at passages 3–9 were cultured in the presence of synovial fluid for 5 days.

2.7. Proliferation assay

Rheumatoid arthritis-derived synovial cells were cultured in the presence of synovial fluid for 5 days. 18 h before the experiment the cells were treated with empty or curcumin loaded hyalurosomes. To avoid non-specific binding, the samples were saturated with Odyssey Blocking Buffer (LI-COR Bioscience) for 2 h with moderate shaking. The plate was washed four times with washing solution (0.1% Tween 20 in PBS) and then incubated for 1 h with Cell Tag 700CW (1:500; LI-COR Bioscience). Cell Tag 700 Stain is a near-infrared fluorescent, non-specific cell stain that accumulates in the nucleus and cytoplasm of cells and provides an accurate label for cell number counting. After 5 washes, the plate was scanned at 700 nm using the Odyssey Infrared Imaging System (LI-COR Bioscience).

2.8. Apoptosis assay

The apoptosis of rheumatoid arthritis-derived synovial cells treated with empty (containing 27 μg and 0.3 μg of P90G and hyaluronan respectively) or curcumin loaded 180P90G hyalurosomes (containing 27 μg , 1.5 μg and 0.3 μg of P90G, curcumin and hyaluronan respectively) was measured after 18 h. Untreated cells were used as negative control. Apoptotic cells were detected with the Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. All samples were analysed with a FACS Calibur flow cytometer. FITC-conjugated Annexin V emission was collected in the FLH-1 channel, and propidium iodide (PI; for the detection of dead cells) emission was collected in the FLH-3 channel. Data were analysed

with Cell Quest software. The percentage of apoptosis was calculated, considering cells in both early (Annexin⁺, PI⁻) and late apoptosis (Annexin⁺, PI⁺).

2.9. Western blots

Rheumatoid arthritis-derived synovial cells were treated with empty (containing 27 μg and 0.3 μg of P90G and hyaluronan respectively) or curcumin loaded 180P90G hyalurosomes (containing 27 μg , 1.5 μg and 0.3 μg of P90G, curcumin and hyaluronan respectively) for 48 h before inhibitors of apoptosis proteins (IAP) detections. Cells were lysed in lysis buffer and the protein concentration was measured by the BCA Protein Assay method (Thermo Scientific, USA) according to the manufacturer's instructions. For IAP and cytokine detection, the cell lysates were separated by SDS-PAGE in 4–12% or 10% Tris-HCl pre-cast gels (Life Technologies, Carlsbad, CA) respectively, and transferred onto nitrocellulose membranes (Life Technologies, Carlsbad, CA). The membranes were blocked for 3 h with 5% non-fat dry milk (Lab Scientific) in washing medium and probed overnight at 4°C with primary antibodies: cIAP1, 1:600 (R&D Systems, Minneapolis), cIAP2, 1:600 (BD Pharmingen, MA, USA), anti IL6, 1:600 and anti IL10, 1:1500 (Prospec East Brunswick, NJ USA), anti TNF- α and anti IL15, 1:1000 (from Peprotec, London UK), whereas β -actin, 1:4000, was used as the loading control. Secondary antibodies were conjugated to horseradish peroxidase (Thermo Scientific, USA), and the gels were developed using Westar ηC (Cyanagen, Italy). Densitometry was performed using Image J Software (National Institutes of Health, Bethesda, USA).

2.10. Quantification of ROS generation

ROS formation was quantified according to the method of Wang and Joseph [31] with some modifications, using the cell permeable, non-fluorescent probe 2.7 dichlorofluorescein diacetate (DCFDA), which is de-esterified intracellularly and turns to highly fluorescent 2.7-dichlorofluorescein upon oxidation. Rheumatoid arthritis-derived synovial cells were seeded in black 96-well plates and pre-treated for 6 h with empty (containing 27 μg and 0.3 μg of P90G and hyaluronan respectively) or curcumin loaded hyalurosomes (containing 27 μg , 1.5 μg and 0.3 μg of P90G, curcumin and hyaluronan respectively). Luperox (600 μM) was used as positive control. The cells were then incubated with 25 M DCFDA at 37°C for 2 h. Fluorescence was measured using a microplate reader (Multilabel counter Victor Wallac 1420, Perkin Elmer, Monza, Italy) at $\lambda_{\text{ex/em}}$ 485/530 nm. The results are reported as fluorescence units (FU)/ μg of cell proteins.

2.11. Statistical analysis

Statistical analysis was performed using Student's-test for matched pairs. Differences with a confidence level of $> 95\%$ were considered statistically significant ($p < 0.05$); the SPSS 21 (IBM) program was used.

3. Results

3.1. Pre-formulation study and vesicle characterization

In the present study, curcumin loaded hyalurosomes have been tailored to be applied directly in the articular joint. As previously found, the incorporation of curcumin in conventional liposomes was difficult and around 80% of polyphenol precipitated in a short time. The addition of sodium hyaluronate allowed the formation of peculiar vesicles, hyalurosomes, which were immobilized in a preformed and structured polymer network and were able to stably incorporate high amount of curcumin [18]. Aiming at finding the vesicles with the most appropriate characteristics, an exhaustive pre-formulation study was carried out using four different phospholipids (P90G, S75, P90H or SL),

Table 1
Composition of curcumin loaded hyalurosomes.

	S75 (mg/ml)	P90G (mg/ml)	P90H (mg/ml)	SL (mg/ml)	Curcumin (mg/ml)	Hyaluronan (mg/ml)
90-S75 hyalurosomes	90	–	–	–	10	2
180-S75 hyalurosomes	180	–	–	–	10	2
360-S75 hyalurosomes	360	–	–	–	10	2
90-P90G hyalurosomes	–	90	–	–	10	2
180-P90G hyalurosomes	–	180	–	–	10	2
360-P90G hyalurosomes	–	360	–	–	10	2
90-P90H hyalurosomes	–	–	90	–	10	2
180-P90H hyalurosomes	–	–	180	–	10	2
360-P90H hyalurosomes	–	–	360	–	10	2
90-SL hyalurosomes	–	–	–	90	10	2
180-SL hyalurosomes	–	–	–	180	10	2
360-SL hyalurosomes	–	–	–	360	10	2

which contain different amounts of phosphatidylcholine and other lipids. In particular, P90G, contains 96% of phosphatidylcholine, ~0.8% of lyso-phosphatidylcholine, 0.20% of tocopherol and other minor components such as fatty acid, cephalin and phosphatic acid; S75, contains 70% phosphatidylcholine, 9% phosphatidylethanolamine, 3% lyso-phosphatidylcholine, triglycerides and fatty acids; SL, contains phosphatidylcholine, phosphatidylinositol, iron, phosphorus and calcium; P90H, contains at least 90% of hydrogenated phosphatidylcholine (with saturated fatty acids). Each phospholipid mixture was used at three different concentrations (90, 180 and 360 mg/ml), while curcumin and sodium hyaluronate were used at fixed concentration (10 mg/ml and 2 mg/ml respectively, Table 1).

Using the smaller amount of all phospholipids, the obtained hyalurosomes were large (> 900 nm) and highly polydispersed (polydispersity index > 0.70), especially those prepared with S75 and SL (> 1200 nm) (Table 2). In addition, in these dispersions curcumin precipitated in a short time confirming the ineffectiveness of this amount of phospholipids to stably incorporate 10 mg/ml of curcumin.

Using higher amounts of phospholipids (180 and 360 mg/ml) the mean diameter of vesicles decreased as a function of the used phospholipid. Vesicles prepared with S75 were the largest irrespective of the used amount of phospholipid (1577, 760 and 867 nm, for 90, 180 and 360 mg/ml respectively $p > 0.05$), while vesicles prepared with 180 and 360 mg/ml of P90H and SL were smaller (~365, ~460 and ~311, ~321 nm, respectively, $p > 0.05$ among vesicles prepared with the two concentrations) but the polydispersity index was too high (> 0.45). The smallest vesicles with the lowest polydispersity index were obtained using 180 mg/ml of P90G (~189 nm and 0.24), while using 360 mg/ml of P90G both parameters slightly increased (~290 nm and 0.41). Additionally, any precipitation of curcumin was detected in these dispersions as well as in that prepared with SL at the highest concentrations.

Taking into account these results, curcumin loaded 180-P90G

hyalurosomes were selected as the most suitable formulation for further studies. To evaluate their morphology, 180-P90G hyalurosomes were observed by cryo-TEM, which represents a reliable technique able to inspect vesicular structures in their native form. As previously found [18], hyalurosomes exhibited a regular spherical shape with a prevalent single membrane surrounding the aqueous core and a mean diameter ~170 nm (Fig. 1).

Curcumin entrapment efficiency was high, as ~88% of the drug was retained in the vesicles.

To evaluate the stability of the selected hyalurosomes as a function of the time and their ability to retain the curcumin on storage, the size, size distribution and entrapment efficiency were monitored during 60 days at $25 \pm 1^\circ\text{C}$ (Fig. 2) [32]. The studied parameters remained constant during all the storage period, indicating a high stability of the system, which did not undergo either vesicle aggregation or fusion nor significant loss of curcumin.

3.2. Antioxidant activity analysis

The antioxidant activity of curcumin loaded hyalurosomes was evaluated by the colorimetric DPPH test and compared with that of a methanolic solution used as reference. The antioxidant activity of curcumin is related to its ability to eliminate free radicals and to prevent lipid peroxidation. Free curcumin in methanolic solution had an antioxidant power of $86 \pm 6\%$, which was not significantly modified by its incorporation in hyalurosomes ($92 \pm 5\%$, $p > 0.05$).

3.3. In vitro studies with rheumatoid arthritis-derived synovial cells

Casnici et al. [33] emphasized the importance of using synovial fluid, from rheumatoid arthritis patients, in *in vitro* studies involving cells obtained from rheumatoid arthritis synovial tissue, as this combination enabled the faithful reproduction of the physio-pathological

Table 2

Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and curcumin retention observed for curcumin loaded hyalurosomes using different type and amount of phospholipids. Mean values \pm standard deviation, obtained from at least 6 independent samples, were reported.

	MD (nm)	PI	ZP (mV)	Precipitation of curcumin
90-S75 hyalurosomes	1577 \pm 228	0.88	-71 \pm 12	Yes
180-S75 hyalurosomes	760 \pm 85	0.60	-81 \pm 6	Yes
360-S75 hyalurosomes	867 \pm 66	0.77	-83 \pm 3	Yes
90-P90G hyalurosomes	919 \pm 51	0.74	-39 \pm 6	Yes
180-P90G hyalurosomes	189 \pm 15	0.24	-35 \pm 6	No
360-P90G hyalurosomes	290 \pm 43	0.41	-38 \pm 6	No
90-P90H hyalurosomes	981 \pm 62	0.82	-23 \pm 18	Yes
180-P90H hyalurosomes	365 \pm 59	0.46	-25 \pm 5	Yes
360-P90H hyalurosomes	460 \pm 75	0.68	-22 \pm 5	Yes
90-SL hyalurosomes	1228 \pm 516	0.83	-54 \pm 2	Yes
180-SL hyalurosomes	311 \pm 86	0.51	-57 \pm 7	No
360-SL hyalurosomes	321 \pm 64	0.56	-72 \pm 11	No

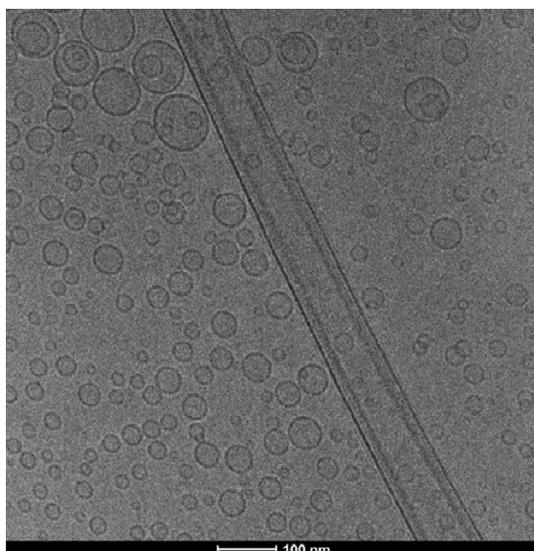


Fig. 1. Representative images obtained by the cryo-TEM observation of curcumin loaded 180-P90G-hyalurosomes. Bars represent 100 nm.

environment of joints. In the light of this, the activity of curcumin loaded hyalurosomes was tested on rheumatoid arthritis-derived synovial cells cultured in synovial fluid using different tests.

3.4. Proliferation assay

The rheumatoid arthritis-derived synovial cells were treated for 18 h with curcumin loaded hyalurosomes (containing 27 µg, 1.5 µg and 0.3 µg of P90G, curcumin and hyaluronan respectively) corresponding to 180P90G hyalurosomes and, considering the well-known efficacy of the hyaluronic acid in alleviating the symptoms of rheumatoid arthritis, the empty vesicles were also tested at the same dilution (Fig. 3). The curcumin loaded vesicles significantly reduced the cell viability ($p < 0.05$) in comparison with untreated cells (negative control) and empty hyalurosomes, confirming their ability to inhibit the proliferation of rheumatoid arthritis-derived synovial cells.

3.5. Apoptosis determination

Since it is well known that in patients with rheumatoid arthritis the pannus cells are resistant to apoptosis, the effect of curcumin loaded hyalurosomes on the apoptosis of rheumatoid arthritis-derived synovial cells was evaluated using the Annexin V staining method (Fig. 4). When these cells were treated for 18 h with curcumin loaded hyalurosomes, the percentage of apoptotic cells, calculated considering cells in both early (Annexin +, PI-) and late apoptosis (Annexin +, PI+) cells, significantly increased from < 10% up to 85% in comparison with both

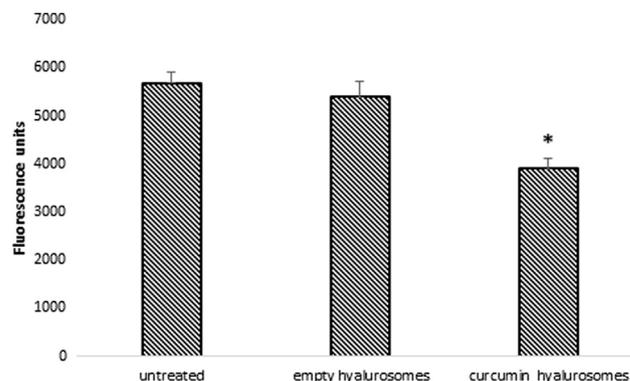


Fig. 3. Viability of rheumatoid arthritis-derived synovial cells untreated (negative control) or treated for 18 h with empty and curcumin loaded 180P90G hyalurosomes. Data were generated with Odyssey infrared platform. Results are expressed as the mean percentage ± standard deviation (error bars) of four individual experiments. * ($p < 0.05$) indicates statistically significant differences compared to untreated cells (negative control).

untreated cells and cells treated with empty hyalurosomes. These results suggested that empty vesicles did not exert beneficial effects on these cells while curcumin loaded hyalurosomes reduced cell viability and stimulated the apoptotic pathway.

Given the lack of activity of empty vesicles, the effect on anti-apoptotic protein expression and anti-inflammatory activity were performed using only curcumin loaded hyalurosomes.

3.6. Expression of anti-apoptotic protein

Considering that inhibitor of apoptosis proteins (IAPs) emerged as important regulators of inflammation signalling and apoptosis, the effect of curcumin on apoptosis proteins was evaluated (Fig. 5). The treatment of rheumatoid arthritis-derived synovial cells with curcumin loaded hyalurosomes downregulated the production of anti-apoptotic IAP1 and IAP2. In particular, the level of IAP1 decreased up to ~0.4 (AU) and that of IAP2 up to ~0.3 (AU). The reduction of both protein production was statistically significant ($p < 0.05$) respect to that obtained for untreated cells (negative control).

3.7. Study of anti-inflammatory effect

Inhibitor of apoptosis proteins play an important role in the production of multiple inflammatory mediators, such as cytokines and chemokines. In particular, the overall effects of an inflammatory response are mainly determined by the equilibrium between pro- and anti-inflammatory mediators, which is imbalanced in the joint of patients affected by rheumatoid arthritis since the patho-physiological process is largely mediated by inflammatory cytokines and other molecules that are able to intensify the catabolic effects. In addition, the

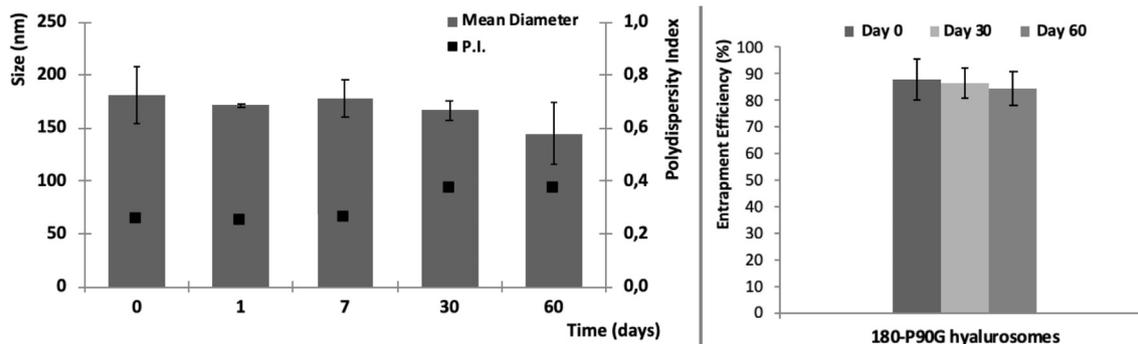


Fig. 2. Size and polydispersity index (A), and entrapment efficiency (B) of curcumin loaded 180P90G hyalurosomes during 60 days on storage at $25 \pm 1^\circ\text{C}$.

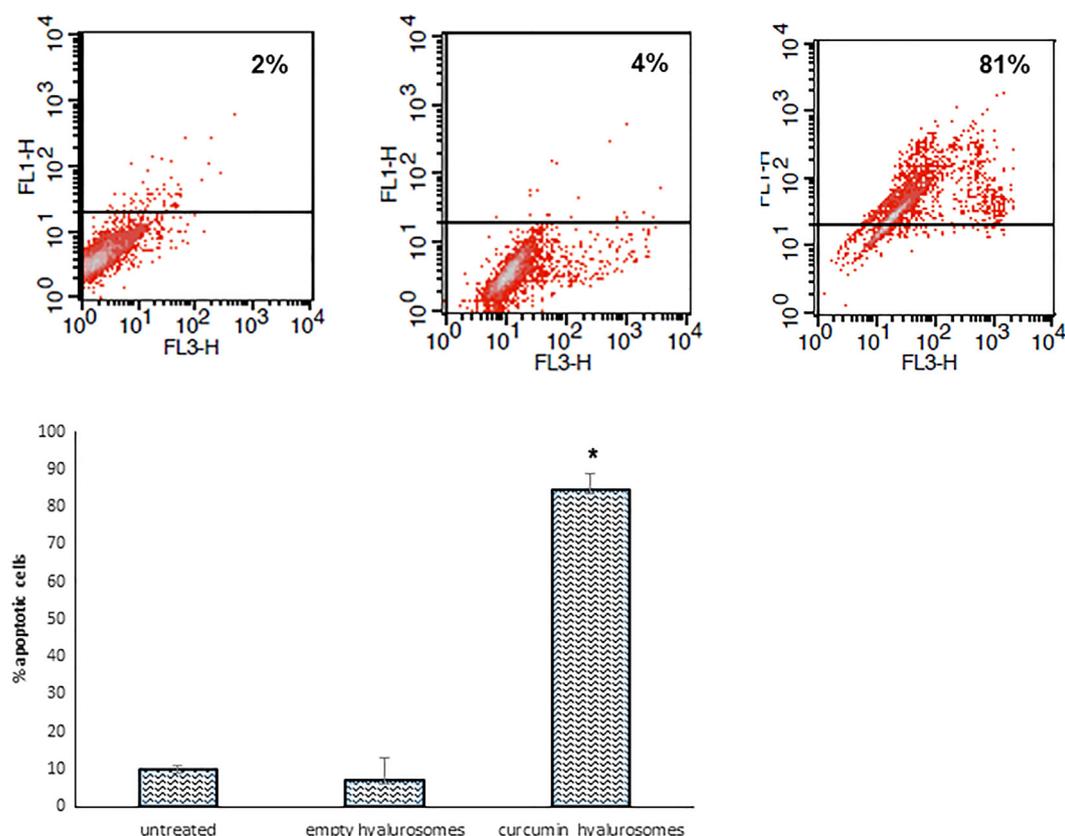


Fig. 4. Values of apoptotic rheumatoid arthritis-derived synovial cells untreated or treated for 18 h with empty and curcumin loaded 180P90G hyalurosomes. Apoptosis was detected with the Annexin V test. (Upper panel) Apoptosis of the untreated cells, treated with empty hyalurosomes and treated with curcumin hyalurosomes. (Lower panel) Percentages of apoptotic cells. Results are expressed as the mean percentage \pm standard deviation (error bars) from five individual experiments. * ($p < 0.05$) indicates statistically significant differences compared to untreated cells.

role of anti-inflammatory cytokines should be considered as it represents the main protection in the joint tissue of rheumatoid arthritis patients. The effect of curcumin loaded 180 P90G hyalurosomes on the activity of pro-inflammatory (IL6, TNF- α , IL15) or anti-inflammatory (IL10) cytokines involved in the pathogenesis of rheumatoid arthritis was evaluated. Actin was used as a loading control (42 kDa; not shown). This treatment induced an effective downregulation of inflammatory cytokines IL6, IL15 and TNF- α , and an upregulation of IL10 (Fig. 6) confirming the positive effect of curcumin 180 P90G loaded hyalurosomes in modulating the inflammatory response.

Results are shown as the ratio of cytokine/actin protein expression \pm standard deviation of four independent experiments. * ($p < 0.05$) indicates statistically significant differences compared to untreated cells (negative control).

3.8. Study of oxidative stress

Another process involved in the pathogenesis of rheumatoid arthritis is the oxidative stress, which has been described as an important cause of destructive proliferative synovitis [34,35]. ROS may be formed in the inflamed joint of patients either by chondrocytes, which are activated macrophages in the synovial membrane, or by activated neutrophils in the synovial cavity [1,8,36]. The treatment of rheumatoid arthritis-derived synovial cells with curcumin loaded hyalurosomes caused a significant reduction in ROS production as compared with the untreated cells, confirming the antioxidant effect of formulation due to the curcumin effect (Fig. 7). In this study untreated cells were used as negative control and cell treated with luperox (600 μ M) were used as positive control.

4. Discussion

In a previous study hyalurosomes demonstrated their optimal performances as carriers for skin delivery of curcumin [18]. In this work, aiming at improving the beneficial effect of curcumin in the treatment of joint of rheumatoid arthritis patients, hyalurosomes were formulated using different kind and amount of phospholipids. The hyalurosomes prepared with 180 mg/ml of P90G were selected as possible carrier for intra-articular region due their small size, homogeneous dispersion and great stability on storage.

Rheumatoid arthritis is characterized by chronic inflammation and hyperplastic growth of synovium that erodes cartilage and bone [5]. Among the inflammatory cell populations that might play a role in rheumatoid arthritis pathogenesis, fibroblast-like synovial cells is a main effector cell type, which initiates the inflammation process through the secretion of diverse inflammatory mediators [37]. Rheumatoid arthritis-derived synovial cells likely play pivotal roles in both the initiation and perpetuation of this pathology since they have been prominently linked to the progressive destruction of articular structures, particularly cartilage [1,34]; moreover, they are the principal cells responsible for hyperplasia because they are resistant to apoptosis, mainly due to upregulation of inhibitor of apoptosis proteins [38]. As previously found, curcumin may inhibit IAP-1 and IAP-2 activities and can sensitize toward TNF- α -induced apoptosis, apparently through suppression of IAP-1/2-dependent receptor-interacting serine/threonine-protein kinase1 (RIPK1) ubiquitination, which is required for canonical NF- κ B activation [39,40]. Furthermore, inhibition of apoptosis proteins influences the production of multiple inflammatory mediators, which are also inhibitors of apoptosis. The treatment of fibroblast-like synovial cells with curcumin loaded hyalurosomes inhibited the

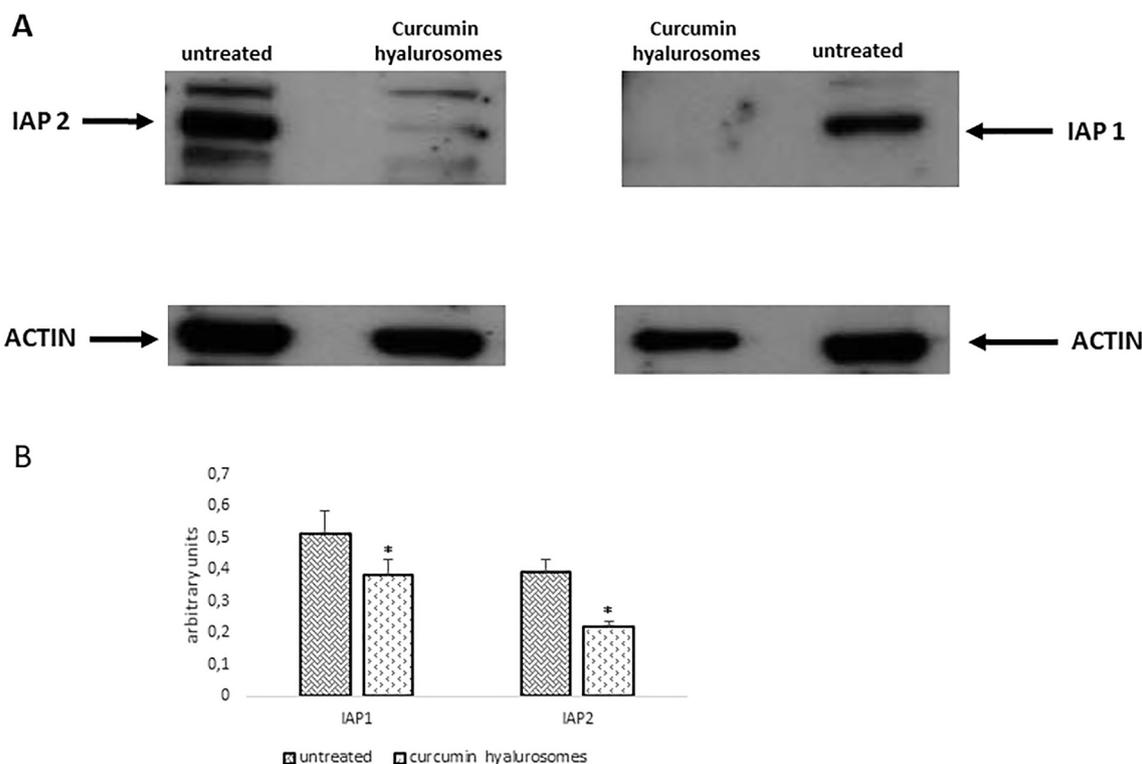


Fig. 5. Levels of inhibition of apoptosis proteins (IAPs) in rheumatoid arthritis-derived synovial cells untreated or treated with curcumin loaded hyalurosomes for 48 h. (A) Immunoblots of the detection of IAP1 (72 kDa) and IAP2 (70 kDa) using β -actin as a loading control (42 kDa). (B) Densitometric analyses of the immunoblots. Results are expressed as the ratio of inhibitor of apoptosis proteins/actin protein expression \pm standard deviation (error bars) of four independent experiments. *($p < 0.05$) indicates statistically significant differences compared to untreated cells (negative control).

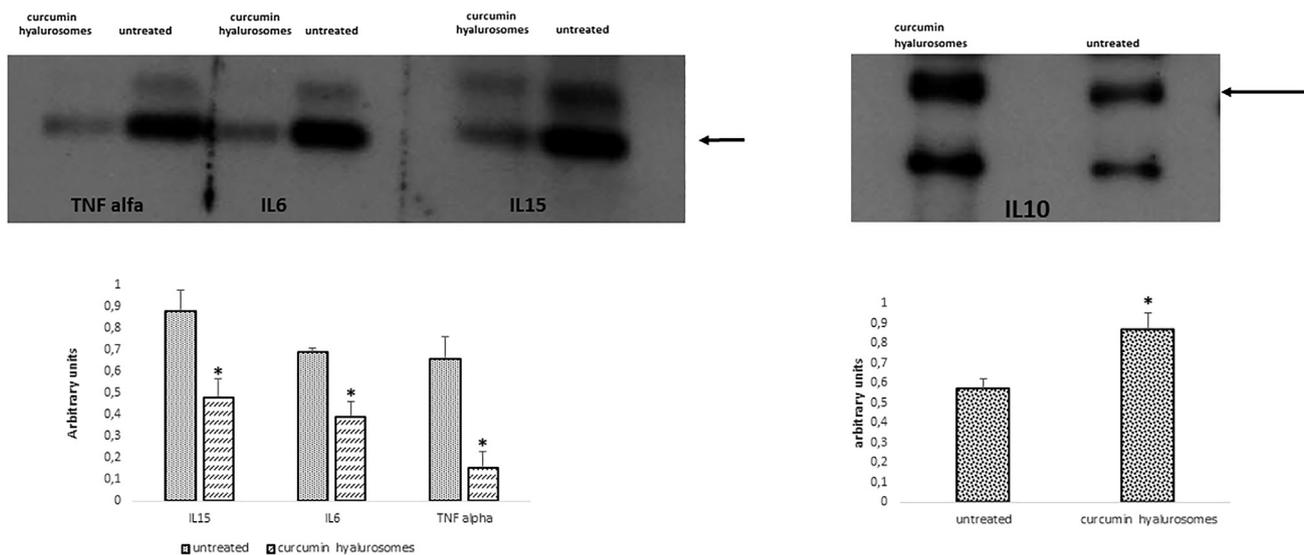


Fig. 6. Cytokine levels measured in rheumatoid arthritis-derived synovial cells untreated or treated with curcumin loaded 180P90G hyalurosomes. (A) Immunoblots showing the detection of cytokines. (B) Densitometric analyses of the immunoblots.

production of IL-15, which is a potent proinflammatory cytokine constitutively expressed on the surface of rheumatoid arthritis-derived synovial cells, which plays a role in the pathogenesis of rheumatoid arthritis. The block of IL-15 secretion is an important goal because it breaks the pro-inflammatory loop and decreases the resistance to apoptosis of fibroblast-like synovial cells [41]. The levels of IL-6 were high in rheumatoid arthritis-derived synovial cells cultured in presence of synovial fluid, which reproduces the microenvironment within inflamed joints, and this is consistent with the hypothesis of the presence of several pro-survival and proliferative factors, secreted by cells

located within the joint [42]. Interestingly, curcumin loaded hyalurosomes were also able to inhibit IL-6 production.

The intense synovial cell hyperplasia and proliferation are both associated with a compensatory anti-inflammatory response characterized by the production of soluble TNF- α receptors, transforming growth factor, IL-1 receptor antagonist, and IL-10. Particularly, IL-10 levels are high in the synovial fluid of patients with rheumatoid arthritis and biologically significant amounts of IL-10 are released in the suspensions of rheumatoid synovial cell cultures [43–45]. Our results demonstrated that curcumin loaded hyalurosomes significantly

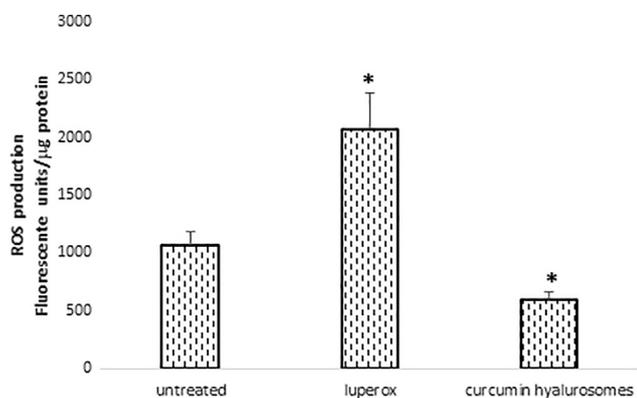


Fig. 7. ROS production detected in rheumatoid arthritis-derived synovial cells untreated or treated with curcumin loaded 180P90G hyalurosomes or luperox (positive control). Results are shown as the mean values of fluorescence units/ μg protein \pm standard deviation of three independent experiments. * ($p < 0.05$) indicates statistically significant differences compared to untreated controls.

increased the production of IL-10, which exerts a dual anti-inflammatory activity, decreasing the secretion of pro-inflammatory cytokines IL-15 and IL-6 and stimulating its own secretion by rheumatoid arthritis-derived synovial cells.

Another key mechanism for the modulatory effects of curcumin loaded hyalurosomes on pro-inflammatory cytokines is the suppression of NF- κ B [46]. Furthermore, the formulation controlled the cytokine-induced oxidative stress and ROS production which are mediators of synovial inflammation, as their excessive production at the site of inflammation contributes to stimulate the inflammatory process. Additionally, ROS may directly be involved in tissue destruction through the activation of extracellular matrix-degrading metallo-proteinases [47]. Inflammatory reaction in the synovium of rheumatoid arthritis patients could be improved by the autocrine or other cytokine-induced production of IL-6 with subsequent generation of ROS in the synoviocytes. In addition, oxidative stress of rheumatoid arthritis synovial tissue can cause DNA damage and suppress the DNA mismatch repair system in cultured synoviocytes [48]. In particular, the overproduction of TNF- α is thought to be the main contributor to increased ROS release in patients with rheumatoid arthritis [49], thus the high ROS levels have been proven to be related to the disease activity/inflammatory-oxidative cascade effects [50]. The control of the systemic inflammation is a therapeutic goal, which not only may guaranty the remission of musculoskeletal symptoms, but also an improvement of the overall health of patient. As curcumin loaded hyalurosomes exert the dual effect of reducing the production of both TNF- α and ROS, thus limiting the joint damage, they may represent a valid local adjuvant for the treatment of rheumatoid arthritis symptoms.

5. Conclusion

A suitable formulation of curcumin loaded in hyaluronan-immobilized phospholipid vesicles called hyalurosomes was prepared for the local treatment of rheumatoid arthritis. The *in vitro* studies using rheumatoid arthritis-derived fibroblast-like synovial cells on synovial fluid underlined the efficacy of curcumin loaded hyalurosomes in stimulating the apoptosis of cells, reducing the oxidative damage in the synovial fluid and the inflammatory response, and improving the production of IL-10. Overall results suggest the potential use of curcumin loaded hyalurosomes in controlling the local cascade effects of rheumatoid arthritis, thus representing a valid alternative for the treatment of this disease.

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