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Curcumin-in-cyclodextrins-in-liposomes: an alternative for osteoarthritis treatment

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Abstract: Osteoarthritis (OA) is one of the most frequent degenerative joint disease characterized by joint pain and stiffness traditionally treated with symptomatic drugs like oral NSAIDs and, in extreme cases, with intra-articular corticoids. However, both these drugs are not exempt from adverse effects. Curcumin (Cur) has proven its anti-inflammatory properties and its potential as anti-osteoarthritic drug. However, its low solubility hinders its usage and limits its therapeutic efficacy. To overcome this issue, drug-in-cyclodextrin double-loaded liposomes (DCL-DL) were developed. These liposomes contained free drug in the lipid bilayer and drug-cyclodextrin complex in the aqueous compartment. The aim of this work was to evaluate the actual effectiveness of Cur-DCL-DL formulations in the OA treatment by intra-articular treatment. For this purpose, the monoiodoacetate (MIA) model of OA pain in rats was used. A single dose of samples containing Cur as DCL-DL, conventional liposomes (SL) and empty liposomes (EL, as control) were injected once intra-articularly. Paw-pressure, beam-balance and incapacitation tests were performed to evaluate OA progression at 7 and 14 days. After ending the assay, animals were sacrificed, and histological evaluation of the ankle-joint tissue was performed. Results showed that DCL-DL significantly reduced pain and ameliorated balance and gait of rats over the 14 days compared to SL. Histological tests showed that DCL-DL had protective properties in some aspects of OA.

Keywords: drug-in-cyclodextrin-in-liposomes; double-loaded liposomes; Curcumin; Osteoarthritis

1. Introduction

Osteoarthritis (OA) is an inflammatory joint disease that affects primarily the elderly. This disease is characterized by progressive degradation of articular cartilage, synovial hyperplasia, osteophyte formation and subchondral bone injury [1].

Currently, OA pharmacotherapy is oriented on pain relief and improving function by means of NSAIDs, COX-inhibitors, weak opioids administrated orally, or hyaluronic acid and glucocorticoids administrated via intra-articular injection [2]. However, except for hyaluronic acid, these drugs have numerous adverse effects. To solve this issue, many researchers propose the use of natural products, such as curcumin.

Curcumin (Cur) is a polyphenol with numerous properties, like anti-inflammatory action. Regarding OA, Cur is able to inhibit IL-1β and TNF-α activation of NF-κB (inactivating multiple pathways of NF-κB activation) and antagonize COX-2 up regulation by IL-1β and TNF-α in chondrocytes [3], inhibit

inflammatory cell proliferation, decrease the expression of IL-1β and TNFα in macrophages [4], etc. However, low water solubility of Cur represents a problem for its pharmacological use. To overcome this issue, a double encapsulation in liposomes was proposed in which Cur is entrapped both as free drug in the lipid compartment and as cyclodextrin complex in the aqueous compartment. This approach (Drug-in cyclodextrin-in liposome double-loaded liposomes, DCL-DL) allows improving Cur release and stability [5].

The aim of the present work was to test the anti-inflammatory properties of Cur when administered directly to the joint (intra-articular injection) in the form of DCL-DL in comparison with single loaded liposomes (SL). For this purpose, DCL-DL, SL and empty liposomes were formulated and characterized. The formulations were tested in vivo by a rat OA model in terms of paw-pressure, incapacitance and beam-balance test. Histological examination was also performed.

2. Experiments

2.1. Materials

Curcumin (Cur), cholesteryl hemisuccinate (Chems) and 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were supplied from Sigma-Aldrich Co (Barcelone, Spain). Hydroxypropyl)-β-cyclodextrin (HPβCD) was a gift of Roquette (Lestrem, France). Monoiodoacetate (MIA) was provided by Sigma-Aldrich (Milan, Italy). Other chemicals were high-quality analytical products. Solvents were HPLC quality.

2.2. Quantification of curcumin

Cur concentration was measured by UV/VIS spectrophotometry, as previously reported in Fernández-Romero et al., 2018 [5]. Cur content was measured by using a Shimadzu 1900 UV-visible spectrophotometer (Shimadzu Italia S.r.l., Milan; Italy). 200-µL of sample was diluted up to 5 mL with a 2% acetonitrile-acetic acid 1:1 v/v mixture, and absorbance was measured at 425 nm. Any interference was observed from other components.

2.3. Liposome preparation

Liposomes were prepared according to Fernández-Romero et al. (2018) protocol [5]. In brief, 0.0125 mmol of CHEMS, 0.064 mmol of DPPC and 0.0027 mmol of Cur were added to a round bottom flask and dissolved in 3.2 mL of methanol and 4.8 mL of chloroform. The mixture was evaporated in a rotary evaporator with a thermostatic bath fixed at 58 °C. The evaporation process was finished when a homogenous film was formed.

For DCL-DL formation, 14.5 mM of HPβCD and 0.4 mM of Cur were dissolved in citric acid-disodium phosphate buffer at pH 5.4. After 72 h of constant stirring, the sample was centrifuged at 1000 rpm 10 min to separate the unbounded Cur. Afterwards, 3 mL of this solution was added to the previously formed film and the system was heated at 58 °C in a thermostatic bath for 5 min and stirred with vortex for 1 min. The process was repeated 5 times.

As the main objective of this work was to test the ability of Cur to ameliorate OA, two control formulations were prepared: one without Cur (EL), and the other single-loaded liposomes (SL). For SL formation, the procedure was similar to that of DCL-DL, but only citric acid-phosphate buffer was added as aqueous solution (Cur concentration 0.4027 mM). Therefore, these liposomes only contained Cur on to the bilayer. For EL formation, Cur was avoided both in the bilayer and the aqueous space without altering the other components. In all cases, formulations were stored at 4 °C.

2.4. Liposome characterization

2.4.1. Liposome size, polydispersity index and zeta potential

Liposome size and polydispersity index (PdI) were measured by dynamic light scattering technique by using Zetasizer Nano-S equipment (Malvern Instrument, Malvern, UK). Size results were expressed as

average liposomal hydrodynamic diameter (nm). PdI values were dimensionless and values below 0.5 were considered indicative of homogenous dispersions.

The Z potential or surface charge of vesicles was measured by correlation spectroscopy from electrophoretic mobility (μ), using the same apparatus mentioned before. Results were expressed as zeta potential (Z, mV) after conversion of μ to Z by Smoluchowsky equation: $Z=\mu\eta/\varepsilon$, were η represents viscosity and ε is the permittivity of the solution. In both cases, samples were prepared equally. 200 μL of samples were diluted with 3.8 mL of citric acid-phosphate buffer.

2.4.2. Encapsulation efficacy

To evaluate the amount of Cur entrapped, the encapsulation efficacy (EE) was measured. An aliquot of each sample was centrifuged at 10000 rpm for 1 h at 4 °C. Afterwards, the supernatant was collected and the pellet was treated with sodium lauryl sulfate followed by three cycles of sonication (10 min) and vortex (1 min). Subsequently, the suspended pellet was analyzed following section 2.2.

2.5. In vivo test

2.5.1. Animals

Male rats (Sprague Dawley, 220-250 g, Envigo, Italy) were housed in cages under controlled conditions (20–24 °C, 50–60% relative humidity, artificial 12-h light–dark cycle). Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. The accommodation was in the Department of Neuroscience, Psychology, Drug Research and Child Health (Florence, Italy), according to European standards as for experimental animals' welfare (European ID-EL 09 BIO 03). The rats were kept at 23 ± 1 °C with a 12-h light–dark cycle, light at 7 a.m., and were allowed *ad libitum* access to tap water and food. All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union Council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the guide for the care and use of laboratory animals of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the animal subjects review board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [6]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.5.2. Osteoarthritis animal model

Unilateral osteoarthritis was induced by injection of MIA into the tibiotarsal joint [7]. Briefly, rats were lightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. 2 mg MIA in 25 μL saline was delivered into the left articular cavity. Control rats were treated with an equal volume of saline.

2.5.3. Treatments

Seven days after MIA or vehicle injection, rats were treated, as was explained in section 2.5.2., with 40 μL of solutions (time 0). Groups were as follow:

Sham group: 25 μL of saline vehicle was injected at time -7 and 40 μL of saline vehicle at time 0.

MIA group: 25 μL of saline vehicle + 2 mg of MIA was injected at time -7 and 40 μL of saline vehicle at time 0.

MIA+ EL group: 25 μL of saline vehicle + 2 mg of MIA was injected at time -7 and 40 μL of empty liposomes (EL) at time 0.

MIA+ SL group: 25 µL of saline vehicle containing 2 mg of MIA was injected at time -7 and 40 µL of single-loaded liposomes (SL) at time 0.

MIA+ DCL-DL group: 25 µL of saline vehicle containing 2 mg of MIA was injected at time -7 and 40 µL of double loaded liposomes (DCL-DL) at time 0.

Paw pressures, beam balance and incapacitance tests were performed 7 and 14 days after Cur intra-articular injection. In addition, histological test was performed.

2.5.4. Paw pressure test

The nociceptive threshold of rats was determined by an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. [1988]. Briefly, a constantly increasing weight was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical weight (expressed in g) was increased until vocalization or withdrawal reflex occurred while rats were lightly restrained. An arbitrary cut-off value of 100 g was adopted [7].

2.5.5. Beam balance test

A rectangular beam (3.2 cm wide, 122 cm long and 63.5 cm tall) was suspended between two tables (105 cm tall for the top of the beam). A black box is placed at the end of the beam as the finish point. Animals were placed perpendicularly on the midpoint of the beam and allowed to traverse the beam for 120 s. A score to the motor abilities of the animal was given: 0, correct gait; 1, clings with the 4 paws; 2, slips with one paw; 3, slips with two paws; falls in a time less than 60 sec [8].

2.5.6. Incapacitance test

Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury [9]. The methodology was described in Di Cesare Mannelli et al (2016) [10]. Data were expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one (Δ Weight).

2.5.7. Histological examination

Animals were killed by cervical dislocation on day 14 after the behavioral measurements. Legs were cut under the knee, flayed and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 48 h at room temperature. Subsequently, samples were decalcified by 0.76 M sodium formate and 1.6 M formic acid solution in H_2O for 4 weeks with a change of solution every 7 days. At the end of decalcification, these samples were routinely dehydrated in alcohol and embedded in paraffin. Sections (6 µM) thick were observed and an histological score (0: absent; 1: mild; 2: moderate; 3: severe) was attributed to the following morphological parameters: (a) inflammatory infiltrate; (b) synovial hyperplasia; (c) fibrin deposition; (d) synovial vascularity; (e) cartilage erosion; (f) bone erosion; (g) joint space [11,12].

2.6. Statistical analysis

All experimental results are given as mean ± S.E.M. Analysis of variance was followed by Fisher's post hoc comparison to verify the significance between two means. Data were analyzed with the StatView software for the Macintosh (1992). P values of less than 0.05 were considered statistically significant.

3. Results

3.1. DCL-DL characterization

Liposomes were characterized in terms of vesicle size, zeta potential and EE. As results showed, SL and DCL-DL were bigger than EL; however, only DCL-DL liposomes showed a significant difference ($p=0.0142$) with the EL system. On the other hand, no statistical significance was found between Z potential of the

different samples. Regarding EE, although SL had a higher percentage of Cur entrapped, there was no statistical significance between DCL-DL and SL.

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

Table 1. Characterization results * p<0.05, **p<0.01, ***p<0.001 compared with empty liposomes (EL).

Liposomes	Particle size ± s.d. (nm)	Z potential ± s.d. (mV)	EE ± s.d. (%)
SL	3259 ± 568	27,2 ± 3,5	94,8 ± 2,8
DCL-DL	4325 ± 462*	31,7 ± 2,8	92,2 ± 3.8
EL	3057 ± 257	24,5 ± 8,3	-

3.2. In vivo test

Table 2 shows the results of the different in vivo tests performed. Paw pressure test measured the force that the animal could stand before vocalizing or moved away from its leg. The more weight the paw was able to handle, less inflamed was the joint [13]. It was clear that MIA+DCL-DL group had a significantly higher pain tolerance when compared with the MIA group, which indicates that DCL-DL were able to reduce joint inflammation. After 7 days of treatment, joint pain reduction was extremely significant in the MIA+DCL-DL group when compared with sham group (p=0.0001), however, on day 14 this difference was reduced, although it was still very significantly different (p=0.0074). Considering MIA+ SL and MIA+ EL groups, results were unexpected. MIA+ SL group showed more pressure tolerance when compared with MIA group at day 7, but this tendency did not continue at day 14. MIA+ EL group, at day 7 showed a lower pressure tolerance when compared with MIA group and highly ameliorated at day 14, resulting in a pressure tolerance very significantly higher than that of OA group at day 14.

Beam balance test studies the ability of the animal to properly walk. As was mentioned before, it is scored 0-4 depending on animal's gait. The higher the score, the worst the gait is MIA+ DCL-DL group showed an extremely significant improvement on walking skills in days 7 and 14 when compared with MIA group. On day 14, animals treated with DCL-DL had the same ability to walk than sham group (p=0.1607). MIA+ EL and MIA+ SL groups did not show significant differences with MIA group except EL group at day 14, which showed significantly better walking abilities than OA group at day 14.

Incapacitance test measured the differences in weight that animals place on their hind limbs. Animals with no limb issues should have minimal differences between both legs. As table 2 depicts, MIA+ EL and MIA+ DCL-DL groups had similar weight differences compared with MIA group. However, MIA+ DCL-DL group showed an extremely significant improvement on it, although, not even at day 14 results were close to those of sham group. Nonetheless, animals treated with DCL-DL ameliorated with time (p=0.0096).

Table 2. In vivo test results. Table shows paw pressure test on treated limb (weight the paw resists before vocalization or movement), beam balance test and incapacitance test (differential weight between non-treated and treated limb measures in grams) *p<0.05, **p<0.01, ***p<0.001 when data were compared with sham group. ^p<0.05, ^^p<0.01, ^^^<0.001 when data were compared with MIA group.

Treatment	Paw pressure test (g)		Beam balance test (0-4)		Incapacitance test (g)
	Day 7	Day 14	Day 7	Day 14	Day 7
Sham	65.4 ± 0.8	67.6 ± 2.1	0.2 ± 0.2	0.3 ± 0.3	5.4 ± 0.6
MIA	41.7 ± 1.7***	46.7 ± 1.7***	3.2 ± 0.4***	2.8 ± 0.3***	51.0 ± 2.6***
MIA+EL	39.2 ± 0.8^	51.7 ± 1.7^^	3.5 ± 0.4	1.9 ± 0.4^	46.8 ± 4.8
MIA+SL	48.3 ± 4.4^	46.7 ± 4.2	2.9 ± 0.3	2.7 ± 1.7	52.3 ± 6.4
MIA+DCL-DL	55.3 ± 1.5^^^	62.6 ± 1.4^^^	1.3 ± 0.3^^^	0.7 ± 0.4^^^	32.5 ± 4.0^^^

After 14 days, animals were sacrificed and histological tests were performed. Results (data not shown) highlighted, as expected, the ability of MIA to trigger OA. All parameters studied were significantly higher when compared with those of the sham group. Regarding MIA+ EL group, no significant differences were found when compared with OA group. MIA+DCL-DL group showed protective properties at least regarding some aspects of OA.

4. Discussion

In the present work, an attempt has been made to elucidate if Cur has anti-inflammatory properties applicable to OA. For this purpose, Cur-in HPβCD-in liposome in a double-loaded system was employed. This formulation was previously developed by our research group [5]; however, major modifications had to be made as the original formula contained didodecyldimethylammonium bromide (DDAB), a cationic lipid that provides the liposome stability over time. Preliminary studies revealed that liposomes with DDAB triggered joint swelling that did not disappear during the experiment (data not shown). On the other hand, positively charged lipids are known for their cell toxicity [14,15] and then, for these reasons, DDAB was removed from the formulations.

Analyzing the physicochemical characteristics, liposomes containing Cur are bigger than those empty. However, no statistical differences were found between SL and DCL-DL, indicating that the presence of Cur in both compartments do not affect the size of the liposomes. Regarding EE, it was higher than expected for DCL-DL. Previous work on this field yielded 52.38% for DCL-DL, which is smaller than EE of DCL-DL in the present study (92.25%), indicating that the absence of DDAB highly improves EE.

The main objective of this paper was to study if the developed innovative Cur liposomal formulation ameliorated OA symptoms. For this, unilateral osteoarthritis was induced in rats by MIA injection on one of their legs. 7 days after injection, OA was established in all cases. Afterwards, a single dose of the different samples was applied, mimicking regular OA treatment with hyaluronic acid or glucocorticoids [2]. Three tests were performed to evaluate animal's walking abilities and inflammation: Randall and Selitto's paw pressure test, beam balance test and incapacitance test.

Randall and Selitto's paw pressure test is based on the premise that inflamed zones have more sensitivity to pain [13]. Thus, when pressure is applied to an inflamed area, the weight that this area can handle is lower than the weight that can handle a non-inflamed area. For comparison purposes, treated and untreated limbs were tested. Non-treated limbs showed similar results in all cases (data not shown). Regarding treated limbs, DCL-DL showed an extremely significant improvement in pressure tolerance, which indicates a marked reduction in joint inflammation. This improvement increases over time, indicating that DCL-DL have better control than SL over Cur release. However, it cannot be said that this system completely eliminates inflammation since there are still significant differences between MIA+ DCL-DL group and sham group at day 14 ($p<0.01$).

To study motor abilities of rats after treatment, a beam balance test was performed. In this case, the ability to walk and balance was measured using different scores. 7 days after treatment, animals treated with DCL-DL exhibited an extremely significant improvement in their walking capacity, mostly grabbing the beam with two paws. On day 14, these animals improved, even more, obtaining scores equal to those of untreated animals. This indicates that DCL-DL reduces inflammation to a point that allows the animal to normally walk.

Incapacitance test is an interesting essay that allows the researcher to evaluate pain. Normally, body weight is equally distributed over both hind legs; however, when one of the paws inflamed, i.e. it is painful to stand on it, the animal naturally compensates by overloading the non-inflamed paw. This compensation was recorded as the difference in weight between them [9,10]. Data showed that only DCL-DL group ameliorated with treatment at days 7 and 14, however, there was still a significant difference with the sham group. This indicates that pain is significantly reduced but has not disappeared.

Surprisingly, SL showed a much lower capability to reduce inflammation compared with DCL-DL. As SL do not contain HPβCD in the aqueous compartment, the amount of Cur able to be released from the

liposomes is inferior to that of DCL-DL [5]. As a result, SL showed only very limited anti-inflammatory properties, evidencing the critical role of an effective drug formulation to fully exploit its therapeutic action.

5. Conclusions

DCL-DL is a novel approach that has been recently developed for allowing Cur intra-articular administration. However, the presence of DDAB in the liposome bilayer of such formulation hinders its use in vivo, due to its irritating action and potential cell toxicity. In the present work, DDAB was removed from the liposomal formulation and the new DCL-DL system ability to ameliorate OA was tested in vivo in a MIA rat model in comparison with a conventional single-loaded Cur liposomal formulation (SL).

From the results obtained, we can conclude that DCL-DL successfully reduced pain and inflammation in OA joint with a prolonged action up to 14 days from administration, differently from SL that exhibited only minimal effects in inflammation reduction and walking ability improvement of the animals.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:
Cur: Curcumin
DCL-DL: Drug in Cyclodextrin-in-liposome double-loaded liposomes
SL: Single-loaded liposomes
EL: Empty liposomes
DDAB: Didodecyldimetilammonium bromide
HPβCD: Hydroxypropyl-β-cyclodextrin
OA: Osteoarthritis
MIA: Monoiodoacetate

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