



# Oral delivery of oleuropein-loaded lipid nanocarriers alleviates inflammation and oxidative stress in acute colitis

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## ABSTRACT

Inflammation and oxidative stress pathways have emerged as novel targets in the management of inflammatory bowel diseases (IBD). Targeting the drug to the inflamed colon remains a challenge. Nanostructured lipid carriers (NLCs) have been reported to accumulate in inflamed colonic mucosa. The antioxidant/antiinflammatory polyphenol oleuropein (OLE) was loaded in NLCs (NLC-OLE). NLC-OLE showed to be more effective in decreasing the TNF- $\alpha$  secretion and intracellular reactive oxygen species (ROS) by activated macrophages (J774) compared to the conventional form of OLE. OLE efficacy was preserved within NLC-OLE ameliorating inflammation in a murine model of acute colitis: reduced levels of TNF- $\alpha$  and IL-6, decreased neutrophil infiltration and improved histopathology of the colon were reported. In addition, NLC-OLE enhanced the ROS scavenging activity of OLE in the colon after oral administration. These data suggest that the proposed NLC-OLE could be a promising drug delivery system for OLE in IBD treatment.

## 1. Introduction

Inflammatory bowel diseases (IBD) are multifactorial immune mediated gastrointestinal disorders. Clinically, they are characterized by chronic but relapsing diarrhoea and rectal bleeding which are usually accompanied by weight loss and abdominal pain. The main idiopathic forms involved in IBD are ulcerative colitis (UC) and Crohn's disease (CD). Whilst inflamed lesions in CD could be widespread to any part of the gastrointestinal tract (GIT), UC is limited to the colon (Kasser et al., 2010).

Current treatment for IBD aims to block the inflammatory pathway with the administration of corticosteroids, sulfasalazine, immunosuppressants and biological drugs (i.e. anti-TNF $\alpha$  or monoclonal antibodies). However, their unspecific targeting along with their undesirable side effects and the high daily doses needed, makes the search for new alternatives an urgent necessity (Stallmach et al., 2010; Yang

and Lichtenstein, 2002; Rogler, 2010).

How are IBD originated still remains unclear but recently, redox impairment has gained special attention as one of the potential etiological factors for IBD development. The GIT is one of the main sites in the organism for the presence of prooxidant molecules. Clinical studies have shown that IBD patients have reduced antioxidant capacity even in the remission phase of the disease. Furthermore, inflammation is known to be caused, in part, by free radicals and thus, their increased amount in IBD together with the pro-inflammatory cytokines (i.e. TNF- $\alpha$ ) have strong repercussion on the establishment/pathogenesis and progression of these diseases (Moura et al., 2015). Given this scenario, it has been recently proposed that treating with antioxidants with additional anti-inflammatory effect might be a promising strategy for designing new therapies. A growing evidence support the use of polyphenolic compounds (i.e. curcumin, oleuropein, ellagic acid) as natural preventive treatments of IBD to relief intestinal inflammation (Beloqui

**Abbreviations:** CD, Crohn's disease; DCFH-DA, 2,7-dichlorofluorescein diacetate; DSS, dextran sulfate sodium; EE, encapsulation efficiency; GIT, gastrointestinal tract; HPLC, high-performance liquid chromatography; IBD, inflammatory bowel diseases; Interleukin-6, IL-6; MPO, myeloperoxidase; NLC, nanostructured lipid carrier; OLE, oleuropein; PDI, polydispersity index; ROS, reactive oxygen species; TEM, transmission electron microscope; TNF- $\alpha$ , tumor necrosis factor-alpha, UC, ulcerative colitis

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et al., 2014; Beloqui et al., 2016; Zatorski et al., 2015; Saldanha et al., 2019; Marín et al., 2013).

Oleuropein (OLE) is one of the most bountiful phenolic acids in olive leaves (*Olea europaea* L). Recently, the European Medicines Agency (EMA) has emitted its own assessment report about the health-promoting properties of this molecule in human health (EMA, 2017). It is widely known for its potent antioxidant and anti-inflammatory activities which seem to be the cornerstone for its multiple pharmacological activities: antiviral, anticancer, antimicrobial, neuroprotective and gastroprotective, among others (Marković et al., 2019; Cavaca et al., 2020).

Whilst a number of encouraging preclinical data have revealed the influence of polyphenols on intestinal inflammation as plant extracts or pure compounds, scarce studies are available concerning isolated oleuropein. As for IBD, particularly OLE has shown to be effective in acute, chronic and colon-cancer associated colitis (Giner et al., 2016, 2013, 2011). Nevertheless, OLE is limited by its poor stability not only against external factors (light, oxygen) but also across the human organism (pH, enzymes). Several studies suggest that OLE suffers a complex biotransformation process during gastric digestion (Corona et al., 2006; González et al., 2019; Lin et al., 2013). Because of this, the efficacy results retrieved from the oral administration of OLE might be lower than those expected and high doses have been used to date for *in vivo* studies.

In this context, nanotechnology has emerged as a promising approach towards IBD treatment and/or prevention. Nanoscale drug delivery systems (DDS) can be specifically designed to accumulate in the inflamed areas of the bowel. The disrupted intestinal barrier along with the overproduction of mucus and the higher amount of immune-related cells can result in a greater accumulation of the nanoparticles in the inflamed colonic tissues as against the healthy ones (Beloqui et al., 2016; Schneider et al., 2010; Collnot et al., 2012; Lamprecht et al., 2001; Youshia and Lamprecht, 2016). Among the existing nano-sized DDS, nanostructured lipid carriers (NLCs) offer special advantages: sustained release, good biocompatibility and biodegradable properties. Moreover, the lipid excipients used for NLC formulation have documented biological anti-inflammatory effects in IBD, (Beloqui et al., 2016; Schneider et al., 2010).

All in all, an optimal and colon-targeted NLC could enhance OLE efficacy at ameliorating inflammatory and oxidative stress symptoms in IBD by protecting the molecule inside the lipid core until its release on the specific site, as well as synergistically improving its anti-inflammatory activity thanks to the lipid excipients.

On this basis, this work aimed to analyze the behavior and efficacy of the anti-inflammatory and antioxidant compound oleuropein (OLE) encapsulated in olive oil-based NLCs, in terms of their potential efficacy for transport OLE to the specific site and preserve its efficacy in IBD. With this purpose OLE-loaded NLCs were evaluated (i) in accordance with their physicochemical characteristics; (ii) *in vitro* by assessing their ability to reduce TNF- $\alpha$  production and intracellular ROS levels from, respectively, LPS-activated and H<sub>2</sub>O<sub>2</sub>-stimulated J774 macrophages; and lastly (iii) *in vivo*, by evaluating the neutrophil infiltration, cytokines concentration (TNF- $\alpha$ , IL-6), ROS concentration as well as by histologically and macroscopically assessing the severity of inflammation in dextran sodium sulfate (DSS)-induced acute colitis mice model.

## 2. Materials and methods

Sources for materials and some methods are shown in the [Supplementary material](#).

### 2.1. Preparation of the formulations

#### 2.1.1. Nanostructured lipid carriers

NLC-OLE were elaborated through the hot melt emulsification method followed by ultrasonication (Huguet-Casquero et al., 2020b,

2020a). Briefly, Precirol ATO® 5 (0.66 g) and Olive oil (0.44 g) were melted at 75 °C to obtain a clear and homogeneous oil phase. Oleuropein (OLE) was uniformly dispersed in deionized water. For aqueous surfactant phase preparation, Poloxamer 188 (0.66%) (w/v) and Tween 80 (1.3%) (w/v) were dispersed in deionized water (15 ml). Prior to the emulsification step, OLE solution and aqueous phase were heated to the same temperature (75 °C) as the lipid phase. Next, OLE solution was added to the lipid phase and immediately after the aqueous phase was added too. The mixture was sonicated for 30 s at 50 W (Branson Sonifier 250, Danbury, CT, USA) and gently shaken by magnetic stirring for 10 min at room temperature. Then, the obtained nanoemulsion was stored for 2 h at 4 °C to give rise to the lipid re-crystallization and the resulting NLC formation. Afterwards, nanoparticles were centrifuged (2500 rpm, 10 min) and washed three times in deionized water using 100-kDa cut-off centrifugal filter units (Amicon, “Ultracel-100 k”, Merck, Millipore, Spain). Blank nanoparticles were prepared as aforementioned without OLE (NLC-Blank).

Finally, a cryoprotectant aqueous solution (trehalose (15% w/w)) was added the resultant NLCs and were subjected to lyophilization for 36 h (Telstar Lyobeta freeze-dryer, Terrassa, Spain).

#### 2.1.2. OLE suspension

An OLE suspension (OLEsus) was prepared as a control for *in vitro* and *in vivo* experiments. For this purpose, OLE was dispersed in deionized water to a concentration of 67 mg/ml.

### 2.2. In vitro cell experiments

#### 2.2.1. Cell culture

J774 murine macrophages were maintained in RPMI 1640 media supplemented with 1% (v/v) PEST and 10% (v/v) FBS at 37 °C in a 5% CO<sub>2</sub> / 95% (v/v) air atmosphere. 75 cm<sup>2</sup> flasks (Corning, Lowell, MA, USA) were used for cell growth.

#### 2.2.2. Cell viability studies

CCK-8 method was used for assessing the cytotoxicity of the formulations in murine macrophages. Briefly, cells were seeded in 96-well microtiter plates (Nunc, Roskilde, DK) and their density was set to 20,000 cells/well. After an incubation period of 24 h (37 °C, 5% CO<sub>2</sub>) cells were treated for 4 h with OLEsus, NLC-OLE and the equivalent unloaded formulation (NLC-Blank) which were diluted in 100  $\mu$ l of cell culture medium, at increasing concentrations (from 7.8 to 500  $\mu$ g/ml of OLE concentration). Dimethyl sulfoxide (DMSO) was used as negative control (dead cells) and fresh medium as positive control (cells alive). The CCK-8 assay was then conducted as previously described by our group (Pastor et al., 2014). The spectrophotometric absorbance was measured at 450 nm/630 nm wavelength (MultiSkan Ex plate reader, Thermo Fisher Scientific, Waltham, MA, USA) and the IC<sub>50</sub> values were calculated. All CCK-8 tests were conducted in triplicate.

#### 2.2.3. Study of cellular antiinflammatory effects

J774 cells were seeded in 24-well plates (200,000 cells/well) and left to adhere for 24 h. Murine macrophages were then treated for 4 h with the studied formulations. Based on the results from the cell viability studies, the following concentrations for each formulation were prepared in 1 ml of culture medium: (i) OLEsus (0.15 mg/ml) (ii) NLC-OLE (0.15 mg/ml of OLE) and the equivalent amount of unloaded nanoformulation. The cell-free supernatants were then collected and macrophages were stimulated with fresh medium containing 0.1  $\mu$ g/ml of LPS for 24 h (Fernandes et al., 2012; Kitamura et al., 2008). Supernatants were subsequently gathered and stored at -20 °C until later assessment. TNF- $\alpha$  levels were quantified using a Mouse TNF- $\alpha$  uncoated ELISA Kit (Thermofisher, Invitrogen Corporation; Paisley, UK) following the manufacturer's instructions.

### 2.2.4. Study of cellular antioxidant effects

Intracellular levels of reactive oxygen species (ROS) were measured using the 2,7-dichlorofluorescein diacetate (DCFH-DA) reagent (Aranda et al., 2013). J774 cells were seeded at 25,000 cells/well in dark, clear-bottom 96-well microtiter plates and left to adhere for 24 h. Cells were then loaded with 200  $\mu$ l of 1 mM solution of DCFH-DA for 40 min at 37 °C. Then, cells were washed once with DPBS and exposed for 4 h to 100  $\mu$ l of OLEsus, NLC-OLE and NLC-Blank formulations dispersed in culture medium. The same non-cytotoxic concentrations used in Section 2.2.3. were selected for this study. Afterwards, cells were washed once and 100  $\mu$ l of a 0.03% hydrogen peroxide solution ( $H_2O_2$ , pro-oxidant agent) in DPBS was added to each well. The microplate was incubated for 30 min at 37 °C.

Fluorescence intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Spectrophotometer SpectraMax M2e & program SoftMax Pro, Molecular Devices, LLC, USA). Data was reported as the intracellular percentage of ROS compared with oxidized control cells ( $H_2O_2$  activated untreated cells). All assays were performed in triplicate, each comprising six replicates of each sample tested.

### 2.3. In vivo efficacy study against acute ulcerative colitis

Animal care and experimental protocols were in accordance with the *Université catholique de Louvain* animal committee (2018/UCL/MD/45). 8-week old male C57BL/6 mice (Janvier Laboratories, FR) weighing 21–26 g were housed under standard conditions, fed with standard laboratory food and supplied with tap water *ad libitum*. Acute colitis was established by the addition of DSS (3% (w/v)) in mice drinking water for the first 5 consecutive days of the study, and colonic symptoms were evaluated 7 days after the beginning of DSS treatment (Wirtz et al., 2017). Animals were randomly separated into six groups (8 mice per group), namely: untreated control group (healthy mice), control DSS group, OLEsus-treated DSS group (0.5 g of OLE/kg), NLC-OLE-treated DSS group (1.7 g of NLC-OLE/kg) and NLC-Blank-treated DSS group (1.2 g of NLC-Blank/kg). NLC-OLE group received the same amount of OLE as the OLEsus group. NLC-Blank group received the same amount of NLC excipients as NLC-OLE group. Formulations were administered by oral gavage during the first five days of DSS-colitis induction. Control groups received deionized autoclaved water given by oral gavage instead of the formulations. Mice were fasted 12 h prior to the administration of the treatments. All animals were sacrificed on day 7 by cervical dislocation. Colons were excised and properly stored for later evaluation.

#### 2.3.1. Clinical disease activity scoring

Severity of colitis was assessed by evaluating weight loss, stool consistency and rectal bleeding as previously described (Beloqui et al., 2016; Melgar et al., 2005). Recorded results were scored on a 0–3 scale as shown in Table 1.

#### 2.3.2. Colon weight/colon length ratio

After 7 days of colitis induction, mice were sacrificed and the luminal contents of the collected colons were rinsed with PBS. Then, colons were weighted and measured to further calculate the weight/

length ratio which is considered a sensitive and reliable marker of the extent and severity grade of the colitis-associated inflammatory status (Alhouayek et al., 2011).

#### 2.3.3. Histological appearance study

Small pieces of the colons (distal regions) from each study group were fixed in 4% buffered paraformaldehyde overnight, washed with ethanol (70% v/v) and embedded in paraffin. For each mouse, two sets of 3 serial sections with a thickness of 10  $\mu$ m were cut 100  $\mu$ m apart, and stained with hematoxylin-eosin for detailed histological study of colonic inflammation.

#### 2.3.4. Myeloperoxidase (MPO) assay in colon tissue

MPO is commonly used as an indicator for neutrophil infiltration which, at the same time, is correlated with the severity of colonic inflammation in UC (Krawisz et al., 1984). Briefly, at the time of necropsy, proximal portions of the colons (~25 mg) were placed on Eppendorf safe-lock tubes, snap frozen in liquid  $N_2$  and stored at  $-80$  °C. For MPO activity investigation, tissue samples were gently homogenized on ice with HTAB buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6). Then, resulting samples were ultracentrifuged at 5000 rpm for 20 min at 4 °C (Eppendorf Centrifuge 5804R) to yield a supernatant fraction of around 300  $\mu$ l. An aliquot of the obtained supernatants (7  $\mu$ l) was placed on 96-well plates (Nunc, Roskilde, DK) along with 200  $\mu$ l of a 50 mM potassium buffer solution supplemented with 0.167 mg/ml of O-dianisidine hydrochloride and 1% (v/v) of hydrogen peroxide. Samples were analyzed in duplicate. Absorbance was measured spectrophotometrically at 460 nm for 30 min (Spectrophotometer SpectraMax M2e & program SoftMax Pro, Molecular Devices, LLC, USA). Resulting data were expressed as MPO units per gram of total protein of sample. The bicinchoninic acid assay (BCA) method was used for protein quantitation in each supernatant sample (ThermoFischer, Pierce™ Biotechnology; Illinois, USA). One unit of MPO activity was defined as the amount needed the degradation of 1 mmol/min of hydrogen peroxide ( $H_2O_2$ ) at 25 °C (Alhouayek et al., 2011).

#### 2.3.5. Determination of inflammatory cytokine levels in colon tissue

The colonic concentration of cytokines (TNF- $\alpha$  and IL-6) was quantified by the sandwich-type ELISA technique following the manufacturer's instructions for the Mouse TNF- $\alpha$  uncoated and IL-6 mouse ELISA kits (ThermoFischer, Invitrogen Corporation; Paisley, UK). Frozen colonic tissue portions of each mice group were vigorously homogenized in 500  $\mu$ l of extraction buffer (DPBS with 1% SDS and 1 tablet of complete protease inhibitor cocktail (Roche Diagnostics, Vilvoorde, BE) per 10 ml of solution) by means of an UltraTurrax (IKA T18 Basic, Staufen, Germany) and ultracentrifuged for 20 min at 5000 rpm at 4 °C. Obtained supernatants were stored at  $-80$  °C until cytokine analysis. Data were normalized with the total protein concentration (BCA method) of each sample.

#### 2.3.6. Determination of ROS levels in colon tissue

The tissue-associated ROS were analyzed to assess the degree of oxidative stress which correlates with the severity of colitis (Moura et al., 2015; Mosińska et al., 2017). Briefly, at the time of necropsy, colon specimens from the central region were snap frozen in liquid  $N_2$  and stored ( $-80$  °C) until further assessment. For ROS quantitation, colon samples were placed in 1000  $\mu$ l TRIS-HCl buffer (50 mM, pH 7.4) on ice and gently homogenized with an UltraTurrax. The homogenate was ultracentrifuged at 5000 rpm for 15 min at 4 °C. A supernatant aliquot (50  $\mu$ l) was added to 96-well black plates (Fluotrac™, Greiner, GE) along with 50  $\mu$ l of a prepared DCFH-DA (10  $\mu$ M) solution and left to react for 30 min at 37 °C, under shaking and dark conditions. The autofluorescence of each tissue homogenate was also assayed and subtracted from the results. Excitation and emission wavelengths were set to 485 nm and 535 nm, respectively, for measuring the fluorescence intensity with a plate reader (Spectrophotometer SpectraMax M2e &

**Table 1**

Assessment of inflammation by means of clinical and macroscopic score.

Score	Weight loss score	Consistency score	Bleeding score
0	None	Normal pellets	None
1	1–5%	Slightly loose faeces	Slightly bloody
2	6–10%	Loose faeces	Bloody
3	11–18%	Watery diarrhoea	Blood in the whole colon

Score reflects degree of weight loss, diarrhea and fecal bleeding score on the day of necropsy and is characterized on a scale of 0–3.

program SoftMax Pro, Molecular Devices, LLC, USA). Data from triplicate assays were expressed as fluorescent intensity units (FU) per mg of total protein for each sample. Protein concentration in homogenates was quantified by the BCA method.

#### 2.4. Statistical analysis

The results are expressed as the mean  $\pm$  standard error of the mean (SEM) unless otherwise stated. GraphPad Prism 6.0 program (GraphPad Software Inc., San Diego, CA, USA) was employed for statistical analyses. Differences were considered statistically significant when  $*p < 0.05$ . The Shapiro-Wilk normality test was applied for normal distribution assessment. For those studies in which normality and homogeneity were confirmed, a one-way ANOVA test of multiple comparisons followed by Bonferroni's post-hoc test was performed. All other analyses were conducted using Student's *t*-test or a Mann-Whitney non-parametric test.

### 3. Results

#### 3.1. Physicochemical properties of NLCs

The physicochemical behavior of NLC-OLE and NLC-Blank is summarized in Fig. 1C. The size was found to be  $\sim 150$  nm and both formulations presented a negative zeta potential. OLE was almost fully encapsulated in the NLCs. A round-shape of the nanoparticles was confirmed by TEM micrographs (Fig. 1A). Previous *in vitro* release studies conducted at pH 7.4 and 37 °C, demonstrated that about 80% of OLE was released in a sustained manner within 27 h (Huguet-Casquero et al., 2020).

#### 3.2. *In vitro* cell experiments in murine J774 macrophages

##### 3.2.1. Cytotoxicity evaluation

The CCK-8 test (Fig. 2A) was carried out to study the possible toxicity of the formulations and thereafter, select the appropriate concentration for antiinflammatory and oxidative stress studies in J774 macrophages. The  $IC_{50}$  of OLEsus was 200  $\mu$ g/ml whereas NLC-OLE showed an  $IC_{50}$  of 680  $\mu$ g/ml. NLC-Blank did not show any cytotoxic effect in the concentration ranges studied (up to 1200  $\mu$ g/ml) demonstrating the safety of the nanocarrier matrix. Therefore, 150  $\mu$ g/ml of

OLEsus and its equivalent amount in NLC-OLE were selected for the forthcoming studies.

##### 3.2.2. *In vitro* antiinflammatory activity evaluation

The ability of the formulations to suppress TNF- $\alpha$  production from J774 cells was assessed by comparing NLC-OLE with OLEsus and NLC-Blank (Fig. 2B). OLEsus did not exert any inhibitory effect in comparison with the control-(+) (LPS stimulated non-treated cells). By contrast, NLC-OLE and NLC-Blank significantly reduced the secretion of TNF- $\alpha$  from the cells under the same studied conditions ( $*p < 0.05$ ). Moreover, both nanoformulations exerted a significantly higher effect when compared to OLEsus ( $^{##}p < 0.01$ ) under the same conditions, but no difference was shown between the nanocarriers.

##### 3.2.3. *In vitro* antioxidant activity evaluation

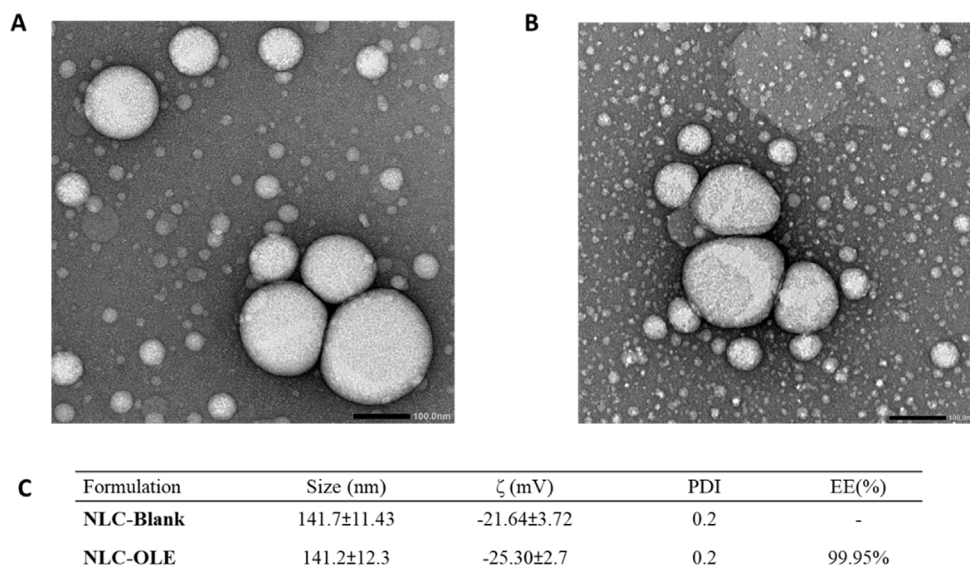
The inhibition of intracellular ROS production by  $H_2O_2$ -stressed murine macrophages was studied by comparing NLC-OLE with NLC-Blank and OLEsus (Fig. 2C). Only NLC-OLE was able to significantly scavenge the generated ROS compared to the control ( $**p < 0.01$ ), enhancing the activity of OLE when encapsulated within NLCs compared to OLEsus ( $^{##}p < 0.01$ ).

#### 3.3. *In vivo* efficacy studies against acute colitis

##### 3.3.1. Histological appearance evaluation and clinical activity determination

The clinical activity score (Fig. 3) and the histological assessment results (Fig. 4) proved the expected effects from the chemical induction of acute colitis in the DSS-groups compared to the untreated control mice (healthy). No significant differences were found between DSS colitis induced groups. The same result was observed regarding the colon weight/length ratio (data not shown).

Regarding histopathology, as shown in Fig. 4A, disrupted mucosa architecture, high leukocyte infiltration as well as submucosal edema were observed in the control-DSS group. For OLEsus and NLC-OLE treated DSS groups (Fig. 4B and D respectively) the colon structure was found to be similar to the healthy untreated control group (Fig. 4C). Compared to the other treatments, NLC-Blank treated DSS group showed a higher infiltration of immune cells (Fig. 4E) but the architecture of the crypts was well preserved in comparison with the control DSS group.



**Fig. 1.** Summary of NLCs characterization: (A) TEM micrographs of NLC-Blank, (B) TEM micrographs of NLC-OLE, and (C) NLCs particle size, zeta potential ( $\zeta$ ), polydispersity index (PDI) and encapsulation efficiency (EE) of each formulation ( $n = 3$ ; data are expressed as the mean  $\pm$  SD).



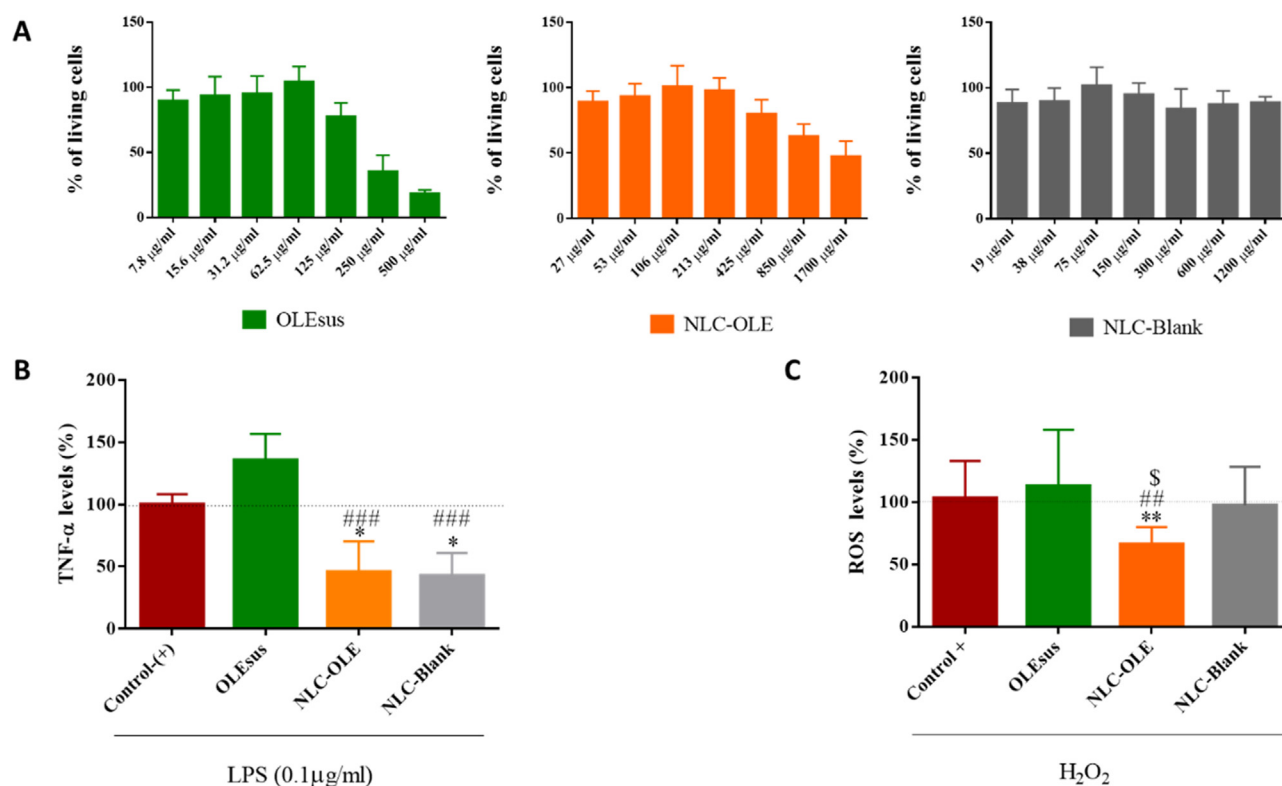


Fig. 2. Summary of the *in vitro* studies in J774 macrophages. (A) Cytotoxicity results, (B) TNF- $\alpha$  quantitation in LPS-activated macrophages and (C) Intracellular ROS quantitation in H<sub>2</sub>O<sub>2</sub>-stressed macrophages of OLEsus, NLC-OLE and NLC-Blank. Data are presented as the mean  $\pm$  SD (\*  $p$  < 0.05 vs Control+. #  $p$  < 0.05 vs OLEsus. \$  $p$  < 0.05 vs NLC-Blank).

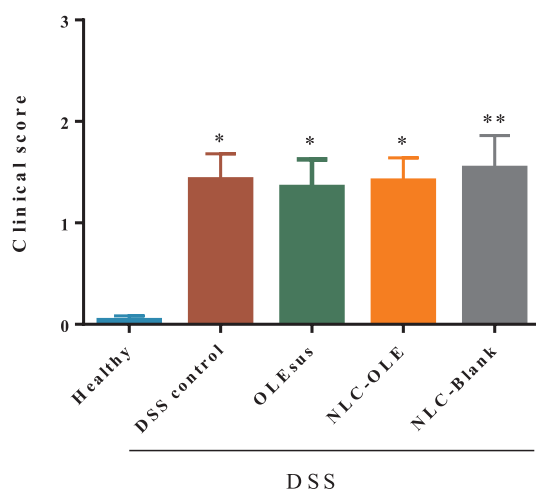


Fig. 3. Clinical activity score after *in vivo* assessment. (n = 8 mice per group).

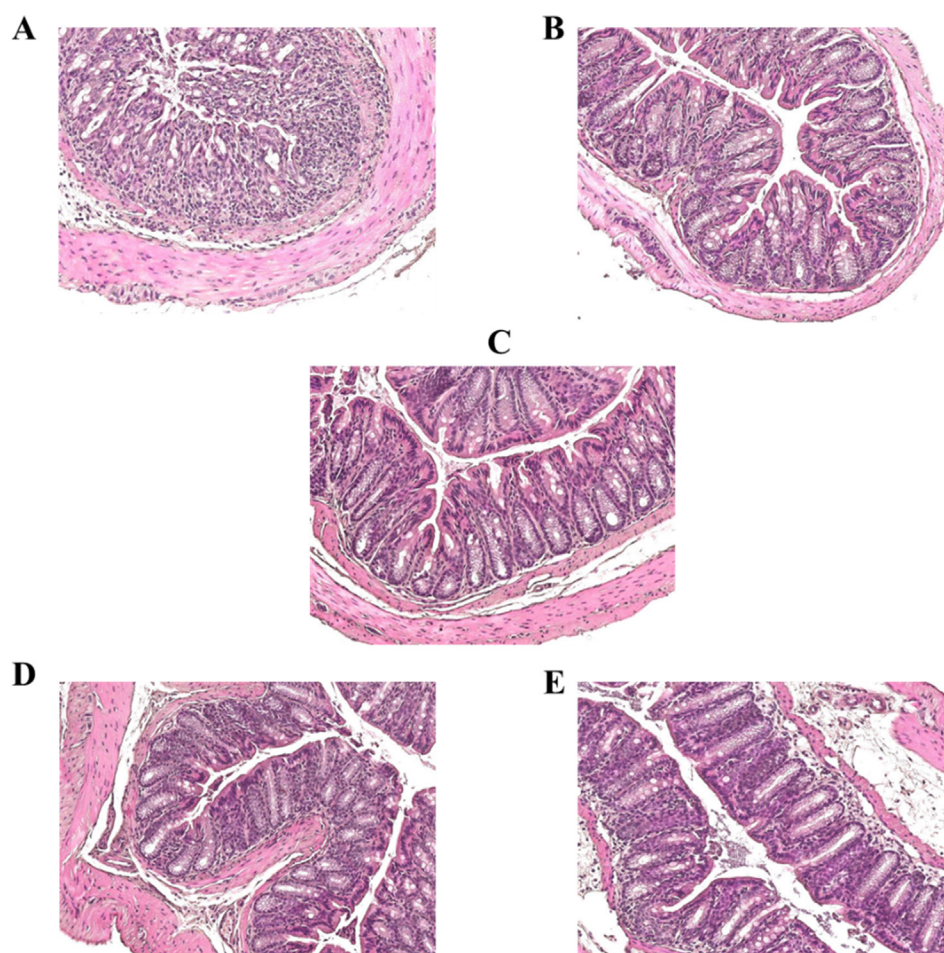
### 3.3.2. Inflammatory and oxidative stress assessment in colon samples

MPO activity, the concentration of the pro-inflammatory cytokine TNF- $\alpha$ , the pleiotropic cytokine IL-6 and total ROS content in the colon are summarized in Fig. 5. In all cases, control-DSS group showed a significant increase in the measured parameters compared to the healthy group (data not shown) and thus confirming the colitis-induction. It is worth to note that MPO activity, TNF- $\alpha$  and IL-6 concentration for the OLEsus and NLC-OLE-treated DSS groups were decreased when compared with DSS group. However, no significant differences were found between OLEsus and NLC-OLE-treated DSS groups. No significant differences were observed in NLC-Blank treated DSS group. Interestingly, only NLC-OLE treatment was able to attenuate oxidative stress by significantly decreasing total ROS generation (\*  $p$  < 0.05)

compared to DSS control, whereas OLEsus and NLC-Blank did not show any significant effect.

## 4. Discussion

ROS mediated oxidative stress together with inflammatory pathways have gained special interest as targets for new drug studies in UC (Moura et al., 2015; Zhu and Li, 2012). OLE, the major polyphenol in olive leaves, is known to inhibit the inflammatory response through several pathways, such as suppressing the secretion of an array of cytokines (i.e. IL-1 $\beta$ , IL-17, TNF- $\alpha$ ) probably, in part, due to its interaction with the nuclear factor (NF)- $\kappa$ B which, indeed, plays an important role in IBD. Additionally, this bioactive compound has shown ROS scavenging ability, although its precise mechanism of action still remains unclear (Marković et al., 2019; Cavaca et al., 2020). Altogether, OLE is a good candidate for IBD management and some authors have revealed its effectiveness in both acute and chronic UC (Giner et al., 2016, 2013, 2011). However, in these studies high doses of OLE were needed to be administered within the food for OLE to be effective. The reason for this could be that OLE, as well as the majority of the anti-inflammatory and antioxidant agents, presents several limiting factors. Particularly, it presents low absorption rates, poor stability after light and oxygen exposure, as well as rapid biotransformation upon gastric digestion and thus, its bioactivity, bioaccessibility and bioavailability seem to be compromised (Corona et al., 2006; González et al., 2019; Lin et al., 2013; Edgecombe et al., 2000). Therefore, targeting the inflamed colonic mucosa with OLE is still a challenge. In this sense, lipid-based nanotechnology seems a promising strategy to overcome these limitations. Among all nanoscale drug delivery systems, NLCs can offer a greater drug loading capacity, an increased retention in the inflamed target site and biological anti-inflammatory properties (Beloqui et al., 2014; Beloqui et al., 2016; Schneider et al., 2010; Youshia and Lamprecht, 2016; Beloqui et al., 2013; Zai et al., 2018). Hence, we



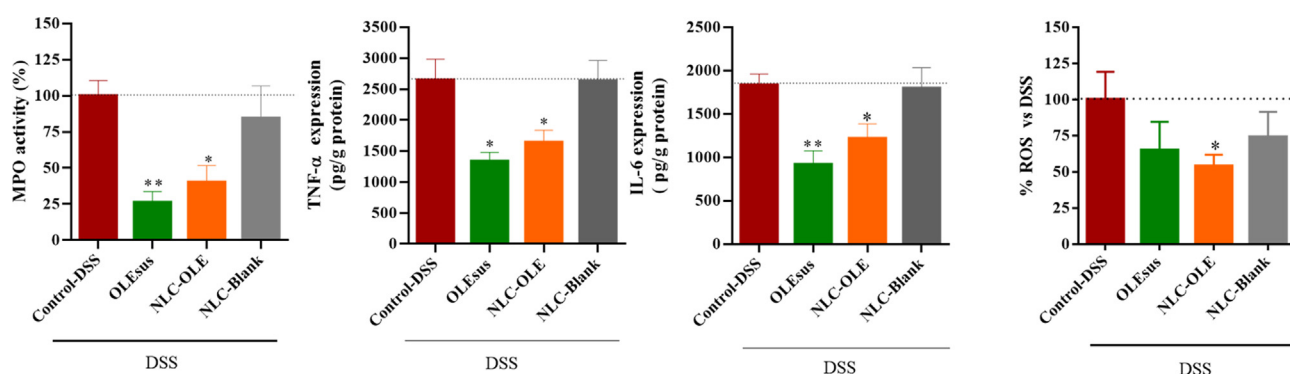
**Fig. 4.** Representative figures of histopathology of the colon tissue after hematoxylin-eosin staining from Control DSS group (A), OLEsus-treated DSS group (B), non-treated healthy group (C), NLC-Ole treated DSS group (D) and NLC-Blank treated DSS group (E). Images are shown at the same magnification (100 $\times$ ).

hypothesized that merging those properties with OLE could lead to a synergistic anti-inflammatory action of the lipid nanoparticles in IBD and a greater targeting to the colon resulting in an improved efficacy of OLE treatment. In this work, nanostructured lipid carriers loaded with oleuropein (NLC-OLE) were assessed both *in vitro* and *in vivo*, with the aim to evaluate its impact on IBD therapy.

NLCs were obtained through the hot-melt emulsification method and showed a mean particle size  $\sim 150$  nm together with a moderately narrow size distribution (PDI  $\sim 0.2$ ). OLEsus was successfully incorporated into the nanocarrier ( $\sim 100\%$ ). Moreover, due to the nature of the lipid core, NLCs exhibited a negatively charged surface

( $\sim -25$  mV) which is known to promote their accumulation in ulcerated tissues via interaction with the positively charged proteins. (Collnot et al., 2012; Belouqui et al., 2016). Therefore, both the nanometer size and surface negative charge together turn NLC-OLE into promising candidates for IBD treatment.

Particularly, Precirol ATO 5 and olive oil were chosen for the lipid core formulation. Precirol ATO 5 has been approved by the FDA for the oral route administration and has been largely used as a component of the lipid matrix for sustained release formulations. As for liquid lipids, the medium chain triglycerides, known under the brand name Miglyol 812, are the most commonly employed. However, in this work, we



**Fig. 5.** Effect of formulations in colitis associated inflammation and oxidative stress. From left to right, MPO activity, absolute levels of TNF-alpha and IL-6 and relative ROS presence in the colon. \*\*p < 0.01, \*p < 0.05 v. DSS group. (n = 7–8).

selected olive oil since it provided our NLCs with a suitable physico-chemical stability. Olive oil is also expected to help enhance OLE availability in the intestine and reduce the possible cytotoxicity effect of residual surfactants that might be present in the final nanoformulation (Talegaonkar and Bhattacharyya, 2019; How et al., 2013).

Innate immunity has an important role in IBD pathogenesis<sup>1</sup>. During active inflammation, particles in the nano-scale range are more easily internalized by a greater number of immune-related cells. Given this, the anti-inflammatory and antioxidant potential of NLC-OLE was evaluated *in vitro*. Firstly, TNF- $\alpha$  production by LPS-stimulated murine J774 macrophages was studied. Surprisingly, we found that OLE itself did not exert any inhibitory effect *in vitro* whereas NLC-OLE significantly inhibited TNF- $\alpha$  secretion (\* $p$  < 0.05). The negligible efficacy of OLEs to suppress TNF- $\alpha$  production in LPS-stimulated macrophages has been also observed by others (Giner et al., 2011; Castejon et al., 2019). Interestingly, NLC-Blank was also able to attenuate TNF- $\alpha$  secretion in our study. These results are consistent with those published by Beloqui et al. (2016, 2013) who found that after 4 h of pre-treatment with budesonide and curcumin loaded NLCs and blank NLCs in LPS-activated J774 cells TNF- $\alpha$  levels were significantly reduced, whereas the active compound alone was not able to exert any effect. Serpe et al. (2010) also observed the ability of solid lipid nanoparticles to significantly decrease the secretion of IL-1 $\beta$  and TNF- $\alpha$  in an *in vitro* model of human IBD whole-blood. Therefore, in light of these results, the nature of the NLCs might boost their ability to inhibit TNF- $\alpha$  secretion from LPS-stimulated J774 cells. Later, we assessed the antioxidant potential of NLC-OLE *in vitro*. It is a well-accepted fact that olive polyphenols are excellent reactive oxygen scavengers (Marković et al., 2019; Cavaca et al., 2020). Our previous studies, demonstrated that OLEs and NLC-OLE antioxidant activity was cell-type dependent. In this work only NLC-OLE showed antioxidant activity in H<sub>2</sub>O<sub>2</sub>-activated J774 macrophages (\* $p$  < 0.05). All in all, the *in vitro* results highlighted the importance of OLE encapsulation within NLCs toward an efficient anti-inflammatory and antioxidant effect.

After assessing the *in vitro* potential of NLC-OLE, their therapeutic efficacy was evaluated in an acute DSS colitis mice model with documented similarity to human UC (Wirtz et al., 2017; Wirtz and Neurath, 2007). DSS is known to induce weight loss, bloody stool and loose feces in mice. From the clinical score results we confirmed that the administration of DSS successfully induced colitis mice. However, no formulation was able to reduce the clinical symptoms (Fig. 3). The DSS colitis model is a well-established *in vivo* model for the study of UC-related innate immune response (Beloqui et al., 2013). During the genesis of active intestinal inflammation, innate response manifests itself as an increased level of leukocyte infiltration in the inflamed mucosa leading to the recrudescence of oxidative stress (Holma et al., 2001). MPO, one of the most abundant neutrophil proteins, is commonly used as standard indicator in feces of IBD patients as well as for colitis assessment in IBD animal models (Mendoza and Abreu, 2009). Precisely, it is mostly used in DSS-induced colitis murine models, where extensive neutrophil infiltration occurs during the entire DSS treatment period, even after it is withdrawn, (Beloqui et al., 2014; Yan et al., 2009). At day 7, both OLEs and NLC-OLE treated DSS groups showed a decrease in MPO activity in comparison with H<sub>2</sub>O-treated DSS group (Fig. 5). DSS-induced damage is also promoted by several anti/pro-inflammatory cytokines. In this regard, at day 7, mice showed a marked secretion of the proinflammatory cytokine TNF- $\alpha$  in colonic tissue which was significantly reduced by NLC-OLE treatment (Fig. 5). Contrary to the *in vitro* results, OLE was effective in TNF- $\alpha$  inhibition *in vivo*. This is in line with other study in which the amelioration of acute colitis by OLE in the *in vivo* model was not consistent with its negligible activity in the *in vitro* models of inflammation (Giner et al., 2011). The secretion of IL-6, a pleiotropic cytokine mainly localized in the intestinal crypts, was also assessed. Pro-inflammatory as well as wound healing activities have been attributed to IL-6 signalling. Particularly, IL-6 has shown to protect intestinal epithelial cells from DSS-induced

apoptosis (Kuhn et al., 2014). Both OLE and NLC-OLE were able to reduce IL-6 when compared to DSS-control group.

As aforementioned, the increased MPO activity could be correlated with the recrudescence of ROS in the inflamed colonic tissue (Holma et al., 2001). Likewise, increased levels of the pro-inflammatory cytokines TNF- $\alpha$  and IL-6 are known to be connected with a higher rate of cellular oxidative stress. Particularly, ROS are known to be induced by IL-6 and TNF- $\alpha$  is known to induce IL-6 secretion through ROS, (Kosmidou et al., 2002; Mosińska et al., 2017). This, together with the *in vitro* proven antioxidant efficacy of NLC-OLE lead us to assume that NLC-OLE could have ameliorated DSS-induced inflammation through its antioxidant activity in the colon.

Aimed to elucidate this, we analyzed the total ROS content in the colon tissue. As expected, DSS-control group presented a significant accumulation of ROS when compared to the healthy-control group (data not shown) and they were significantly reduced by NLC-OLE (\* $p$  < 0.05). Meanwhile, OLE slightly reduced oxidative stress but the differences did not reach statistical significance compared to the control. This, again, highlights the importance of the encapsulation of OLE towards an enhanced pharmacological activity in the murine colitis model.

It is worth noting that no significant therapeutic effect was observed for NLC-Blank treated DSS group in the any of the *in vivo* studies indicating that the nano-sized lipid carrier has no influence on the therapeutic activities of NLC-OLE.

All these results from the *in vivo* experiments were in line with those from the histological assessment of the colitis associated inflammatory injury (Fig. 4). DSS-induced colitis (Fig. 4A) clearly modified the architecture of the colon tissue, with a reduction of the protective epithelial layer and the number of goblet cells as well as an increased infiltration of immune-related cells. For OLE-treated and NLC-OLE treated DSS groups (Fig. 4B and 4D, respectively) adequate colon structure was observed when compared to the healthy control group (Fig. 4C). As expected, NLC-Blank treated group showed a higher infiltration of immune cells (Fig. 4E) but the architecture of the crypts well preserved compared to the control DSS group. Therefore, NLC-OLE demonstrated to maintain the anti-inflammatory potential of OLE and enhanced its ROS scavenging effects after oral administration *in vivo*.

To the extent of our knowledge, this is the first study evaluating the *in vivo* efficacy of OLE loaded NLCs in a murine model of acute colitis. *In vitro*, NLCs inhibited TNF- $\alpha$  production by activated murine macrophages and the encapsulation of OLE into the lipid core lead to an efficient antioxidant activity in stressed macrophages. Our findings from the *in vivo* study showed the ability of orally administered NLC-OLE to alleviate inflammation and oxidative stress in DSS-induced acute colitis as confirmed by the enhanced histological appearance of the colonic samples which was further confirmed by the reduced MPO activity, decreased tissue-associated TNF- $\alpha$  and IL-6 levels and the inhibited ROS-generation in NLC-OLE treated mice colons. The discussed results highlight and justify the combination of olive oil and Precirol formulated within NLCs for the delivery of OLE or other antioxidant/anti-inflammatory phenolic compounds in IBD therapy. On a wider level, future research is also needed to determine the precise mechanism of action and pharmacokinetics of NLC-OLE as well as the rationale for the enhancement of the antioxidant activity of OLE.

#### CRedit authorship contribution statement

**Amaia Huguet-Casquero:** Conceptualization, Investigation, Writing - original draft, Visualization, Formal analysis. **Yining Xu:** Investigation. **Eusebio Gainza:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jose Luis Pedraz:** Conceptualization, Methodology, Resources, Data curation, Supervision, Writing - review & editing, Funding acquisition. **Ana Beloqui:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing - review & editing,



Supervision, Funding acquisition.

## 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2020.119515>.

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