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EVALUATION OF THE EFFECT OF LIPOSOMES LOADED WITH CHLOROGENIC ACID IN TREATMENT OF 2,4,6-TRINITROBENZENESULFONIC ACID-INDUCED MURINE COLITIS

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Crohn's Disease (CD), one of the types of inflammatory bowel disease, poses a significant challenge to modern healthcare. This condition severely impacts patients' quality of life, and its incidence is continuously rising. Despite constant research, current treatment options are limited and largely unsuccessful and result in serious side effects, therefore new therapy alternatives are needed. Liposomal formulation provides a new hope for disease management. In our study, we characterized the anti-inflammatory activity of mesalazine (5-ASA) and chlorogenic acid (CGA) encapsulated in liposomal formulation in the animal model of CD. Liposomes were obtained by thin film hydration method and characterized in terms of suspension stability and particle size and distribution. Colitis was induced in mice by intracolonic (i.c.) administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS). The effect of treatment with liposomal suspensions of 5-ASA and CGA was evaluated macroscopically and by measuring myeloperoxidase (MPO) activity. We observed that liposome-encapsulated 5-ASA (5 mg/kg), but not CGA (20 mg/kg) attenuated colitis as evidenced by a decreased macroscopic and microscopic scores. It may be hypothesized that the composition of liposomal lipid bilayer as well as the switch in macrophage populations leading to unfavorable accumulation of anti-inflammatory agents in the cells may underly the efficiency of obtained liposomes and need to be taken into consideration in further studies on drug delivery.

Key words: liposomes, Crohn's disease, inflammatory bowel disease, murine colitis, chlorogenic acid, mesalazine, myeloperoxidase

INTRODUCTION

Crohn's Disease (CD), next to ulcerative colitis (UC), is one of the two main types of inflammatory bowel disease (IBD). The morbidity for CD is still rising, especially in developing countries, therefore CD becomes a problem of global importance. Of note, its incidence is highest between the second and fourth decade of life; moreover, in developed countries the rise in occurrence is most significant in children, which makes this disease a major burden to healthcare systems (1, 2). Although the pathogenesis of CD has not yet been clearly identified, many factors that influence its development have been recognized. In addition to immunological and genetic factors, environmental influence is considered particularly important (3). Therefore the rise in IBD prevalence is expected to continue (1, 2).

Conventional IBD therapy is based on aminosalicylates (*e.g.* mesalazine, 5-ASA), corticosteroids, immunosuppressive and biological agents. Although remission can be obtained in most cases, this chronic disease requires prolonged treatment, which leads to severe side effects. Due to exacerbations, which often

require hospitalization, as well as high costs of therapy and significantly decreased patients' quality of life, new therapeutic options are required (1, 4).

Liposomal delivery could increase the efficiency of the treatment and allow for dose reduction, consequently diminishing adverse effects. Liposomes are small vesicles with an aqueous core and lipid bilayer on the outside. Owing to this structure, the delivery of both hydrophilic and hydrophobic compounds is possible. Liposomes have been studied as a drug delivery system for decades, primarily with a goal to improve cancer therapy (5). Their characteristics proved valuable, especially due to increased selectivity and accumulation at the target location, significantly reduced toxicity with equal or enhanced efficacy, increased solubility, especially in the case of hydrophobic drugs, as well as a possibility to control and extend the release of encapsulated substances (6, 7). As a consequence, several liposomal formulations are already commercially available (e.g. Doxil and Myocet, which are formulations of doxorubicin, or Marquibo, which contains vincristine), while others are at the stage of clinical trials (6, 7). As liposomes have been reported to possess mucoadhesive properties, they are believed to have potential in the treatment of gastrointestinal diseases (8).

Chlorogenic acid (CGA) is a plant-derived compound, which has long been known in traditional Chinese medicine. CGA has been recognized for its anti-inflammatory, antioxidant, anticarcinogenic and anti-bacterial activity (9, 10). Recently CGA has also been reported to influence lipid and glucose metabolism and to exert anti-diabetic and anti-obesity effect (11-13).

We previously demonstrated the action of CGA in an animal model of colitis (14). In this study, we compared the antiinflammatory activity of 5-ASA and CGA encapsulated in liposomal formulation in the animal model of IBD.

MATERIALS AND METHODS

Drugs and reagents

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, USA). CGA (purity 98 % by high-performance liquid chromatography) was extracted from honeysuckle flowers and purchased from Nanjing Zelang Medical Technology Co (Nanjing, Jiangsu, China) by one of the authors (C.C.). All other drugs and reagents, unless stated otherwise, were purchased from Sigma-Aldrich (Poznan, Poland).

Liposome preparation

Liposomes were obtained through thin film hydration method. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol and phosphatidylethanolamine (PE) were mixed in 85:50:15 mass ratio, dissolved in chloroform and dried to create a thin lipid layer, which was then hydrated with a solution of a tested compound (5-ASA, CGA) dissolved in 0.9 % sodium chloride. The mixture was incubated for 30 min at room temperature, with intermittent vortexing every 5 min, submitted to 5 cycles of freezing in liquid nitrogen and thawing in 40°C in a water bath and sonicated with the use of a bath sonicator for 10 min. Finally, liposomes were extruded through 0.2 μ m Nucleopore Track-Etch Membrane using Avanti Mini Extruder. Liposomes were used for delivery experiments immediately after preparation or stored at 4°C not longer than 7 days.

Liposome characterization

Size measurements were performed using dynamic light scattering (DLS) using Malvern Zetasizer Nano-ZS ZEN3600 (Malvern Instruments, Malvern, UK). Before each analysis, samples were dispersed in 0.9% NaCl and equilibrated for 120 s at 25°C. The tested samples (1 ml) were measured in disposable glass cuvettes at the temperature of 25°C, and this temperature was actively maintained within 0.1°C in the sample chamber. Samples were irradiated with red light (HeNe laser, wavelength $\lambda = 633$ nm) and the intensity fluctuations of the scattered light (detected at a backscattering angle of 173°) analyzed to obtain an autocorrelation function. The following assumptions were made in the analysis: the viscosity of the solution was assumed to be same as of water, corrected for temperature ($\eta = 0.888$ mPa.s); the solution refractive index was that of water (n = 1.33). Data were acquired in the automatic mode.

The zeta potential of liposomes solution was measured using a Zetasizer Nano ZS ZEN 3600 (Malvern Instruments, Malvern, UK). The liposome solutions were diluted 10 times with 0.9% NaCl solution. The measurement was carried out at 25°C in a 1.0 ml polycarbonate cuvette DTS1070. All measurements were made in triplicate for each sample.

Animals

Male balbC mice obtained from the Animal House of the University of Lodz, Poland, weighing 20 - 25 g (7 – 8 weeks of age), were used for the study. The animals were housed at a constant temperature ($22 - 23^{\circ}$ C) and maintained under a 12-h light/dark cycle with constant access to laboratory chow and tap water.

All procedures were approved by the Local Ethical Committee for Animal Research at the Medical University of Lodz (#36/LB98/2018). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Induction of colitis

Colitis was induced by i.c. administration of trinitrobenzenesulfonic acid (TNBS), as described previously (14). Briefly, mice (n = 6 - 8 per experimental group) were anesthetized with isoflurane (Aerrane, Baxter, Deerfield, USA) and TNBS (4 mg in 0.1 ml of 30% ethanol in 0.9% NaCl) was administered into the colon (3 cm proximally to the anus, 100 µl) through a catheter. Mice from control group received 100 µl of 0.9% NaCl alone. Mice were sacrificed by cervical dislocation on day 7 and colonic damage was evaluated.

Pharmacological treatment

Treatment was administered i.c. twice daily from day 3 to day 6 after colitis induction. Liposomal suspensions contained 0.9% NaCl (TNBS + LIPO-NaCl), 5-ASA at the dose of 5 mg/kg (LIPO-5-ASA) and CGA at the dose of 20 mg/kg (LIPO-CGA). Doses for 5-ASA and CGA encapsulated in liposomes were selected based on previously published data and our preliminary experiments (14, 15).

Control and TNBS groups received 0.9% NaCl alone (100 $\mu l,\,i.c.).$

Macroscopic score evaluation

For total macroscopic score evaluation, the colon was rapidly removed, opened longitudinally and rinsed with phosphate buffered saline (PBS) to remove the fecal contents. The wellestablished semiquantitative scoring system was used taking into consideration the following parameters: colon length (cecum to rectum, cecum excluded), ulcer score, adhesion, presence of hemorrhage, fecal blood, diarrhea and bowel thickness.

For scoring colonic shortening and ulcer, the following scale was used: shortening of the colon - 1 point for > 15%, 2 points for > 25% (based on a mean length of the colon in untreated mice of 7.89 ± 0.17 cm; n = 6); ulcer - 0.5 points for each 0.5 cm. The presence of adhesion, hemorrhage, fecal blood, or diarrhea increased the score by 1 point for each additional feature. The colonic wall thickness was measured in millimeters. A thickness of n mm corresponded to n scoring points.

Microscopic score evaluation

Segments of the distal colon (approx. 0.5 cm in length) were isolated and stapled flat, mucosal side up, onto cardboard and fixed in 10% neutral-buffered formalin for 24 h at 4°C. After subsequent dehydration in sucrose, samples were embedded in paraffin, sectioned at 5 μ m and mounted onto slides. Then, sections were stained with hematoxylin and eosin and examined using Zeiss Axio Imager setup (Jena, Germany).

A microscopic damage score was determined in a blinded fashion using the scoring system as follows: presence (score = 1) or absence (score = 0) of goblet cell depletion, the presence (score = 1) or absence (score = 0) of crypt abscesses, the destruction of mucosal architecture (normal = 1, moderate = 2, extensive = 3), the extent of muscle thickening (normal = 1, moderate = 2, extensive = 3), and the presence and degree of immune cell infiltration (normal = 1, moderate = 2, transmural = 3).

Determination of tissue myeloperoxidase activity

To assess the granulocyte infiltration through quantification of myeloperoxidase activity, a standardized method has been used (14). Briefly, colon sections (approx. 30 mg) were isolated and homogenized in hexadecytrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0; 50 mg tissue/mL). The homogenate was centrifuged 15 min, $13200 \times g$, 4°C). Seven µl of the supernatant and 200 µl of 50 mM potassium phosphate buffer (pH 6.0), supplemented with 0.167 mg/mL of O-dianisidine hydrochloride and 0.05 µL of 1 % hydrogen peroxide were added on a 96-well plate. Absorbance was measured at 450 nm (iMARK Microplate Reader, Biorad, Hertfordshire, UK). All measurements were performed in triplicate. Myeloperoxidase (MPO) activity was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 µmol hydrogen peroxide to water in 1 min at room temperature.

Statistical analysis

The results are presented as mean \pm standard error of the mean (SEM). The analysis was done in Prism 5.0 (GraphPad

Table 1. The values of parameters characterizing liposomal suspension. Data represent mean \pm SEM.

Parameter	Result	Comment
polydispersity index	0.186 ± 0.002	homogenous particle distribution
particle size	163.4 ± 1.4 nm, 99.9 ± 0.04 %	
zeta potential	$-15.5\pm0.05\ mV$	moderate stability



Fig. 1. The effect of liposomes on colonic inflammation in TNBSinduced mouse model of colitis. Comparison of macroscopic score (A), ulcer score (B), bowel thickness (C), colon length (D), adhesion score (E) and myeloperoxidase (MPO) activity (F) for the following groups: control, animals with TNBSinduced colitis (TNBS), animals with TNBS-induced colitis with liposomes treated containing 0.9% NaCl (TNBS + LIPO-NaCl), mesalazine (TNBS + LIPO-5-ASA) and chlorogenic acid (TNBS + LIPO-CGA). Data represent mean + SEM; n = 6 - 8animals per experimental group. P < 0.05, P < 0.01, P < 0.001, 0.001, 0.001, 0.001as compared to control.

Software Inc., La Jolla, CA, USA) using one-way ANOVA followed by Newman-Keuls post hoc test. P values < 0.05 were considered statistically significant.

RESULTS

The obtained liposomal suspensions were homogenous and moderately stable

Liposomes were characterized in terms of polydispersity index, particle size and zeta potential (*Table 1*). The polydispersity index value equaled 0.186 ± 0.002 , indicating homogenous preparation with a narrow range of particle distribution. The size of the particles was 163.4 ± 1.4 nm, $99.9 \pm 0.04\%$.

Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are considered stable. In our study,

the measured zeta potential equaled -15.5 ± 0.05 mV, which indicates the moderate/short term stability of the tested solution.

Liposome-encapsulated 5-ASA, but not chlorogenic acid, alleviated inflammation in TNBS-induced model of colitis

Treatment with liposome-encapsulated 5-ASA (5 mg/kg) twice daily i.c. attenuated colitis, as evidenced by a decreased macroscopic score (5.60 ± 0.75 versus 5.28 ± 0.69 for TNBS-versus 5-ASA-liposome-treated mice; *Fig. 1*). Moreover, this liposomal suspension reduced ulcer score (1.46 ± 0.23 for mice which received 5-ASA-loaded liposomes versus 1.50 ± 0.29 for TNBS-treated mice) and adhesion score (0.92 ± 0.21 for mice treated with 5-ASA-loaded liposomes vs. 0.95 ± 0.21 for TNBS-treated mice).

Surprisingly, although CGA was previously shown to alleviate colitis (14), our liposomal formulation of CGA (20

Fig. 2. Representative micrographs of haematoxylin and eosin-stained sections of distal colon obtained from the TNBS-induced colitis model and microscopic total damage score. ${}^{SP} < 0.05$, ${}^{SP} < 0.01$, as compared with control mice, ${}^{**P} < 0.01$, as compared with TNBS-treated mice. Data represent mean \pm SEM of 6 – 8 mice per group.

mg/kg, twice daily, i.e.) aggravated inflammation (the macroscopic score for this group was 8.18 ± 0.82 , the ulcer score 2.22 ± 0.28 , the adhesion score 1.78 ± 0.22 and bowel thickness 1.84 ± 0.16 mm).

Neither of the liposomal formulations had impact on colon length. Furthermore, the results of MPO activity did not show any specific trend.

Microscopic evaluation of the specimens obtained from the mouse colon is in line with the macroscopic results. The tissues from untreated animals contained intact epithelium, low neutrophil infiltration and correct muscle architecture. Samples presenting microscopic damage (TNBS and LIPO-CGA) were characterized by disturbed mucosal architecture, thickening of muscle layer and extensive leukocyte infiltration. Finally, a decrease in colitis-related changes for LIPO-5-ASA group, and an increase for LIPO-CGA were observed (*Fig. 2*).

DISCUSSION

The issue of liposomal drug delivery in IBD has attracted significant attention. Many studies report that the properties of the liposomes, in particular the particle size and zeta potential, determine their biological activity, which should be taken into consideration while adapting the preparation to our needs (16, 17). Noteworthy, liposomes were shown to have colonic adhesion properties, which suggests their potential use in IBD treatment (18). The charge of liposomes is crucial for their adhesive interactions with intestinal mucosa. Jubeh et al. stated that positively charged liposomes have best adherence to healthy colonic mucosa, while in the case of inflamed mucosa, anionic liposomes are preferred (19). Moreover, it was noted that the attachment of liposomes to epithelium was charge-density dependent: the higher the charge density, the better the adherence. The cationic liposomes are believed to react with negatively charged sialic acid within the mucus lining. The authors suggested that high concentration of eosinophil cationic protein, reported in IBD patients due to significant eosinophil infiltration, may cause the accumulation of anionic liposomes (19).

Additionally, high expression of transferrin was observed in the mucosa at low pH of intestinal lumen, typical for the inflammatory conditions. It was suggested that transferrin is the factor responsible for increased liposomal adherence: in acidic environment transferrin is positively charged, which enables binding of the anionic liposomes (20). Transferrin receptors (TfRs) are markedly overexpressed in various cancer cell types, therefore TfR-targeted liposomal formulation was already described as a promising delivery system in cancer treatment (5). TfR expression is also elevated in the inflamed colonic mucosa in comparison to healthy tissue. The overexpression is related to the mechanism of inflammation - proinflammatory cytokines, TNF-a in particular, increase TfR levels. Anti-TfR antibodies conjugated to liposomes increased liposome accumulation 4fold in the rat inflamed mucosa in comparison to healthy tissue. An additional benefit is that TfR-targeted liposomes undergo endocytosis upon binding the receptor (21).

In the search for better drug delivery options in IBD, we decided to explore the opportunity given by liposomal delivery system. Our liposomal formulation proved to have the desired characteristics, as evidenced by polydispersity index and particle size. The parameters we obtained were in the same range as those reported in literature regarding liposomal delivery reported by experts in the field of liposomal delivery (19, 22-25). Of note, in further studies we should aim at increasing the zeta potential to ensure better stability of the preparation (6).

To assess the therapeutic potential of obtained liposomal formulations, we used 5-ASA and CGA. The former is a

therapeutic agent commonly used in IBD and regarded as a model drug in colitis (18, 26-29). Kesisoglou *et al.* observed that liposomes loaded with 5-ASA accumulated at the mucosal surface but, most likely, did not enter the tissue, therefore the metabolite level was not increased as compared to 5-ASA solution. Our results showed a similar tendency - although we observed a decrease in macroscopic score, colitis was not substantially alleviated (18).

Plant-derived polyphenols, due to their antioxidative and anti-inflammatory properties, show beneficial effects in studies pertaining to colitis, e.g. oligonol, extracted from lychee fruit, significantly ameliorated exacerbations in a relapse model of colitis (30). Chlorogenic acid is a polyphenolic compound of plant origin, which has been known to possess antiinflammatory properties and has been tested in gastrointestinal disorders. CGA was previously reported to diminish TNBSinduced colitis through inhibition of neutrophil infiltration and NF-kB activation, and the i.c. treatment was more effective than the oral administration (14). More recently, in dextran sulfate sodium (DSS) model of colitis, CGA administered p.o. reduced severity of inflammation through inhibition of pro-inflammatory and apoptotic signaling pathways (31). Moreover, when CGA was added to the diet, attenuation of colitis was accompanied by a favorable modification of gut microbiota: a decrease in Firmicutes and Bacteroidetes, and augmentation in Akkermansia municiphila (32). Feng et al. reported enhanced bioavailability and antioxidant activity of liposome encapsulated CGA (22). The liposomes were administered orally and predominantly accumulated in the liver (22).

In our current study, the liposomal formulation aggravated the inflammation. Such result could be explained by toxicity, already observed at high doses of CGA (33-36) which may result from a shift in macrophage populations. In line, Bartneck *et al.* reported that dexamethasone-containing liposomes caused a shift from M2 to pro-inflammatory M1 phenotype of macrophages (37). The authors hypothesize that contrary to passive diffusion of the free drug, which causes an antiinflammatory effect, the phagocytosis of liposomes leads to intracellular accumulation and toxic concentration of the glucocorticoid in the macrophages, which then activate their danger sensing and thereby release IL-1 β (37).

The unfavorable effect of our liposomal formulations may also be related to the composition of the lipid bilayer and the resulting surface charge, the choice of the lipids and proportion between the chosen components, since every modification of these parameters influences the properties of liposomes (6). Additionally, although the dosage was based on our previous experiments, the change of formulation may alter the needed amount of drug per dose. Finally, a different route of administration for a similar preparation than that used by Feng *et al.* may have resulted in a completely opposite systemic effect.

In summary, our study showed that liposomal preparation of 5-ASA, but not CGA alleviated inflammation in the mouse model of TNBS-induced colitis, which proves its potential as a novel form of therapy in IBD and paves the way for further research. There is still room for improvement of our liposomal formulation, *e.g.* through increasing the specificity by targeted delivery or increasing its stability. To diminish the uptake of liposomal particles by macrophages, the liposomal surface can be modified with inert polymers such as polyethylene glycol (38, 39).

Finally, as oral delivery is the preferred route of administration ensuring highest patient compliance, delayed release dosage forms of liposomal preparations need to be investigated.

Authors' contributions: JK, JF - study design; all - data collection and data interpretation; JK, JF - statistical analysis;

JK, JF - literature search; BK, AG, JF - funds collection. All authors have accepted final version of the manuscript.

Acknowledgements: Supported by grant from the Medical University of Lodz (#503/1-156-04/503-11-001 to JF). JF, JK, PP and DT are all participants of the KUMPEL program from the Medical University of Lodz.

Conflict of interests: None declared.

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Received: March 10, 2019 Accepted: April 29, 2019

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