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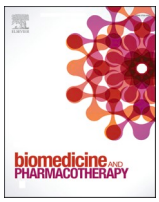

Beclomethasone loaded liposomes enriched with mucin: A suitable approach for the control of skin disorders

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ABSTRACT

Inflammatory skin disorders are the fourth leading cause of chronic non-fatal conditions, which have a serious impact on the patient quality of life. Due to their treatment with conventional corticosteroids, which often result in poor therapeutic efficacy, relapses and systemic side effects from prolonged therapy, these diseases represent a global burden that negatively impacts the global economy. To avoid these problems and optimize corticosteroid benefits, beclomethasone was loaded into liposome formulations specifically tailored for skin delivery. These formulations were enhanced with mucin (0.1 and 0.5 % w/v) to further ensure prolonged formulation permanence at the site of application. The addition of 0.5 % w/v mucin resulted in the formation of small unilamellar vesicles and multicompartment vesicles. Liposomes and 1mucin-liposomes were smaller (~48 and ~61 nm, respectively) and more monodispersed (PI ~ 0.14 and ~ 0.17, respectively) than 5mucin-liposomes, which were larger (~137 nm), slightly polydispersed (PI ~ 0.23), and less stable during storage (4 months in the dark at 25 °C). Liposomes were negatively charged (~ −79 mV) irrespective of their composition, and capable of incorporating high amount of beclomethasone (~ 80 %). In vitro studies on skin fibroblasts and keratinocytes confirmed the high biocompatibility of all formulations (viability ≥ 95 %). However, the use of mucin-liposomes resulted in higher efficacy against nitric oxide production and free radical damage. Finally, topical applications using 12-O-tetradecanoylphorbol-13-acetate-injured skin in vivo experiments showed that only the mucin-enriched formulations could restore healthy conditions within 4 days, underscoring promise as a treatment for skin disorders.

1. Introduction

Human skin acts as the main barrier between the external and internal environment, thus preventing the passage of dangerous substances, protecting the body from infection and external injuries, regulating body temperature, reducing water loss, and enabling sensations such as touch, heat, and cold to be perceived [1]. The integrity of this barrier is essential, and any dysfunction increases the risk of developing infectious and inflammatory chronic or acute conditions [2,3]. Indeed, even if these disorders primarily affect the skin, they represent a common global public health concern due to their potential association with psychological and social complications [3]. Despite the large range of co-morbidities that they might cause, there is sometimes a misconception among the general public and some healthcare providers

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that consider them to be "just simple skin diseases", less severe than other "conventional diseases", thus underestimating that suitable and effective therapies are needed from their onset.

These chronic inflammatory skin disorders are immune-mediated and characterized by similar histopathological features: a cascade release of cytokines that initiate an inflammatory response, which contributes to the development of distinct clinical manifestations of various dermatoses [4]. They can be heterogeneous, chronic-intermittent, and eczematous. In addition, they may start at infancy or early childhood, and persist for a large part of life, thus deteriorating its quality and leading immune dysfunction, alteration of skin microbiome, and reduction of skin barrier effectiveness, due to structure modification and sometime loss of the stratum corneum, which is the first limiting barrier for the entrance of external molecules [5,6]. Considering the damaged state of the skin affected by inflammatory disorders, topical formulations should be carefully balanced to avoid inducing further inflammatory response or irritation. Therefore, current topical treatments, mostly based on the application of glucocorticoids with anti-inflammatory, antiproliferative, and immunosuppressive effects, are limited and sometimes ineffective. Moreover, their chronic topical use is associated with systemic absorption and significant side effects, such as adrenal atrophy, osteoporosis, or induced glaucoma. These effects are mainly related to the high doses required and the increased frequency of application [7,8]. Therefore, advanced therapeutic approaches to reduce the dose and avoid systemic circulation of corticosteroids could significantly improve treatment efficacy and patient compliance.

One strategy to deliver glucocorticoids to the skin is their ad hoc formulation in specific nanocarriers, which can effectively promote their efficacy and reduce or even avoid their side effects. In recent years, several different nanotechnology-based drug delivery systems were developed and tested for the topical application of glucocorticoids. Among them, phospholipid vesicles, whose structure and composition resembled that of cell membranes, have been largely used due their versatility, biocompatibility, and effectiveness [9]. Different liposomes were previously assayed for the in vivo skin delivery of several corticosteroids, such as hydrocortisone, hydrocortisone acetate, betamethasone, and triamcinolone [10–13]. Hydrocortisone and dexamethasone were loaded in transferosomes, special phospholipid vesicles modified with an edge activator, which improved their skin accumulation and the in vivo therapeutic risk-benefit ratio [14]. Hydrocortisone, dexamethasone, or triamcinolone-acetonide were also loaded in other transferosome formulations and, when locally administered, were biologically active at doses several times lower than those currently used for the topical treatment of skin diseases [15]. In the last decades, the skin delivery performances of liposomes and transferosomes have been further improved by the addition of new components such as water-cosolvents, surfactants, polymers, and/or peptides. Among the water-cosolvents, ethanol and glycerol have been widely investigated because of their ability to fluidize phospholipids bilayers, which in turn leads to soft vesicle able to promote topical drug delivery [16]. In addition, glycerol provides higher stability to the formulations and synergistic moisturizing properties to the skin [17]. As for polymers, mucins possess barrier, moisturizing, gelling and lubricating properties [18]. These properties make them suitable and cost-effective additive candidates for 1) providing appropriate spreadability of the formulation on the skin, 2) generating a higher localized concentration of the drug at the application site, and 3) preventing runoff [19]. This ultimately allows for better management of inflammation and promotes healing of the affected skin [20]. In the literature, different studies have dealt with vesicles improved with ethanol and/or glycerol and polymers. However, these improved vesicles have not yet been tested for the skin delivery of corticosteroids [21–24]. With that in mind, in the present study, beclomethasone, one of the glucocorticoids commonly used for local treatment of skin disorders, was loaded into specially formulated phospholipid vesicles. The moisturizing properties of glycerol, the penetration-enhancing properties of ethanol, and the bioadhesiveness of mucin were synergistically combined to enhance the liposome carrier capabilities. Liposomes were prepared by the one-step sonication method, avoiding the use of organic solvents, and their size, surface charge, and entrapment efficiency were then assessed. The in vitro biocompatibility towards fibroblasts and keratinocytes, and the ability to protect macrophages from nitric oxide overproduction and damage were also evaluated. Finally, the ability of formulations to promote healing and re-epithelialization of skin injured by 12-O-tetradecanoylphorbol 13-acetate (TPA) was assayed in vivo in a mouse model.

2. Materials and methods

2.1. Materials

Lipoid S75 (soybean phospholipids with 70 % phosphatidylcholine, S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Mucin from porcine stomach (type II, 640 kDa), beclomethasone, 12-O-tetradecanoylphorbol 13-acetate (TPA), and all other products of analytical grade were purchased from Merck (Milan, Italy).

Cell medium, foetal bovine serum, penicillin, streptomycin, fungizone, and all the other reagents for cell studies were purchased from Thermo-Fisher Scientific Inc. (Waltham, MA, US). The commercial Menaderm Simplex® cream, containing 0.025 % of beclomethasone, was bought in a local pharmacy.

2.2. Preparation of liposomes

Beclomethasone (0.25 mg/mL) was dissolved in a blend composed of water, ethanol and glycerol (1:0.5:0.5), and S75 (120 mg/mL) to prepare liposomes, or S75 (120 mg/mL) and mucin at 2 concentrations (0.1 and 0.5 % w/v) to prepare mucin-liposomes; mixtures were allowed to hydrate for 2 h at room temperature (25 ± 1 °C) (Table 1). Following the preparation phase, blends were sonicated (3 seconds on and 2 seconds off, 20 cycles; 14 μm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK). Empty formulations were also prepared and used as references.

2.3. Characterization of liposomes

Cryogenic electron transmission microscopy (cryo-TEM) analyses were performed using a Tecnai F20 TEM (FEI Company). Briefly, 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). Samples were then observed in a low dose mode (200 kV) and at ~ −172 °C [25].

Liposomes average diameters and polydispersity indexes, as a measure of the homogeneity of the dispersion, were determined by photon correlation spectroscopy (Zetasizer ultra; Malvern Instruments, Worcestershire, UK). The Zetasizer ultra was also used to measure vesicle surface charge (zeta potential) by their electrophoretic mobility in dispersion according to the mixed-mode measurement-phase analysis (M3-PALS). Each sample was properly diluted (1:100) to be optically clear and to avoid the attenuation of the laser beam by the particles along with the reduction of scattered light that can be detected [26].

To eliminate the unloaded drug from dispersions, vesicles (1 mL) were loaded into dialysis tube (Spectra/Por® membranes, 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, the Netherlands) and maintained at room temperature (25 ± 1 °C) in 1 litre of distilled water for 2 h, refreshing water after 1 h. Drug loading efficiency (E%) was expressed as the percentage of the concentration of drug found in dispersion after purification versus that before purification. The concentration of beclomethasone was quantified by high performance liquid chromatography (HPLC) after disruption of vesicles with methanol (1:1000). Analysis was performed at 240 nm using a chromatograph Alliance 2690 (Waters, Milano, Italy)

Table 1
Composition of liposomes prepared with S75, mucin and beclomethasone, and hydrated with a blend of water, glycerol and ethanol (1:0.5:0.5 v/v).

	Beclomethasone mg/mL	S75 mg/mL	Mucin mg/mL	Water mL	Glycerol mL	Ethanol mL
liposomes	0.25	120	-	1	0.5	0.5
1mucin- liposomes	0.25	120	1	1	0.5	0.5
5mucin- liposomes	0.25	120	5	1	0.5	0.5

equipped with a Xselect C18 column (3.5 μm, 4.6 × 150 mm, Waters, Milano, Italy). The mobile phase was a mixture of water, acetic acid, and acetonitrile (30.97:0.03:69 v/v), delivered at a flow rate of 1 mL/min. The experiments were performed in triplicate for each sample.

A stability study was also carried out by monitoring the average size, polydispersity index, and surface charge of the liposomes stored at room temperature (25 ± 1 °C) and in the dark for 5 months.

2.4. In vitro cytotoxicity of formulations in keratinocytes and fibroblasts

Immortalized human keratinocytes and primary mouse embryonic fibroblasts (ATCC collection, Manassas, VA, USA) were grown as monolayers in 150 cm^2 flasks, incubated with 100 % humidity and 5 % carbon dioxide at 37 °C. Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10 % foetal bovine serum, penicillin, streptomycin, and fungizone, was used as culture medium. Cells were seeded into 96-well plates (5×10^4 cells/well) and incubated untouched. After 24 h, cells were treated for 48 h with beclomethasone dispersed in water, ethanol, and glycerol (1:0.5:0.5 v/v) or loaded in liposomes. Samples were previously diluted with the cell medium to reach different drug concentrations (0.25, 0.5, 2.5, 5 μg/mL). At the end of the experiments, cells were washed with warmed phosphate-buffered solution and their viability was measured using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] (MTT) colorimetric assay. MTT solution dissolved in phosphate-buffered saline (100 μL, 0.5 mg/mL final concentration) was added to each well, and cells were incubated for 3 h. Next, the formed formazan crystals were dissolved in 100 μL of dimethyl sulfoxide and spectrophotometrically quantified at 570 nm by using a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy). Results are expressed as percent of cell viability in comparison with non-treated cells (100 % viability).

2.5. In vitro capability of formulations of inhibiting nitric oxide generation in macrophages

Immortalized mouse macrophages (RAW 264, ATCC collection, VA, USA) were preincubated (2×10^5 cells/well) with either dispersion (water, glycerol and ethanol (1:0.5:0.5 v/v)) or beclomethasone loaded liposomes (2.5 and 5 μg/mL) for 1 hour. At the end of preincubation, lipopolysaccharide (1 μg/mL final concentration) was added to each well, and cells were then incubated for 24 h at 37 °C and 5 % carbon dioxide. After incubation, cell culture medium (200 μL) was withdrawn, transferred into a new 96-well plate, and mixed with Griess reagent solution (100 μL). After 5 min of incubation at 25 °C in the dark, the absorbance was measured at 540 nm by using a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.p.A., Bernareggio, Italy). Different solutions of sodium nitrite in culture media, ranging from 0 to 100 μM, have been used to build a standard curve. The amount of nitrite produced by macrophages damaged with lipopolysaccharide and treated with beclomethasone formulations was calculated as the ratio between the released nitrite from treated cells versus that released from control cells (damaged with lipopolysaccharide and untreated), considered as 100 % of nitrite released. As for the macrophages attached to the bottom of each well, after washing them with fresh medium, the number of viable cells was measured by means of the MTT colorimetric assay, adding 100 μL of reagent (0.5 mg/mL in phosphate-buffered saline, final concentration) in each well, as reported above (paragraph 2.4).

2.6. In vivo evaluation of protective effect of liposomes against skin damages

Female CD-1 mice (5 and 6 weeks old, 25 and 35 g) were obtained from Harlan Laboratories (Barcelona, Spain) and acclimatized for 1 week. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the Ethics Committee of the University of Valencia (protocol code 2017/VSC/PEA/00148). The mice were divided in groups (n = 4), including undamaged animals (positive control), animals damaged with TPA, and treated with saline (negative control). The back skin of mice was shaved (~ 2 cm^2) and TPA (6 μg/40 mL, 20 μL) dissolved in acetone was subsequently applied to induce cutaneous inflammation and ulceration (day 1). Beclomethasone in dispersion (water, glycerol and ethanol (1:0.5:0.5 v/v)) or loaded in liposomes (100 μL), and a commercial cream (Menaderm Simplex®) with the same concentration were topically applied until fully adsorbed 3 and 6 h after TPA application. The treatment was repeated daily for additionally 2 days. On day 4, mice were sacrificed by cervical dislocation, and their damaged dorsal skin areas were excised and weighted to measure oedema formation. Myeloperoxidase activity, a quantitative assessment of neutrophil infiltration into the skin, was evaluated as previously reported [27]. Briefly, the skin biopsies have been homogenized and centrifuged, then the supernatant (10 μL) has been incubated with hydrogen peroxide (0.052 % v/v, 40 μL), phosphate-buffered saline (pH 5.4; 20 μL), and tetramethylbenzidine in 8 % aqueous N,N-dimethylformamide (18 mM, 20 μL). Finally, the reaction was stopped by adding 50 μL of sulfuric acid (2 N), and the absorbance of the coloured solution was measured spectrophotometrically at 450 nm. The myeloperoxidase activity was calculated from the linear portion of a standard curve and expressed as ng/mL by using the same conditions and dilution factors.

2.7. Histological examination

Skin biopsies (~ 2 cm^2) were excised from mice treated region, after 72 h of treatment (on day 4) and maintained in formaldehyde (10 % v/v). Tissue samples were processed routinely and embedded in paraffin wax. Longitudinal Section (5 μm) were stained with hematoxylin and eosin. The tissues were observed under a light microscope (DMD 108 Digital Micro-Imaging Device, Leica, Wetzlar, Germany).

2.8. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used for multiple comparisons of means, and the Tukey's test and Student's t-test were performed to substantiate differences between groups using GraphPad Prism 9. The differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. Preparation and characterization of liposomes

A pre-formulation study was carried out to select the most

homogeneous liposomal dispersions formed by stable and small vesicles. Different concentrations (from 30 to 120 mg/mL) of phospholipids, Lipoid S75, and ratio of water, ethanol and glycerol were tested to select the final formulation capable of stably loading 0.25 mg/mL of beclomethasone, which is the amount conventionally used in commercial topical formulations.

Dispersions were obtained by the one-step sonication method, which avoids the use of organic solvents and ensures the formation of homogeneously dispersed small vesicles [28]. The energy provided by sonication as a function of cycles (more than 10 and less than 50) and amplitude (12–15 μm) was varied to find standardized conditions that allowed the preparation of monodisperse formulations with distribution uniformity visible to naked eyes. At the same time, the concentration of phospholipid, being the key parameter, was progressively increased from 30 to 120 mg/mL. It was observed that using lower amounts of S75 (30, 60, and 90 mg/mL), irrespective to the performed sonication, a precipitate was found immediately after sonication. Only using 120 mg/mL of phospholipid, it was possible to stably load 0.25 mg/mL of beclomethasone inside the vesicles and precipitate was never observed. A mixture of water, ethanol and glycerol (1:0.5:0.5 % v/v) was chosen to stabilize and improve the system, taking advantage of the synergistic and beneficial properties provided by glycerol and ethanol, as described in previous studies [29,30]. This specific mixture produced vesicles of small size (~50 nm), with a very low polydispersity index (~0.14) and no detectable precipitate. These characteristics were observed not only immediately after preparation, but also over time by visual inspection (Table 2). The selected liposome formulation was enriched with mucin at two different concentrations (1 and 5 mg/mL), due to its mucoadhesive and moisturizing properties [29,31]. Empty vesicles, with and without mucin, were prepared as well and used as control. As shown in Table 2, the mean diameter of empty liposomes was ~ 49 nm, and the loading of beclomethasone did not cause any significant increase of vesicle size, which was ~ 48 nm ($p > 0.05$ between the two values). The size of empty liposomes enriched with 1 mg/mL of mucin slightly increased up to ~ 59 nm ($p < 0.05$ versus the size of empty liposomes), and that of empty liposomes enriched with 5 mg/mL of mucin strongly increased up to ~ 117 nm, indicating an important effect of this concentration of polymer on vesicle assembling. The behaviour of the liposomes enriched with 1 mg/mL of mucin was comparable to that of the empty 1 mucin- liposomes when beclomethasone was loaded; they were sized ~ 60 nm ($p > 0.05$ versus the size of empty 1mucin- liposomes), while those enriched with 5 mg/mL of mucin were much larger, measuring ~137 nm ($p < 0.05$ versus the corresponding empty one). Thus, the mucin affected the vesicle assembling as a function of the used concentration, and 5 mg/mL was a critical concentration because caused a significant variation of the packing associated with a reduction of the curvature radius. The loading of beclomethasone allowed the formation of more homogenous vesicles as all formulations (liposomes, 1mucin- liposomes and 5mucin- liposomes) were monodispersed (polydispersity index ≤ 0.23) while the corresponding empty ones were slightly polydispersed (polydispersity index ≥ 0.27). All vesicles were highly negatively charged regardless of their composition, which should positively affect dispersion stability due to the high electrostatic repulsion among vesicles. All vesicles were capable of incorporating high amount of beclomethasone as the loading efficiency of liposomes was ~ 70 %, and that of mucin- liposomes, irrespective of mucin concentration, was ~ 84–85 % ($p > 0.05$ versus the two values), probably because the polymer stabilizes the bilayer favouring the retention of beclomethasone.

Cryo-TEM observation of beclomethasone loaded liposomes confirmed the actual formation of lamellar vesicles and disclosed their structure. Liposomes and 1mucin- liposomes were mostly unilamellar (Figs. 1A and 1B) while 5mucin- liposomes were differently aggregated, since small unilamellar vesicles coexisted with multicompartment vesicles where smaller vesicles could be found inside the larger ones (Fig. 1C).

The stability of the vesicles over time was evaluated storing them at 25 °C for 5 months, and measuring their size, polydispersity index, and surface charge at scheduled intervals (Fig. 2). The surface charge of vesicles was stable during the storage, and it is not reported.

The mean diameter of beclomethasone loaded liposomes and 1mucin- liposomes remained almost unchanged during the five months of storage (≈55 nm, $p > 0.05$ among the values). The average diameter of 5mucin- liposomes, which was the larger one after preparation (~ 137 nm), slowly increased as early as the first month, and continued until the fifth month, reaching ~ 200 nm ($p < 0.05$ versus the initial value), which is nevertheless considered suitable for skin administration. In contrast, the polydispersity index of all stored formulations remained almost constant, confirming a homogeneous system regardless of the presence of mucin. So did the zeta potential of all formulations, which remained strongly negative (~ −80 mV) during the entire storage period (data not shown, $p > 0.05$).

Table 2
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and loading efficiency (E%) of freshly prepared empty and beclomethasone loaded liposomes, 1mucin- liposomes and 5mucin- liposomes. Each value represents the mean value ± standard deviation of at least six replicates (n=6).

	MD (nm)	PI	ZP (mV)	E (%)
Empty liposomes	*49 ± 4	▾0.23 ± 0.01	♪-54 ± 6	-
Empty 1mucin- liposomes	*59 ± 4	§0.32 ± 0.01	✳∞-77 ± 9	-
Empty 5mucin- liposomes	#117 ±5	0.27 ± 0.01	∞-74 ± 3	-
Beclomethasone liposomes	*48 ± 9	♥0.14 ± 0.05	✳∞-82 ± 5	©70 ± 3
Beclomethasone 1mucin- liposomes	*61 ± 5	♥0.17 ± 0.02	∞-71 ± 5	●84 ± 4
Beclomethasone 5mucin- liposomes	°137 ± 7	▾0.23 ±0.03	✳-84 ± 3	●85 ± 2

The same symbol (*, §, #, °, ♪, ∞, ▾, ♥, ●, ©, ✳) indicates values that are not statistically different ($p > 0.05$).

3.2. Biocompatibility of formulations

The viability of the human keratinocytes (Fig. 3A) incubated for 48 h with beclomethasone in dispersions or loaded in liposomes, 1mucin- liposomes, and 5mucin- liposomes at the highest dilutions (0.25 and 0.5 μg/mL of drug in cell medium) was, in general, ≈ 90 % ($p > 0.05$ versus control, non-treated cells) without statistical differences between samples, confirming high formulation biocompatibilities. The only difference was detected using the drug dispersion at the lowest dilutions (2.5 and 5 μg/mL of beclomethasone in cell medium), as the cell viability was slightly lower (~ 85 %, $p < 0.05$ versus control). Incubating fibroblasts with beclomethasone in dispersion or loaded in liposomes, biocompatibilities were confirmed (Fig. 3B). The viability of cells incubated with liposomes was ≈ 99 % ($p > 0.05$ versus control), in almost all cases. Biocompatibility slightly decreased to ~ 82 % ($p < 0.05$ versus viability of cells incubated with beclomethasone dispersion) when 1 and 5mucin- liposomes were used at the lower dilution (5 μg/mL of beclomethasone in cell medium). However, also these formulations may be considered biocompatible as mortality was always less than 20 % (Fig. 3B).

3.3. Inhibition of nitric oxide generation in cells and protective effect of the formulations against damages induced by nitrites

The nitric oxide is expressed in large amount in activated inflammatory cells stimulated with lipopolysaccharide and its overexpression is associated with the pathogenesis of acute and chronic inflammatory conditions of the skin. The ability of beclomethasone loaded in

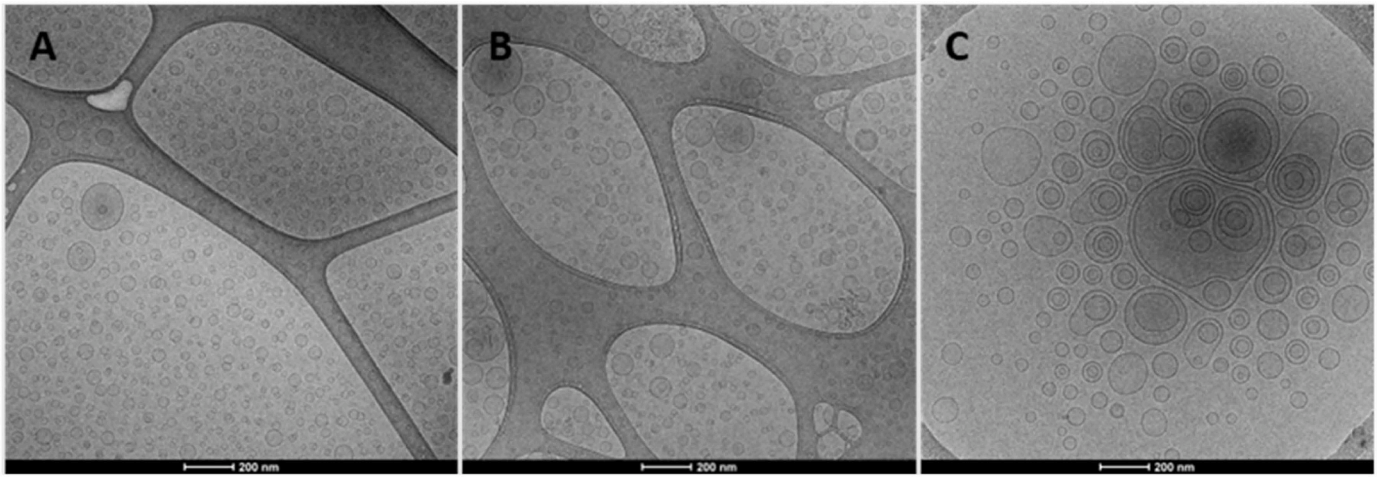

Fig. 1. Representative cryo-TEM images of liposomes (A), 1mucin-liposomes (B) and 5mucin-liposomes (C), all loading beclomethasone (0.25 mg/mL).

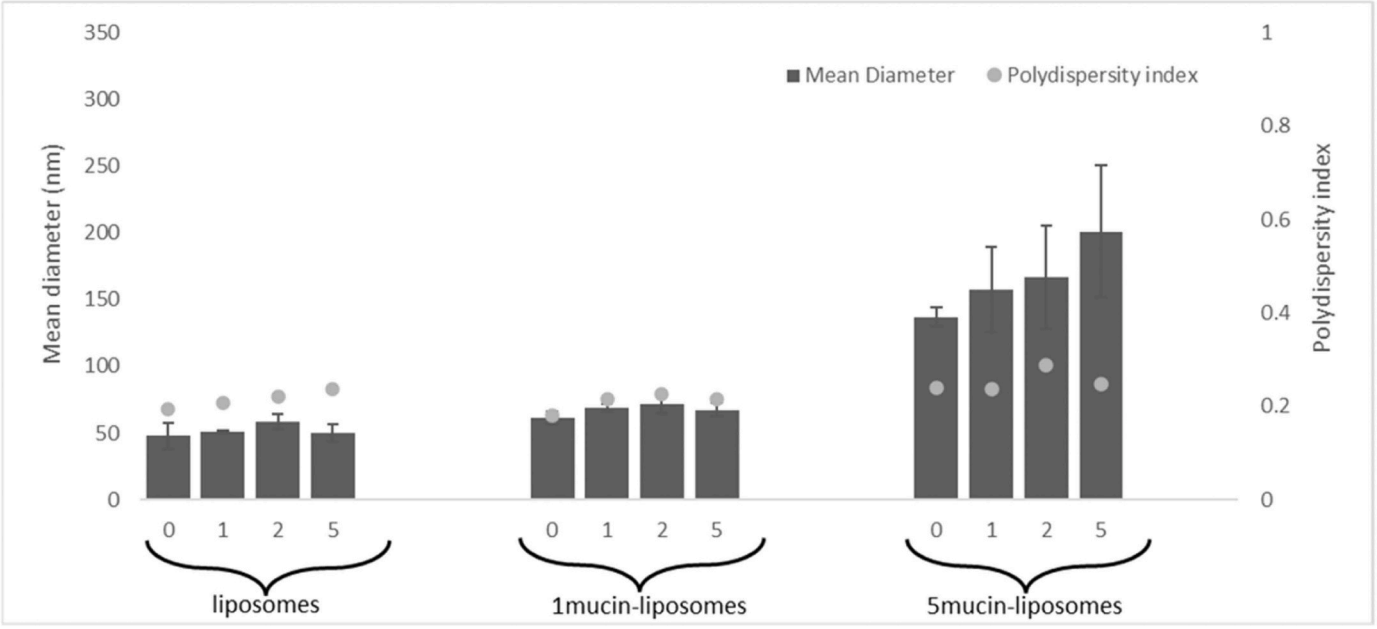

Fig. 2. Mean diameter (nm) and polydispersity index of beclomethasone loaded liposomes, 1mucin- liposomes, and 5mucin- liposomes stored for 5 months at 25 °C. Mean values (bars) ± standard deviations are reported (n = 3). The same symbol (* ° $^{+}$) indicate values that are not statistically different from each other ($p > 0.05$).

liposomes, 1mucin- liposomes, and 5mucin- liposomes to reduce nitric oxide production and inflammatory conditions in macrophages was then evaluated (Fig. 4). The treatment of macrophages with lipopolysaccharide led to a significant reduction of their viability, which decreased to ~ 65 % ($p < 0.05$ versus control) (Fig. 4A). The pre-treatment of cells with beclomethasone in dispersion sufficiently protect them, as the viability was ~ 80 %, irrespective of the dilution evaluated ($p > 0.05$ versus viability of cells damaged with lipopolysaccharide and untreated; $p < 0.05$ versus control). On the other hand, the pre-treatment of macrophages with beclomethasone loaded in liposomes, irrespective to mucin concentration, completely protected the cells, as the viability was ~ 100 % ($p > 0.05$ versus control; $p < 0.05$ versus viability of cells treated with dispersion). The release of nitric oxide followed a similar pattern as the pre-treatment of cells with beclomethasone in dispersion did not effectively reduce the amount of nitric oxide released (~ 80 %, $p < 0.05$ versus control; $p > 0.05$ versus LPS), while using liposomes, it was significantly reduced (~ 60 %, $p < 0.05$ versus value obtained with dispersion) especially when the lower concentration of mucin was used. Results confirmed the capability of the beclomethasone loaded liposomes and mucin- liposomes of inhibiting the toxic effect provided by the treatment with lipopolysaccharide and ensuring the inhibition of all the processes associated with inflammation (Fig. 4B).

3.4. *In vivo healing potential of liposomes*

The potential of beclomethasone loaded liposomes and mucin- liposomes to protect the skin and counteract the pro-inflammatory effect induced in mice with TPA was evaluated. It was topically applied on shaved skin of mice to induce damages, oedema, infiltration of inflammatory cells, and loss of *stratum corneum* or ulceration. The damaged and untreated skin had a diffuse lesioned area with several crusts, covered with necrotic and desquamated tissue (Fig. 5, untreated). The treatment of this damaged skin with beclomethasone in dispersion (0.25 mg/mL) slightly inhibited stratum corneum loss and crust formation as it appeared less damaged than that untreated, even if some crusts were still noticeable (Fig. 5, dispersion). On the contrary, the treatment with

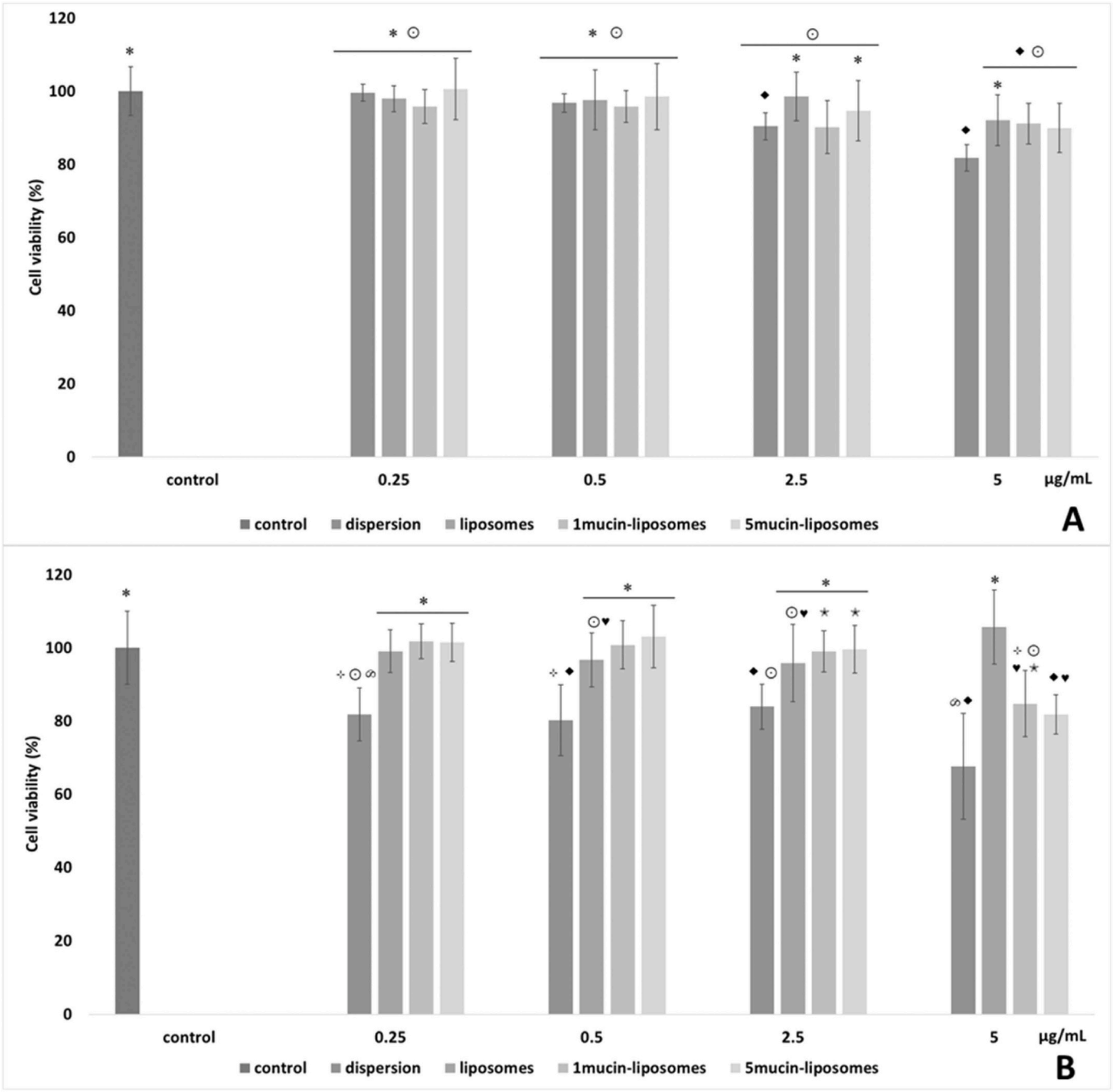

Fig. 3. Viability of keratinocytes (panel A) and fibroblasts (panel B) incubated for 48 h with beclomethasone in comparison with non-treated cells (control) in dispersion or loaded in liposomes, 1mucin- liposomes and 5mucin- liposomes diluted in the cell medium to reach 0.25, 0.5, 2.5 and 5 µg/mL of drug. Data are reported as mean values ± standard deviations (n = 9). Same symbols (*, ⊙, ◆, ♥, ♣, ÷, ★) indicates values that are not statistically different from each other ($p > 0.05$).

beclomethasone loaded in liposomes and mucin- liposomes reduced the pro-inflammatory effect of TPA, especially in the case of 5mucin-liposomes, allowing a complete re-epithelialization of the treated area, which, at visual inspection, appeared similar to that of untreated mice (Fig. 5). The treatment with Menaderm Simplex® cream, used as commercial reference, also significantly reduced the skin damages. However, in this case, a marked *oedema* was still visible under visual inspection and the skin appeared very thin and dry.

To confirm the beneficial effect of beclomethasone loaded liposomes and to assess the degree of skin inflammation, the inhibition of oedema (which is indicative of fluid retention and swelling), and the myeloperoxidase production (an enzyme released by neutrophils and macrophages), were quantified. Oedema and myeloperoxidase release are both predictive of skin damage and inhibition of normal re-epithelialization and restoration of physiological conditions (Fig. 6 A and B). The myeloperoxidase concentration in the tissue inflamed by TPA and untreated was ~ 258 ng/mL ($p < 0.05$ versus healthy skin). The treatment with beclomethasone in dispersion, loaded in liposomes, and in Menaderm Simplex® allowed a decrease of this value up to ~ 111 ng/mL, ~ 112 ng/mL, and ~ 110 ng/mL, respectively ($p < 0.05$ versus healthy skin), and the application of beclomethasone loaded in 1 and 5mucin- liposomes, led to a further inhibition of the myeloperoxidase production (~ 88 and ~ 66 ng/mL respectively, $p < 0.05$ versus untreated skin and healthy skin) (Fig. 6A). Moreover, the *oedema* was

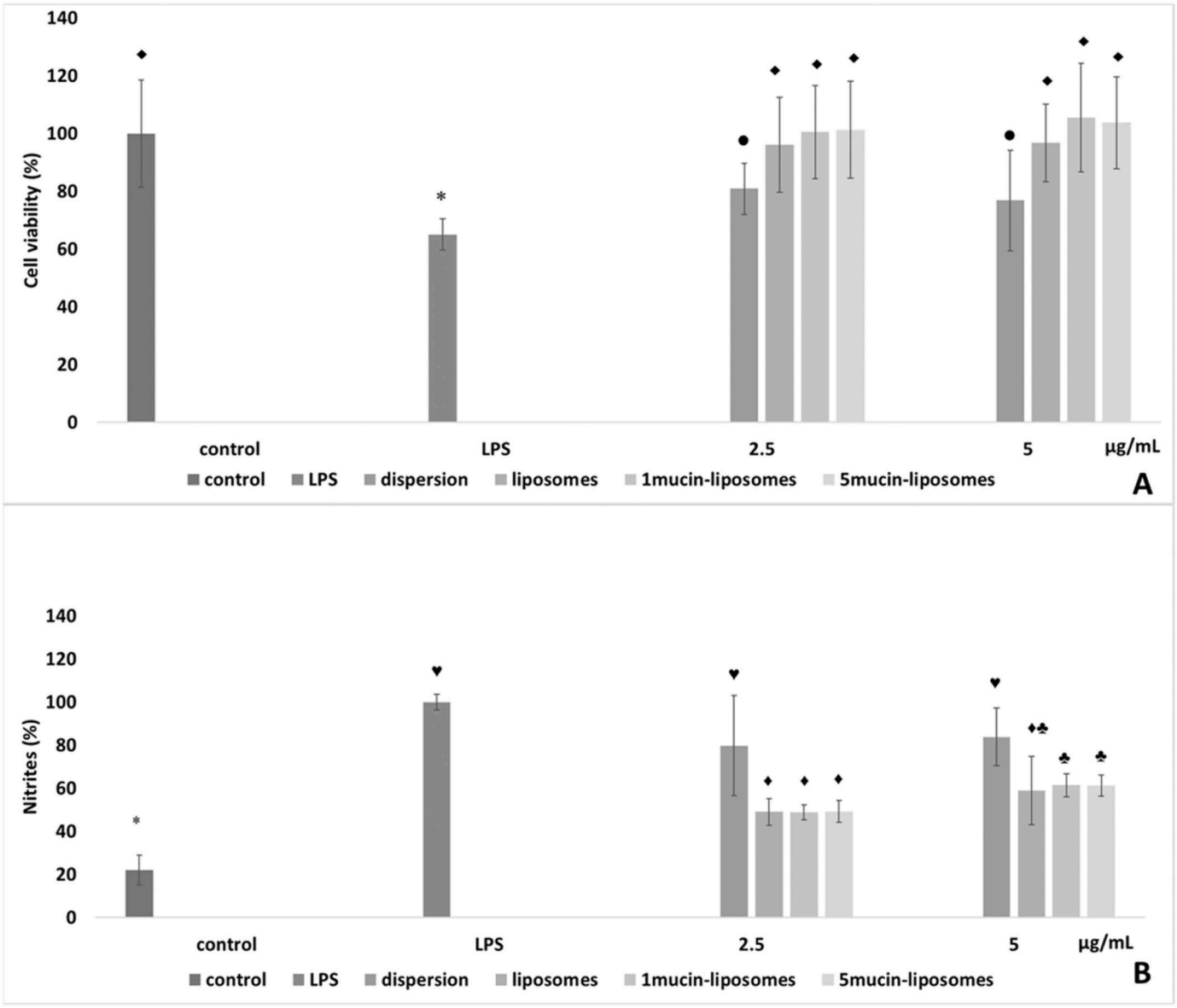

Fig. 4. Viability (A) and release of nitric oxide (B) from macrophages damaged with lipopolysaccharide (LPS) and pre-treated with beclomethasone (2.5 and 5 µg/mL) in dispersion or loaded in liposomes, 1mucin-liposomes and 5mucin-liposomes in comparison with non-treated cells (control). Data are reported as mean values (n = 9) ± standard deviations (error bars). The same symbol (* ♥ ◆ ♣ ●) indicate values that are not statistically different from each other ($p > 0.05$).

drastically reduced by the treatment with liposomes and mucin-liposomes, regardless of their composition, as the weight of the skin differed from that of untreated mice, especially when mucin- liposomes were used (oedema inhibition ~ 66 %, $p < 0.05$ versus untreated skin, Fig. 6). The in vivo results confirmed the protective effect of beclomethasone, formulated in Menaderm Simplex® cream and the improved efficacy obtained with the delivery in mucin- liposomes.

3.5. Histological examination

Histological analysis (Fig. 7) confirmed the macroscopic observation (Fig. 5). In fact, the TPA-damaged and untreated skin had numerous major pustules with involvement of the entire epidermis and severe exocytosis (Fig. 7A). The inflammatory infiltrate (circular areas in epidermis and dermis) in all cases was formed by mononuclear cells, such as monocytes, macrophages, neutrophils, and eosinophils. In addition, oedematous fluid and mild inflammatory infiltrate in dermis and hypodermis were also observed. However, topical application of beclomethasone loaded 5mucin-liposomes effectively reduce the damages induced by TPA, exerting comparable results to those of the Menaderm Simplex®, as all of them exhibited slight or moderate inflammatory infiltration in dermis and slightly in hypodermis, especially for 0.5 mucin where only a slow inflammation in dermis was detected, confirming the protective effect of beclomethasone formulated in Menaderm Simplex® cream and in mucin-liposomes (Figs. 7B and 7C).

4. Discussion

The treatment of chronic disorders at skin level, associated with inflammation processes, requires careful tailoring of formulation properties to optimize the effect and avoid the failure of the therapy. The use of glucocorticoids, which are considered the topical drugs of choice for these disorders, requires high concentrations in the treated area for prolonged periods, so novel approaches are needed to enhance their effects, while reducing or avoiding the onset of adverse phenomena.

Liposomes have been considered for years ideal carriers for the

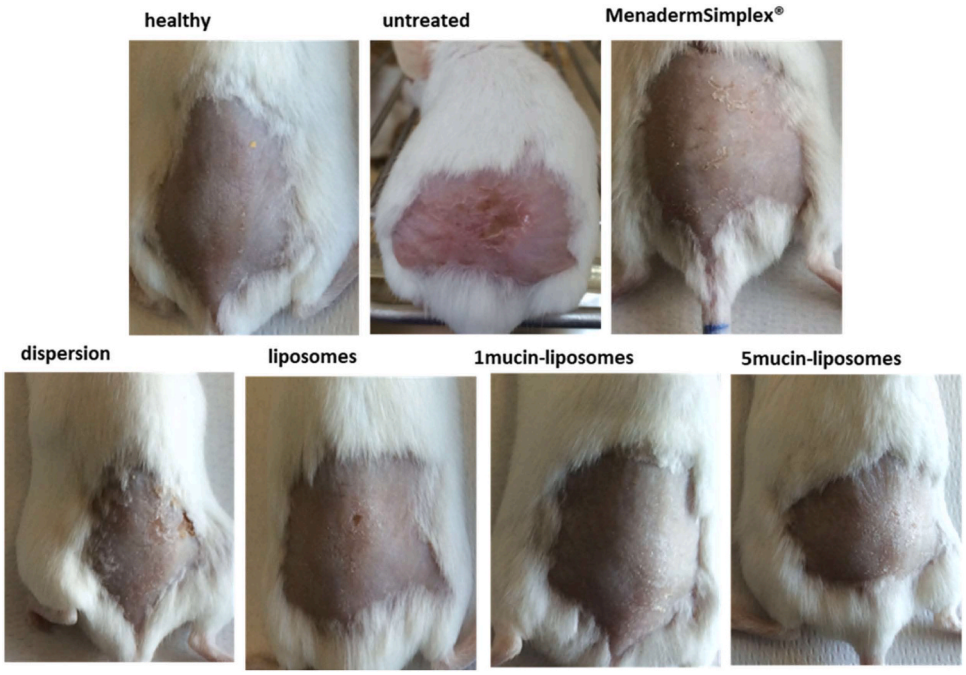

Fig. 5. Representative pictures of dorsal skin areas either not damaged (healthy) or damaged with TPA and treated with saline, untreated, or treated with Menaderm Simplex® cream, used as commercial reference, or with beclomethasone in dispersion or loaded in liposomes, 1mucin-liposomes or 5mucin-liposomes.

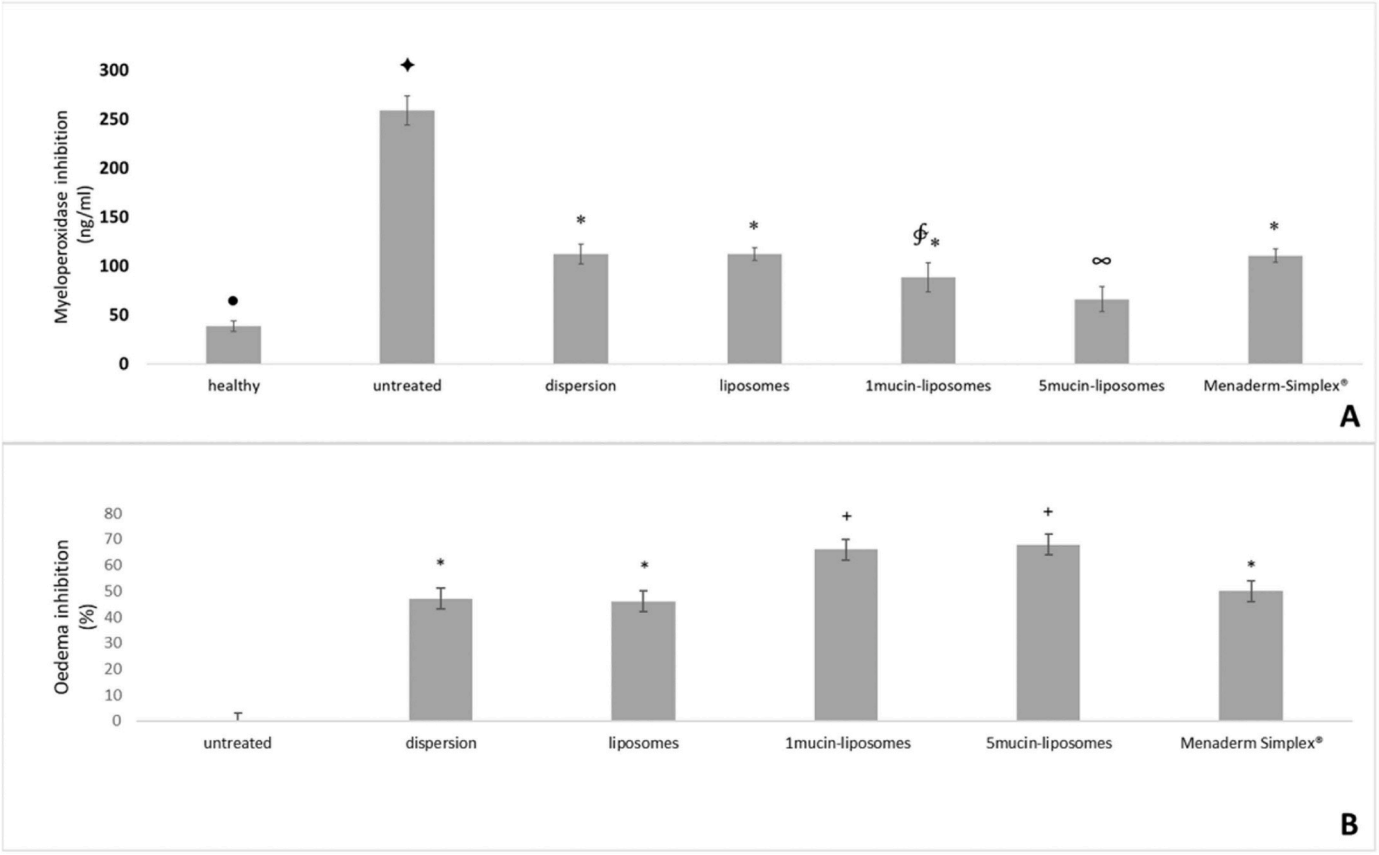

Fig. 6. Myeloperoxidase concentration (A) and *oedema* inhibition (B) induced in mice skin damaged with TPA (untreated) and treated with saline (healthy), beclomethasone in dispersion or loaded in liposomes, 1mucin-liposomes and 5mucin-liposomes or Menaderm Simplex® cream. Data are reported as mean values ± standard deviation (error bars). Same symbols (●, ✦, *, ◆, ∮, +,) indicates values that are not statistically different from each other ($p > 0.5$).

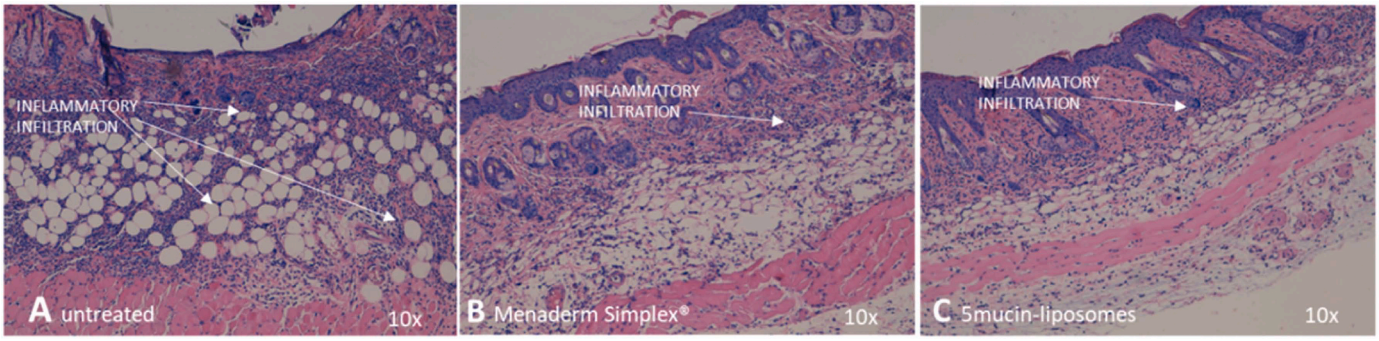

Fig. 7. Representative images of the histological analysis of mouse skin damaged by TPA (untreated) and treated with a commercial cream (Menaderm Simplex®) or beclomethasone loaded in 5mucin-liposomes.

delivery of a wide variety of molecules at skin level as they are able to interact with it, favouring the passage and accumulation of the drug in its different layers [32]. The optimal size for a preferred deposition at epidermis and dermis level is around 100 nm, as reported by other authors [33,34]. Despite their advantages, if not properly formulated and tailored, nanocarriers may pose potential risks, facilitating the penetration of the payload deep into the skin or even to the systemic circulation, which in this case should be avoided [33,34].

Considering this, in this study beclomethasone loaded mucin-enriched liposomes were specifically designed for the treatment of cutaneous and semi-chronic inflammatory disorders, aiming at improving the residence of the payload to the injured site, thus avoiding undesired distribution [32]. Consistently with one of our previous works, natural-based phospholipids, water, glycerol, and ethanol (1:0.5:0.5 % v/v) were mixed to obtain versatile and stable formulations, suitable for skin delivery, in one, easy, and low-dissipative step [30]. Innovative systems were developed adding mucin, which thanks to its bioadhesiveness was expected to prolong the residence time of the formulations on the skin improving the efficacy of beclomethasone [35–37]. From the technological point of view, it was successfully loaded in all the liposome formulations as suggested by the unchanged highly negative surface charge in comparison with empty vesicles, which was due to the negative group of zwitterionic phosphatidylcholine at skin pH (5.5), and because the loading efficiency (E%) was always higher than 70 %, reaching ≈ 85 % when mucin was added to the formulation [38–40]. All the vesicles were small and homogeneously dispersed. Only those containing the higher amount of mucin were larger and less homogeneous, probably because the used concentration (5 mg/mL) was critically affecting vesicle assembling and structure, since small unilamellar vesicles coexisted within multicompartment vesicles. Previous findings have suggested that multicompartment vesicles may be ideal systems for favouring membrane diffusion (e.g. skin, intestine, mucosae) and facilitating the accumulation of the payload at the desired site [41]. Indeed, it seems that the larger and outer vesicles adhering to the skin may interact with it, freeing the inner vesicles, which can then penetrate into the skin already perturbed by the larger ones [41,42]. When mucin was added to the formulation, also the loading efficiency (E%) was found to be higher (≈ 85 %). According to some authors, polymer molecular weight, branching, electrical charge, and hydrophobicity could affect not only release behaviour and delivery capabilities of phospholipid vesicles but also their loading, improving their physico-chemical properties and performances [43].

In vitro studies performed on the most representative cells of the skin (*i.e.*, keratinocytes and fibroblasts), confirmed the high biocompatibility of formulations and their capability of reducing macrophages' over-expression of nitric oxide, which is associated with the pathogenesis of acute and chronic skin inflammatory disorders [44]. Consistently with previous studies, liposomes counteracted the damages and death of cells injured with lipopolysaccharide, probably thanks to their ability to interact with macrophages, to promote cell adhesion and contact, and to release of beclomethasone where it can exert its beneficial activity [45].

Based on these findings, the actual potential of these innovative systems in protecting the skin from TPA-induced damages and inflammation was evaluated in mice and compared to the commercial cream, used as reference[46]. The well-known efficacy of the commercial cream is due to its healthy occlusive properties that promotes hydration at skin level, which in turn leads the perturbation of that barrier and facilitates the passage of the payload. Liposomes loading beclomethasone, especially mucin-liposomes, were as effective as the commercial cream reducing tissue damages and preventing the loss of the most superficial layers of the skin caused by the daily application of TPA, in agreement with previous findings [46]. It is likely that the phospholipids induce the secretion of endogenous hyaluronic acid, hence accelerating the healing process [43]. By contrast, oedema and myeloperoxidase inhibition were effectively reduced only when mucin-liposomes were applied on the injured skin. This was visually confirmed by the almost complete re-epithelialization of the treated area. Considering all the results, the efficacy of beclomethasone loaded mucin-liposomes is due to a dual action: the adhesive property of the mucin, which in turn improve the residence time of the vesicles in the skin, and the delivery of beclomethasone in the injured area. In vitro and in vivo studies have confirmed the achievement of balanced formulations in which the combination of mucin and phospholipids improved the delivery properties of formulation and the effectiveness of beclomethasone at the target area.

5. Conclusion

In this study, the improved effect provided by mucin-enriched liposomes in the delivery of beclomethasone on skin disorders associated with inflammatory processes was demonstrated. Indeed, the therapeutic properties of beclomethasone were enhanced especially when it was delivered in mucin-liposomes, which could retain the payload over time increasing its in vitro and in vivo biological activity. The topical application of mucin-liposomes may be of value in innovative and alternative dermal approach for the topical treatment of skin disorders, improving adhesion and avoiding the common disadvantages associated with the application of glucocorticoids on the skin.

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CRediT authorship contribution statement

Amparo Ruiz-Sauri: Validation, Methodology, Investigation, Data curation. **Xavier Fernandez-Busquets:** Methodology, Investigation. **Maria Manconi:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Maria Letizia Manca:** Writing – review & editing, Validation, Supervision, Data curation,

Conceptualization. **Iris Usach:** Writing – original draft, Investigation. **José Esteban Peris:** Validation, Supervision, Conceptualization. **Donatella Valenti:** Methodology, Investigation. **Octavio Diez-Sales:** Supervision, Methodology, Investigation. **Matteo Aroffu:** Writing – review & editing, Validation, Data curation. **Mohamad Allaw:** Writing – review & editing, Formal analysis, Data curation. **Matteo Perra:** Writing – review & editing, Writing – original draft, Validation, Data curation. **Biancamaria Baroli:** Writing – review & editing, Validation, Supervision, Data curation. **Ines Castangia:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

Author declares that there are no conflicts of interest.

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