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ORIGINAL RESEARCH

Factors influencing transfection efficiency of pIDUA/nanoemulsion complexes in a mucopolysaccharidosis type I murine model

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Abstract: Mucopolysaccharidosis type I (MPS I) is an autosomal disease caused by alpha-L-iduronidase (IDUA) deficiency. This study used *IDUA* knockout mice as a model to evaluate whether parameters such as dose of plasmid and time of treatment could influence the transfection efficiency of complexes formed with PEGylated cationic nanoemulsions and plasmid (pIDUA), which contains the gene that encodes for IDUA. Formulations were composed of medium chain triglycerides, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(amino[polyethylene glycol]-2000), 1,2-dioleoyl-sn-glycero-3-trimethylammonium propane (DOTAP), glycerol, and water and were prepared by the adsorption or encapsulation of preformed pIDUA–DOTAP complexes by high-pressure homogenization. A progressive increase in *IDUA* expression was observed with an increase in the dose and time of transfection for mice treated with both complexes (adsorbed and encapsulated), especially in the liver. Regardless of the complex administered, a significant increase in IDUA activity was detected in lungs and liver compared with nontreated MPS I when a dose of 60 µg was administered and IDUA activity was measured 7 days postadministration. Tissue sections of major organs showed no presence of cell necrosis, inflammatory infiltrate, or an increase in apoptosis. Furthermore, immunohistochemistry for CD68 showed no difference in the number of macrophage cells in treated and nontreated animals, indicating the absence of inflammatory reaction caused by the treatment. The data set obtained in this study allowed establishing that factors such as dose and time can influence transfection efficiency in different degrees and that these complexes did not lead to any lethal effect in the MPS I murine model used.

Keywords: cationic nanoemulsions, DSPE-PEG, MPS I, plasmid, pIDUA

Introduction

Gene therapy has emerged as a promising strategy for a wide range of inherited and acquired diseases.[1] For successful gene therapy, genes have to be delivered to the nucleus of target cells in a safe and effective manner. In the past few decades, a number of viral and nonviral carriers that can be used to transfer foreign genetic material into cells have been developed aimed at enhancing gene transfer in vitro and in vivo.[2,3] Viral vectors have been widely used to achieve efficient gene transfer. However, issues related to safety, risk of insertional mutagenesis, and the strong immune response generated by these vectors have limited their application.[1]

Nonviral gene carriers have been extensively investigated as alternatives to viral vectors for gene delivery since they offer considerable advantages such as safety and easy production.[3,4] Cationic nanoemulsions have been considered as potential nonviral nucleic acid delivery systems.[5] We recently described the design of PEGylated cationic

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nanoemulsions as a delivery system for a plasmid containing the gene that codes for α-L-iduronidase (pIDUA).[6] A deficiency of this enzyme results in the lysosomal storage of the glycosaminoglycans, thereby causing the lysosomal storage disorder mucopolysaccharidosis type I (MPS I). The overall results showed that intravenous administration of pIDUA/PEGylated cationic nanoemulsions (obtained by adsorption or encapsulation of preformed pIDUA-cationic lipid complexes) can significantly increase IDUA activity and expression in different organs, especially in the lungs and liver.[7]

Some well-documented studies have demonstrated that the single or multiple intravenous administration of cationic lipid–DNA complexes is often related to a dose- or time-dependent effect, in terms of transfection efficiency and toxicity, especially in the liver and lungs.[8–10] Similarly, the aim of this study was to evaluate the effect of the dose (30 and 60 μg) and time (2 or 7 days postinjection) on transfection efficiency of pIDUA/PEGylated cationic nanoemulsion complexes in a MPS I murine model. The gene expression and IDUA activity were evaluated after the intravenous administration of complexes obtained by the adsorption or encapsulation of preformed pIDUA–cationic lipid complexes into the oil phase of nanoemulsions. The potential toxicity of such complexes on main organs (ie, lungs, spleen, liver, and kidneys) was also evaluated.

Materials and methods

Materials

Medium-chain triglycerides (MCTs), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(amino[polyethylene glycol]-2000) (DSPE-PEG) were purchased from Lipoid (Ludwigshafen, Germany). Glycerol and chloroform were obtained from Merck (Rio de Janeiro, Brazil). 4-Methylumbelliferylalpha-L-iduronide was purchased from Glycosynth (Warrington, UK), and TRIzol® Reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Platinum SYBR Green qPCR SuperMix-UDG kit was obtained from Thermo Fisher Scientific, and High-Capacity cDNA Reverse Transcription Kit was purchased from Thermo Fisher Scientific. All other chemicals were of analytical grades.

Preparation of PEGylated cationic nanoemulsions

Blank formulations were composed of 1.6% (w/w) MCT, 0.364% (w/w) DOPE, 0.036% (w/w) DSPE-PEG, 0.028% DOTAP, 2.25% (w/w) glycerol, and Milli-Q® water up to 100% (PEGylated nanoemulsion [NEP]). The lipids were dissolved in chloroform and evaporated under reduced pressure (50°C). The lipid film was hydrated with the water phase containing glycerol, vortexed for 3 min, and left overnight. Next day, the formulations were vortexed for another 2 min and sonicated for 15 min at 37°C. Finally, the emulsions were passed through high-pressure homogenization equipment at 10,000 psi for 10 cycles.

Preparation of pIDUA/PEGylated cationic nanoemulsion complexes

The plasmid pIDUA containing the human *IDUA* cDNA under the control of pCMV promoter was associated with nanoemulsions either by adsorption (NEP/pIDUA$_A$) on NEP or by incorporation of hydrophobic pIDUA–DOTAP complex into formulations during the nanoemulsion preparation (NEP/pIDUA$_E$). For the adsorption of pIDUA, a solution of pIDUA was added to nanoemulsions at room temperature and left for 30 min. For the encapsulation of pIDUA, the hydrophobic pIDUA/DOTAP complex (prepared as previously described in detail by Fraga et al[7]) was added into the lipid mixture during nanoemulsion preparation followed by the high-pressure homogenization procedure. NEP/pIDUA$_A$ and NEP/pIDUA$_E$ were prepared at +4/− charge ratio (charge ratio of cationic lipid/DNA phosphate groups).

Physicochemical characterization

The droplet size, polydispersity index, and ζ-potential of blank PEGylated nanoemulsion and complexes were determined by photon correlation spectroscopy (PCS) at 90° and electrophoretic mobility (3000HS Zetasizer). The samples were adequately diluted and analyzed just after preparation and after 2 months of storage at 4°C.

In vivo experiments

Experiments were performed as previously described by Fraga et al.[6,7] Briefly, four groups of mice (4–6 months) received 200 μL of complexes containing 30 or 60 μg of pIDUA obtained by adsorption (NEP/pIDUA$_A$, n=3/group) or encapsulation (NEP/pIDUA$_E$, n=3/group) through intravenous injection in the tail vein. We compared these groups with nontreated MPS I mice of same age (MPS I, n=5). Animals were euthanized 2 (60 μg) or 7 days (30 and 60 μg) after injection by cervical dislocation under anesthesia. Liver, lungs, kidneys, and spleen were isolated, and a piece of each was flash frozen in liquid nitrogen for biochemical analysis and mRNA extraction, with the rest used for histology. Before euthanizing the mice, serum was collected by retroorbital puncture. Data of mice receiving 30 μg and euthanized 2 days postinjection were compared with our previous work, which followed the same protocol.[7]

Gene expression assay to evaluate *IDUA* transcription levels from the plasmid was performed by real-time polymerase chain reaction with Platinum SYBR Green qPCR SuperMix-UDG kit. Approximately 30 mg of flash frozen tissues were used for mRNA extraction with TRIzol® Reagent, and the conversion of RNA to cDNA was performed using High-Capacity cDNA Reverse Transcription Kit. *GAPDH* was used as a reference gene to account for any variance in the quality of mRNA and the amount of input cDNA. The following primers were used: forward *IDUA* 5′-CACTGGCTGCTGGAGCTT-3′, reverse *IDUA* 5′-GCTCAAACCCTGGGAGGA-3′; forward *GAPDH* 5′-CCCATCACCATCTTCCAGG-3′, reverse *GAPDH* 5′-CATATTTGGCAGCTTTCTCC-3′. The polymerase chain reaction was carried out according to the manufacturer's instructions, and the results were presented as described by Livak and Schmittgen.[11]

For IDUA activity measurements, ~30 mg of tissues were homogenized in distilled water. IDUA activity assay was performed incubating protein extracts with fluorescent substrate 4-methylumbelliferyl α-L-iduronide at 37°C for 1 h in sodium formate buffer (pH 2.8) with some modifications.[12] Fluorescence was measured at 365 nm (excitation) and 450 nm (emission) using a fluorescence spectrophotometer, SpectraMax M2. Results are expressed as nanomole per hour per milligram of protein. Protein content was measured using the method described by Lowry et al.[13]

Histological analysis

Tissues were fixed in buffered formalin and processed and embedded in paraffin wax. Thin cross-sections were submitted to routine histological processing, stained with hematoxylin–eosin (H–E), and analyzed for signs of toxicity.

Immunohistochemistry

Immunohistochemistry for CD68 was performed in kidneys, liver, spleen, and lungs samples using specific antibody (mouse polyclonal anti-CD68; Abcam, Cambridge, UK). A horseradish peroxidase-conjugated antirabbit IgG was used as secondary antibody. The slides were analyzed by a researcher blinded to the groups. Cells positively stained for CD68 were analyzed in 3–5 fields per slide, with a 200× magnification. Results are expressed as the number of CD68-positive cells per square millimeter of tissue sections.

Ethics

All experiments were approved by the ethics committee of our institution (Research Ethics Committee of Hospital de Clínicas de Porto Alegre – permit numbers 09-0334 and 12-0477). Animal procedures were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, monitored by our veterinarian, and designed to minimize animal suffering.

Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Differences were considered to be significant at $P<0.05$. Analysis of the data was performed using Student's *t*-test on IBM® SPSS® Statistics, v 18.0 (Quarry Bay, Hong Kong).

Results

Physicochemical characterization

Table 1 exhibits the droplet size, polydispersity index, and ζ-potential of NEP/pIDUA$_A$ and NEP/pIDUA$_E$.

As can be seen, the high-pressure homogenization procedure yielded monodisperse nanoemulsions (polydispersity index <0.2), exhibiting a mean droplet size of ~200–300 nm. Regardless of the method of incorporation (NEP/pIDUA$_A$ or NEP/pIDUA$_E$), an inversion of ζ-potential was detected from +30 to ~−10 mV. Such a result suggests that, at least partially, a part of the plasmid pIDUA is located at the oil/water interface of nanoemulsions.

In vivo experiments

Figure 1 exhibits the relative expression of *IDUA* by quantitative real-time polymerase chain reaction in different organs (lungs, spleen, liver, and kidneys) of MPS I mice. The Ct value of nontreated MPS I mice for *IDUA* expression was used to calibrate relative expression calculations (varied from 38 to 40). *IDUA* was expressed in main organs of treated MPS I mice. As shown in Figure 1, *IDUA* expression was higher in the lungs when the dose was increased from 30 to 60 μg and it was maintained after 7 days postinjection in animals treated with NEP/pIDUA$_A$ (1A). A progressive increase in *IDUA* expression was observed with an increase in the dose and time of transfection for mice treated with both complexes (NEP/pIDUA$_A$ and NEP/pIDUA$_E$), especially in the liver and kidneys. A higher *IDUA* expression in the spleen was detected in animals treated with 30 and 60 μg of pIDUA 7 days after the injection, regardless of the complex administered.

Table 1 Physicochemical properties of blank nanoemulsion and complexes

Code	Droplet size (nm)	PI	ζ-potential (mV)
NEP	225.8±1.5	0.10±0.06	+14.1±1.4
NEP/pIDUA$_A$	263.9±20.2	0.05±0.03	−9.8±6.7
NEP/pIDUA$_E$	242.5±2.1	0.12±0.04	−8.4±1.1

Note: Results represent the mean ± standard deviation of three experiments.
Abbreviations: NEP, PEGylated nanoemulsion; PI, polydispersity index.

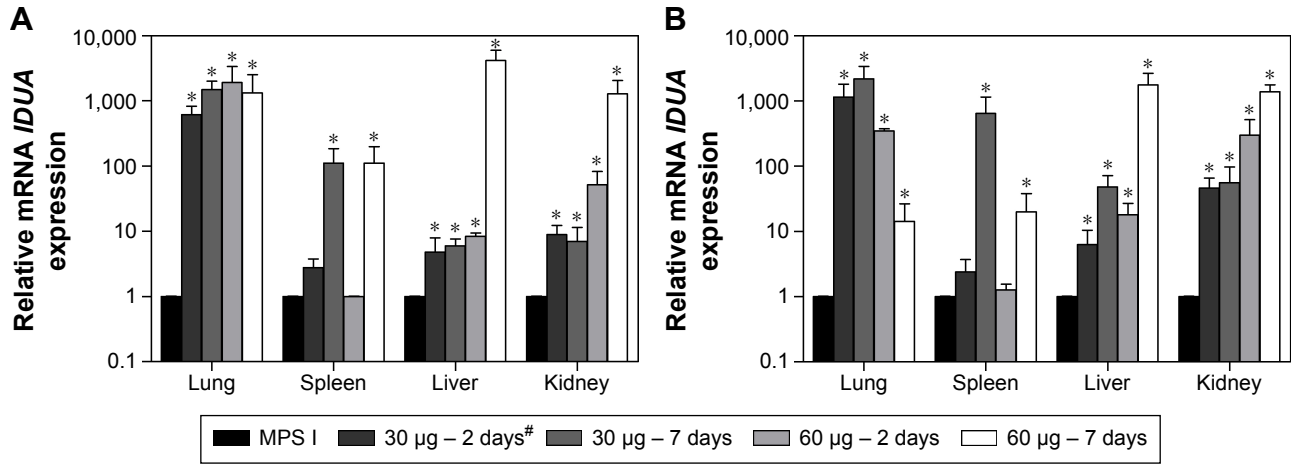

Figure 1 Relative expression of *IDUA* by real-time RT-qPCR in different organs of MPS I mice treated with NEP/pIDUA$_A$ (**A**) and NEP/pIDUA$_E$ (**B**).
Notes: Mice were treated with 30 µg of pIDUA (dark gray bars) or 60 µg of pIDUA (light gray bars) and euthanized 2 days after intravenous injection or 30 µg of pIDUA (medium gray bars) or 60 µg of pIDUA (white bars) and euthanized 7 days after intravenous injection. Nontreated MPS I mice were used as control (black bars). Expression ratios are relative to *GAPDH* endogenous control. Values represent the mean ± standard error of the mean and are presented in log scale. *Difference between treated and nontreated MPS I mice (Student's *t*-test, $P<0.05$). #Data from Fraga et al.[7]
Abbreviations: MPS I, mucopolysaccharidosis type I; RT-qPCR, quantitative real-time polymerase chain reaction.

In similar experimental conditions, IDUA activity was measured in the main organs and compared with nontreated MPS I mice, which were used as negative controls (Table 2). The results showed an increased IDUA activity in the lungs after the injection of 30 µg after 2 days postadministration (1.36 nmol/h/mg of protein) for NEP/pIDUA$_A$.

Regardless of the complex administered, a significant increase in IDUA activity ($P<0.05$) was detected in lungs and liver compared with nontreated MPS I when a dose of 60 µg was administered and IDUA activity was measured 7 days postadministration. Furthermore, significant IDUA activity ($P<0.05$) was present in the liver in all treatment conditions in accordance with the increase in *IDUA* expression in this organ.

Table 2 IDUA activity in different organs of MPS I mice treated with NEP/pIDUA$_A$ or NEP/pIDUA$_E$

Treatment	IDUA activity (nmol/h/mg of protein)			
	Lung	Spleen	Liver	Kidney
30 µg/2 days[a]				
NEP/pIDUA$_A$	1.36±0.13*	0.10±0.01	0.73±0.03*	0.59±0.10*
NEP/pIDUA$_E$	0.39±0.07	0.17±0.02*	1.10±0.03*	0.59±0.02*
30 µg/7 days				
NEP/pIDUA$_A$	0.24±0.02	0.10±0.02	0.52±0.07	0.18±0.04
NEP/pIDUA$_E$	0.27±0.04	0.09±0.03	0.67±0.09*	0.18±0.01
60 µg/2 days				
NEP/pIDUA$_A$	0.28±0.03	0.14±0.01	0.48±0.06	0.24±0.03
NEP/pIDUA$_E$	0.27±0.05	0.18±0.05	0.61±0.03*	0.28±0.03
60 µg/7 days				
NEP/pIDUA$_A$	0.41±0.04*	0.12±0.05	0.55±0.05*	0.29±0.10
NEP/pIDUA$_E$	0.42±0.06*	0.03±0.008	0.54±0.04*	0.23±0.03
MPS I (nontreated)	0.26±0.02	0.10±0.01	0.35±0.06	0.25±0.03

Notes: Results represent the mean ± standard error of the mean. *Difference between treated and nontreated MPS I mice (Student's *t*-test, $P<0.05$). [a]Data from Fraga et al.[7]
Abbreviations: MPS I, mucopolysaccharidosis type I; NEP, PEGylated nanoemulsion.

Histological and immunohistochemistry analysis

Finally, the potential toxicity of pIDUA/PEGylated cationic nanoemulsion complexes was evaluated in dissected major organs, such as liver, lungs, kidneys, and spleen, 7 days postadministration of 60 µg of pIDUA (Figures 2 and 3). Tissue sections of major organs showed no presence of cell necrosis, inflammatory infiltrate, or an increase in apoptosis. Only mild hydropic degeneration was detected in the liver when compared with nontreated MPS I mice, regardless of the type of complex (NEP/pIDUA$_A$ or NEP/-pIDUA$_E$) that was administered intravenously (Figure 2). Immunohistochemistry for CD68, a macrophage lineage cell marker, showed no quantitative difference ($P>0.05$) in the number of these cells in treated and nontreated animals, indicating the absence of inflammatory reaction caused by the treatment (Figure 3).

Discussion

We have recently described an increase in IDUA activity and expression in different organs after the intravenous administration of pIDUA/PEGylated cationic nanoemulsion complexes.[7] However, in this study, our aim was to investigate the effect of dose and time on these parameters.

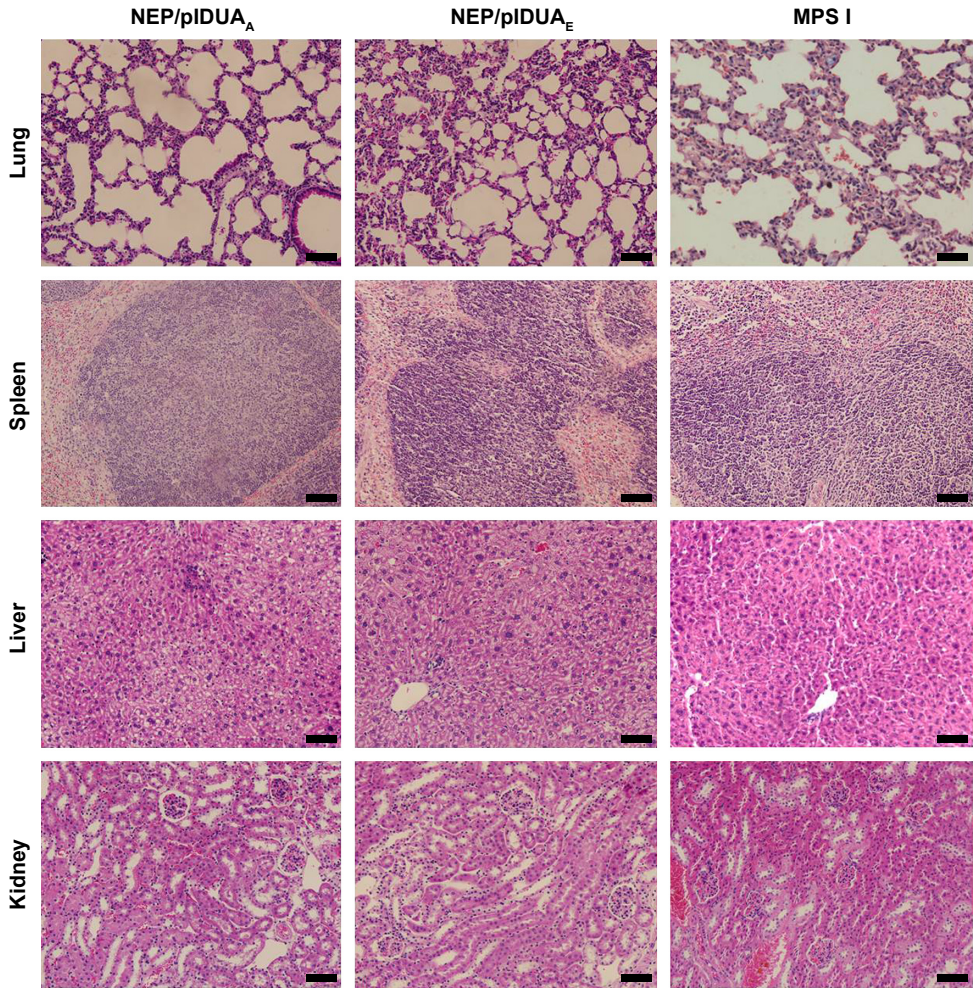

Figure 2 Representative histological sections stained with H–E of different organs of treated groups (NEP/pIDUA$_A$ and NEP/pIDUA$_E$) and of negative control (MPS I).
Notes: Scale bars in figure represent 100 µm. Magnification ×200.
Abbreviations: H–E, hematoxylin–eosin; MPS I, mucopolysaccharidosis type I; NEP, PEGylated nanoemulsion.

Since transfection efficiency is a measure prone to many variable factors, including those related to the properties of vectors, in this study, we evaluated the physicochemical properties of complexes.

The results of physicochemical properties are in agreement with those observed in our previous studies.[6,7] The main physicochemical properties of complexes remained similar after 60 days of storage at 4°C. This could be related to the stability provided by the PEG moiety of DSPE-PEG, since its steric hindrance may prevent the aggregation and neutralization of positive charge of pIDUA/PEGylated cationic nanoemulsion complexes.[14]

To assess the carrier's ability to efficiently transfect cells in vivo, the relative quantification of *IDUA* and *GAPDH* gene expressions in the organs of animals in the treatment groups was performed using cDNA samples from different tissues. Mice were treated with NEP/pIDUA$_A$ or NEP/pIDUA$_E$ at a final dose of 30 or 60 µg and euthanized 2 or 7 days postinjection. Animals treated with 30 µg of pIDUA and euthanized 2 days after the injection were from our previous work, performed under the same conditions,[7] and were included in this study for comparison purposes. That allowed us to show a progressive increase in *IDUA* expression with an increase in the dose and time of transfection, for mice treated with both complexes (NEP/pIDUA$_A$ and NEP/pIDUA$_E$). Information about the effect of time and dose can vary greatly in the literature and depends on the model used. Our results follow in line with those reported by Kim et al[8] and Yoo et al,[10] in which an increase in pDNA dose leads to higher gene expression, showing that an effect of dose and time seems to occur.

We have previously speculated that the increased IDUA activity in the lungs 2 days after the administration of 30 µg of NEP/pIDUA$_A$ results from a possible lung embolization

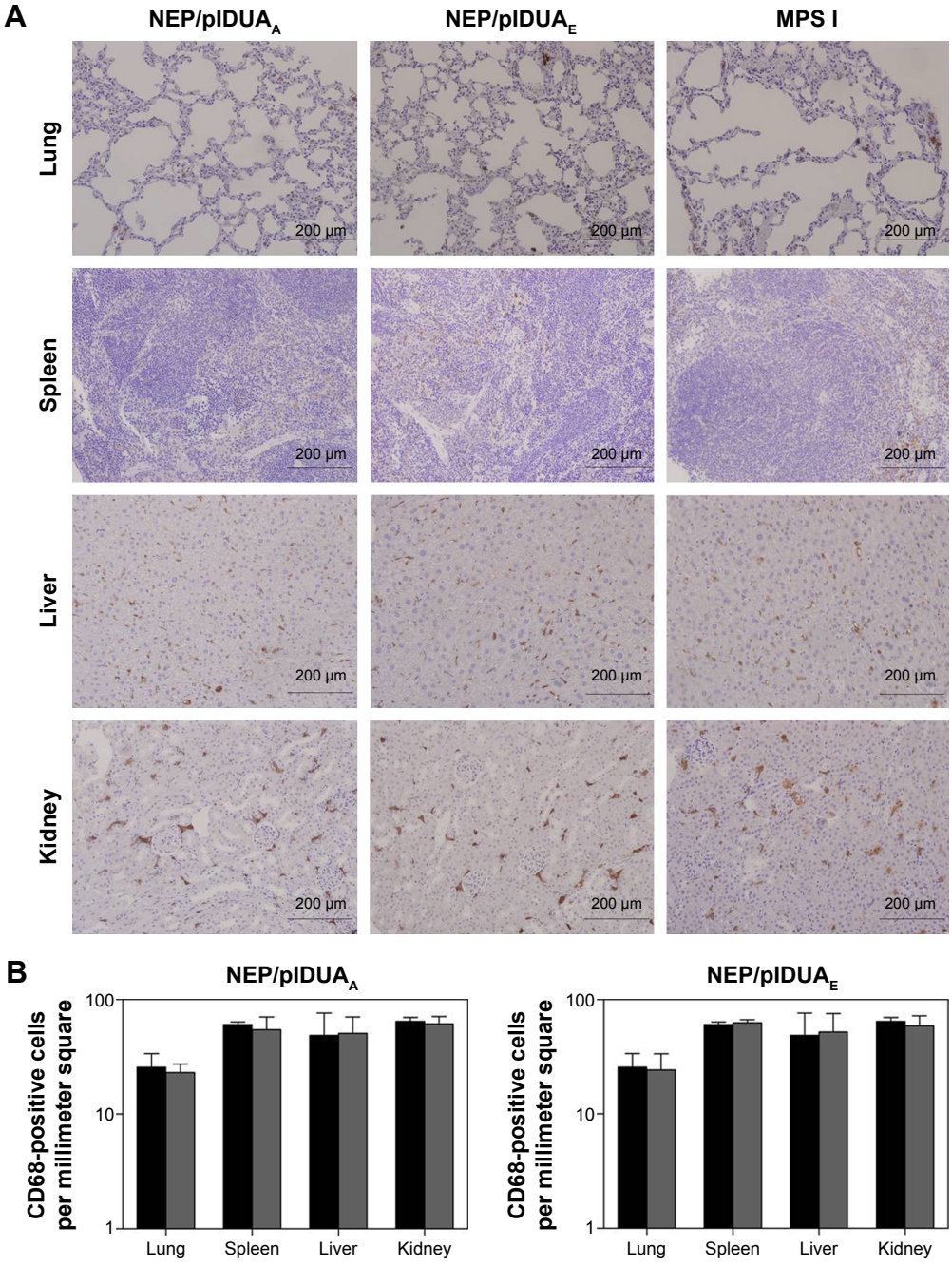

Figure 3 CD68 immunohistochemistry.

Notes: (**A**) Representative sections of different organs of treated groups (NEP/pIDUA$_A$ and NEP/pIDUA$_E$) and negative control (MPS I). Magnification ×200. (**B**) Quantification of CD68-positive cells per millimeter square of tissue. Treated mice are in dark gray bars, and nontreated MPS I mice are in black bars. Values represent the mean ± standard error of the mean.

Abbreviations: MPS I, mucopolysaccharidosis type I; NEP, PEGylated nanoemulsion.

of this complex that was not observed when pIDUA was encapsulated (NEP/pIDUA$_E$).[7]

Although previous studies have shown that a dose of 60 µg of pDNA in a similar formulation using DOTAP can cause a widespread toxicity and architectural damage[9] and these authors also used a charge ratio of +4/−, our histological and immunohistochemistry evaluations did not show any signs of toxicity of the evaluated complexes on the MPS I mice model at this plasmid dose; maybe this difference was due to the use of DSPE-PEG in our formulation.

Conclusion

The set of results demonstrates that dose and time might have an effect on transfection efficiency to a different extent in

each organ. In the lungs, the expression and activity were influenced by the mode by which pIDUA was associated with PEGylated cationic nanoemulsions. A significantly higher *IDUA* gene expression and IDUA activity were detected in the liver 7 days postadministration of the highest pIDUA dose. In these conditions, there was no evidence of a deleterious effect of the complexes on different organs of MPS I mice.

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Disclosure

The authors report no conflicts of interest in this work.

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