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Research Article

Nebulization of Risedronate Sodium Microspheres for Potential Attenuation of Pulmonary Emphysema: a Promising New Insight of Alveolar Macrophage Apoptosis

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Abstract. Risedronate sodium (RS) is a potent nitrogen-containing bisphosphonate which is known to induce osteoclast apoptosis. As a drug repurposing approach, the current work explored the potential of nebulizable RS-chitosan (CS) microspheres to induce alveolar macrophage apoptosis. RS-CS microspheres were assessed for lung deposition, cytotoxicity, and cellular uptake percentage in Calu-3 cells. The potential of nebulizable microspheres for treating elastase-induced emphysema in rats was investigated, compared to RS marketed oral tablets®, with respect to histopathological, immunohistochemical, and flow cytometric studies. The *in vitro* lung deposition pattern suggested deep alveolar deposition of RS microspheres, with respect to high FPF% and suitable MMAD (66% and 1.506 μm, respectively, at a flow rate of 28.3 L min^{-1}). No apparent cytotoxicity was observed, with a cell viability > 90%. The inhalation of RS-CS microspheres was suggested to inhibit airspace enlargement and lung rarefaction after elastase instillation and reduce the macrophage accumulation in alveolar parenchyma. Immunohistochemical and cytometric analyses revealed significant low expression levels of CD68 and CD11b surface markers, respectively, with significantly ($P < 0.05$) lower detected numbers of intact alveolar macrophages following inhalation of RS-CS microspheres. The nebulization of RS-CS microspheres could induce apoptosis in alveolar macrophages and be promisingly adopted for attenuation of pulmonary emphysema.

KEY WORDS: risedronate sodium; microspheres; pulmonary emphysema; alveolar macrophages; lung deposition.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major human chronic inflammatory lung disease. It is considered the third global main cause of death [1]. The pathophysiology of COPD is related to the triggered inflammatory responses in the larger airways after inhalation of toxic gases and/or particles (chronic bronchitis), in addition to a continuous destruction of the alveolar epithelium (emphysema). Symptoms of COPD include suffering from cough and dyspnea which result from the elevation of mucus secretion, and the airflow obstruction with air trapping [2]. Emphysema — by itself — is a powerful risk factor for lung cancer, whether it is smoking-associated or not [3]. COPD involves many types of inflammatory cells, where alveolar macrophages are vital effector cells since they generate inflammatory mediators such as cytokines, reactive oxygen species, chemokines, proteases, and growth factors. As the severity of COPD worsens, the amount of alveolar macrophages and their elastinolytic activity in the pulmonary parenchyma, airway, and bronchoalveolar lavage fluid and in sputum increase [4]. However, few studies have shown that attenuating active alveolar macrophages in patients suffering from COPD is a viable choice [5, 6].

In macrophage cell lines, bisphosphonates were shown to cause apoptosis [6, 7]. The ability of the monocyte-macrophage-lineage cells to take up adequate quantities of bisphosphonates is thought to be supported by their pinocytotic behavior [8]. According to Ueno *et al.* [5], nitrogen-containing bisphosphonates like alendronate have similar impacts on alveolar macrophages. Alendronate inhibits the mevalonate pathway, inducing caspase-3 dependent alveolar macrophage apoptosis, weakening phagocytosis, and *in vitro* cell viability and migration. In a parallel line, it inhibits the

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NF-kB signaling pathway, which decreases the development of inflammatory cytokines (such as TNF-a, IL-1b, IL-6, and MCP-1) and MMPs (such as MMP-9) induced by LPS in alveolar macrophages.

Risedronate sodium (RS) is a more potent nitrogen-containing bisphosphonate which is known to induce osteoclast apoptosis through the loss of isoprenylated proteins. It inhibits farnesyl pyrophosphate synthase (FPP) enzyme, as a mevalonate pathway key enzyme, causing a disruption in geranylgeranyl pyrophosphate (GGPP) and FPP (isoprenoid intermediates) synthesis that are essentially required for small GTP-binding proteins to function properly and offer adequate subcellular localization [7, 9]. Accordingly, RS repurposing approach was investigated in the current work for the treatment of pulmonary emphysema via induction of alveolar macrophage apoptosis.

The development of a safe and effective inhalation therapy depends not only on a pharmacologically active molecule, but also on a well-designed delivery system. Given the anatomy of the airways, it has long been assumed that droplets and/or particles having an aerodynamic diameter of 1–3 μm will be deposited in the alveolar zone, while those with a larger aerodynamic diameter will be deposited predominantly in the tracheobronchial region. It is obvious that the delivery system's size and density would be crucial in achieving an adequate therapeutic outcome [10]. Various attempts were reported to encapsulate drugs to micro-sized formulations for pulmonary delivery using different biodegradable, biocompatible polymers like acrylates, cellulose derivatives, polysaccharides, and polyesters like poly-lactic-co-glycolic acid (PLGA) [11]. However, the hydrophobic nature of PLGA limited the loading efficiency of hydrophilic drugs like bisphosphonates. Furthermore, the slow degradation rates (months) result in a "slow release" of the drug payload and possible potential for polymer accumulation [12]. Alternatively, the hydrophilic and mucoadhesive nature of chitosan (CS), a natural polysaccharide, triggered the development of several studies exploring its potential as a promising inhalable drug delivery carrier [10, 13, 14]. CS particles bind to macrophage mannose receptors, allowing the particles to be phagocytosed by macrophages, prior to degradation by lysozymes and N-acetyl-D-glucosaminidase in phagosomes [15–17]. In our previous work [18], RS-CS microspheres were developed and optimized for the nebulizable delivery of RS. Herein, the potential of these microspheres for targeting the alveolar macrophages and the subsequent attenuation of pulmonary emphysema were explored. Based on the well-known RS induction of osteoclast apoptosis in animal models and humans, the aim of this work was to investigate the possibility of RS to be therapeutically effective in the treatment of pulmonary emphysema through the induction of alveolar macrophage apoptosis.

MATERIALS AND METHODS

Materials

Risedronate sodium hemi-pentahydrate and Actonel® tablets were gifted by Sanofi (Cairo, Egypt). Low molecular weight chitosan flakes (average M_w 150 kDa, deacetylation degree ≥75%), Pluronic® F127, Dulbecco's Modified Eagle's Medium (DMEM), Insulin, Penicillin/streptomycin 100x, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin, Xylazine hydrochloride, Ketamine hydrochloride, Porcine Pancreatic Elastase (PPE), 3% Hydrogen Peroxide, Collagenase type II, and Fetal bovine serum were purchased from Sigma-Aldrich (St Louis, MO). Glacial acetic acid and dimethylsulfoxide (DMSO) were obtained from El Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt). Calu-3 cell line was purchased from ATCC (Manassas, VA). Normal Horse Serum was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Avidin/Biotin Blocking Kit, Biotinylated Horse Anti-Mouse IgG (H+L), R.T.U. Vectastain Elite ABC Reagent, and DAB chromogen were purchased from Vector Laboratories (Burlingame, CA). Mouse anti-rat CD68 and mouse anti-rat CD11b monoclonal antibodies were purchased from AbD Serotec (Raleigh, NC). Purified Mouse IgG1 Isotype Control Serum was purchased from BD Biosciences (San Jose, CA). Mouse anti-rat Fc Block antibody was purchased from BD Pharmingen (San Diego, CA). All other chemicals were of analytical grade and used as received.

Preparation of RS-CS Microspheres

The optimized RS-CS microspheres were prepared according to a crosslinker-free ionotropic gelation technique, as previously described [18]. Briefly, CS solution (1%, w/v) was prepared by dispersing CS flakes in acetic acid solution (1% v/v) and stirring at 450 rpm (Stuart magnetic stirrer, Bibby Scientific Ltd, UK) at room temperature till complete dissolution. RS solution (1%, w/v) was developed by dissolving RS in an aqueous solution of Pluronic® F127 (2%, w/v). RS-CS microspheres were developed at room temperature (25±1°C) via drop-wise addition of RS solution to CS solution (RS: CS ratio of 1:7) and homogenization (Silent Crusher M homogenizer, Heidolph, Germany) at a speed of 14000 rpm for 5 min. Finally, the resulting RS-CS microsphere dispersion was concentrated to the required volume (containing 5 mg RS) for further use.

In Vitro Characterization of RS-CS Microspheres

Aerosolization Studies

The *in vitro* aerosolization studies were carried out using Andersen Cascade Impactor (ACI) (Copley Scientific Ltd, Nottingham, UK). The impactor includes 8 stages with plates being staged in place. As previously reported [19, 20], the impactor was kept in a refrigerator at 5 °C for 90 min prior operation to reduce the aerosolized particles' evaporation effect [21]. The test was carried out within 5 min from the impactor's removal from refrigerator. RS-CS microsphere dispersion was delivered via a commercially available jet nebulizer at a rate of 7 L min^{-1}, and the duration of the nebulization process was < 5 min.

In a brief, 2 mL of the freshly prepared RS-CS microsphere dispersion was transferred to the reservoir of an air jet nebulizer (LC Plus, Pari Respiratory Equipment, Midlothian, VA) that is attached to the induction port of the ACI. In accordance with the standard methodology for nebulizers (set by the European Committee for

Standardization), ACI was operated at 15 L min^{-1} and 28.3 L min^{-1} using a vacuum pump (High-capacity Pump Model HCP5, Critical Flow Controller Model TPK, Copley Scientific Ltd, Nottingham, UK) and the aerosol was drawn through the impactor until dryness, with gentle tapping of the nebulizer wall during the sputtering period to maximize the output. Nebulization was continued for an extra 20 s, after the nebulization of microspheres, to ensure a complete impaction of particles on the respective stages. The effective cut-off diameters (ECD) for ACI stages at 15 L min^{-1} and 28.3 L min^{-1} inhalation flow rates are depicted in Table I [22]. The deposited microspheres at each ACI stage, as well as those remaining on the mouth piece adaptor, induction port, and the nebulizer, were collected using 0.1N HCl to ensure solubilization of RS-CS microspheres [23]. Phosphate buffered saline (PBS) solution (0.154 mM, pH 7.4) was added to extract RS since CS is insoluble at pH values greater than 6.5 [22, 24]. The resulting dispersion was centrifuged and the supernatant was filtered (0.45 μm membrane filter) and analyzed for drug content spectrophotometrically at 262 nm [18, 25–28].

Data analysis was carried out using Copley Inhaler Testing Data Analysis Software (CITDAS, Copley Scientific, Nottingham, UK). The emitted dose (ED) was calculated as the difference between the initial and the remaining amount of the microsphere dispersion in the nebulizer chamber. The accumulated mass of microspheres retained at each respective stage (expressed as a percentage of total mass recovered in the impactor) was plotted against the stage's cut-off diameter (between stages 2 and 3). The fine particle fraction (FPF), the mass median aerodynamic diameter (MMAD), and the geometric standard deviation (GSD) were estimated from the plot. The FPF was measured as the percentage of the microspheres emitted from the inhaler with an aerodynamic diameter of less than 5 μm. The MMAD was determined as the particle size at which the line crosses the 50% mark, while the GSD was defined as:

$$\mathrm{GSD} = (\mathrm{Size\ X}/\mathrm{Size\ Y})^{1/2}$$

where size X is the particle size at which the line crosses the 84% mark and size Y is the particle size at which the line crosses the 16% mark [27, 29, 30].

Table I. The ECD in μm for the ACI Stages at 15 and 28.3 L min^{-1} Inhalation Flow Rates [22, 56]

Stage	ECD at 15 L min^{-1}	ECD at 28.3 L min^{-1}
Stage 0	12.4	9
Stage 1	8.0	5.8
Stage 2	6.5	4.7
Stage 3	4.5	3.3
Stage 4	2.9	2.1
Stage 5	1.5	1.1
Stage 6	1.0	0.7
Stage 7	0.6	0.4
Filter	0	0

Cytotoxicity Studies

The concerns regarding the safety and clearance of the employed polymers for pulmonary drug delivery should be carefully evaluated with respect to polymer type and concentration. The cytotoxicity of the microspheres was determined via MTT assay in Calu-3 cells. The cells were maintained with regular passage in DMEM supplemented with heat-inactivated fetal bovine serum (10%, v/v), insulin (10 μg/mL), and penicillin/streptomycin 100× (1%, v/v) in a humid atmosphere (95% RH) at a temperature of 37 °C with carbon dioxide (5% v/v) (Humid CO_2 incubator, Shel lab 2406, USA) [25, 26]. The cells were seeded at a cell concentration of 1×10^4 cells per well in 100 μL of growth medium in a 96-well plate. Several microspheres' dispersions were prepared in serial concentrations ranging from 0.1 to 1000 μg/mL in a volume of 100 μL of the growth medium, and were then added to the cell monolayers and incubated for 1 day. Twenty microliters of diluted MTT solution in PBS (5 mg/mL, pH 7.4) was added to the seeded cells, and incubated for 4 h at 37 °C. After that, the medium was detached and the formed formazan crystals were solubilized in 100 μL of DMSO. The absorbances were measured using a Tecan Sunrise® microplate reader (Tecan, Hombrechtikon, Switzerland) at 570 nm [25]. The wells including cells, not the samples, were used as control and the baseline was obtained using the culture medium [31].

The equation below was used to calculate the percentage of cell viability [25]:

$$\textit{Cell viability}\% = \frac{A(test)}{A(control)} \times 100$$

where A (test) is the measured absorbance for each concentration of the test substance, and A (control) is the measured absorbance for the untreated cells that were incubated with the medium only. The latter reading corresponds to 100% cell viability.

Cellular Uptake Studies

The cellular uptake efficiency of RS from RS-CS microspheres in Calu-3 cells was assayed by HPLC, as previously described [18]. The cell culture was similarly prepared to that mentioned in cytotoxicity MTT assay. A volume of RS-CS microspheres containing 250 μg/mL of RS was seeded in million Calu-3 cells. The microsphere dispersion was added into the wells and was incubated at 37 °C for 3 h. To optimize the amount of drug taken up by cells, an incubation period of 3 h was selected. The control treatment included the addition of free RS to the wells. To extract the microspheres that had adhered to the cell surface, each well was washed three times with PBS (pH 7.4). The cell culture media was transferred and centrifuged at 16,000 g for 5 min to extract cells after cell detachment by trypsin digestion for 5 min. The cells were re-suspended in PBS (0.5 mL) and extracted with 1 mL 0.1 M NaOH to determine the amount of RS taken up by the cells. The assay was carried out three times, with three separate replicates of each experiment [32].

In Vivo Characterization of RS-CS Microspheres Via Pulmonary Emphysema Model in Rats

Animals

Eight-week-old pathogen-free male Wistar albino rats weighing 300–350 g were derived from the animal house of the Research Institute of Ophthalmology (Giza, Egypt). The animals were housed individually in stainless steel cages at 25 ± 2°C, relative humidity range of 40–60% and alternating 12-h light/dark cycles. They were provided with standard chow and drinking water *ad libitum*. Seven days was allowed for acclimatization before any progression in experimental work. The protocol of the *in vivo* studies was approved (Approval No. PI 1583) by Research Ethics Committee for experimental studies, Faculty of Pharmacy, Cairo University, Egypt (REC-FOPCU).

The pulmonary emphysema model in rats was used to evaluate the role of RS on alveolar macrophages. The pulmonary emphysema was induced via intratracheal (IT) administration of PPE (330 μg/kg). The rats were anesthetized with xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) intraperitoneal injections [33]. The tracheas were exposed by surgical resection and pierced with a 26-gauge needle for IT administrations, and the neck wound was closed with sterile sutures. The intratracheal administration of PPE and other treatments was achieved via Micro Sprayer® (Penn-Century Inc., Philadelphia, PA). For the following *in vivo* characterization studies, the rats were randomized into four groups, as follows: negative control group members (group I, n = 12) were intratracheally treated with sterile PBS; (II) positive control group members (group II, n = 12) were intratracheally treated with PPE in sterile PBS solution; RS-orally treated group (group III) members (group III, n = 8) were intratracheally treated with PPE, followed by oral administration of an aqueous dispersion of crushed Actonel® tablet (500 μg/kg/day); and (IV) RS-intratracheally treated group members (group IV, n = 8) were intratracheally treated with PPE, followed by intratracheal administration of RS-loaded microsphere dispersion (500 μg/kg/day). RS was administered twice, 1 week after elastase instillation (when the inflammatory response was robustly induced), and after another week [5]. RS was administered twice, 1 week after elastase instillation (when the inflammatory response was robustly induced), and after another week [5]. Four rats were euthanized from groups I and II only under general anesthesia after 24 h of elastase instillation for conducting the histopathological studies. Eight rats were euthanized from all groups under general anesthesia after 3 weeks of elastase instillation for conducting the immunohistochemical and flow cytometric studies [34, 35].

Histopathological Studies

The histopathological examinations were performed as a pilot study intended to verify the validity and reliability of the elastase induced emphysema model. Four rats from group I and group II were euthanized 24 h post-elastase administration. Formalin-fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, then cleared in xylene, embedded in paraffin, cut to 4-μm-thickness sections, and finally stained with hematoxylin and eosin (H & E) for light microscopy examination (Axiostar Plus, Zeiss, Oberkochen, Germany) [36].

Immunohistochemical Studies

Lung tissues were fixed in neutral buffered formalin solution (10%), embedded in paraffin and sectioned at 4 μm thickness. The sections were deparaffinized with xylene, and treated with hydrogen peroxide (3%, 15 min) to block the activity of endogenous peroxidase, prior to incubation in trypsin solution (0.1%, 20 min). The subsequent steps for CD68 consisted of protein blocking with normal horse serum (10%) for 20 min, avidin block for 15 min, and biotin block for 15 min. Tissue sections were incubated with primary antibody at 1:500 dilution for 15 min, horse anti-mouse secondary antibody at a 1:1000 dilution for 30 min, Vectastain R.T.U. Elite Label for 30 min, DAB chromogen in the dark for 6 min, and counterstained with hematoxylin for 20 s. To ensure consistency between different runs, a tissue with a known staining pattern and intensity was used as a positive control. The primary antibody was replaced with IgG1 control serum at the same working concentration for negative controls [37, 38]. Lung tissue samples were examined using a light microscope equipped with a high-resolution camera (DP50, Olympus, Tokyo, Japan) at different magnification powers. All images were analyzed using Image J software v.1.48 (National Institute of Health, Bethesda, MD).

Flow Cytometric Studies

The stromal lung cells were extracted as previously explained by Ueno *et al.* [5], with minor modifications. The lung tissue was sectioned into small pieces (~ 1 mm^3) and centrifuged at 100 g for 4 min after the attached organs (thymus, heart, and vessels) were separated under microscope. The floating fragments were isolated from the blood cells-containing precipitate and were incubated in collagenase type II solution in Tyrode buffer (2 mg/mL) for 20 min with gentle stirring. The digested tissue was centrifuged and a 70-μm mesh was used to filter the re-suspended resultant pellet containing stromal vascular (SV) fraction in PBS. The dissociated SV cells were then washed two times in PBS, incubated in erythrocyte-lysing buffer for 10 min, and re-suspended in PBS supplemented with 3% fetal bovine serum. Fc Block antibody was added at 60 ng per 1×10^6 cells, and then incubated for 15 min on ice. The isolated cells were incubated with CD11b monoclonal antibody, and analyzed by flow cytometry using a Canto II flow cytometer (BD Biosciences, San Diego, CA), associated software (BD FACS Diva, BD Biosciences, San Diego, CA), and flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

Statistical Analysis

The mean values were analyzed for their statistical significance using ANOVA using Minitab® 17 software (Minitab Inc.; State College, PA, USA). *P*-value ≤ 0.05 was chosen as the criterion for statistical significance of data.

RESULTS AND DISCUSSION

In Vitro Characterization of RS-CS Microspheres

Aerosolization Behavior

The *in vitro* aerosol performance of inhalable multiparticulate systems is one of the crucial factors that influence their *in vivo* lung deposition [27]. The generated aerosol droplets after nebulization can deposit at various regions within the lower respiratory tract, including primary bronchi, bronchi, bronchioles, and eventually alveoli, depending on particle size. Herein, the ACI was investigated as an aerodynamic classifying system for the inhaled RS microspheres [28]. Aerosol particle deposition at each stage of ACI is expected to mimic the deposition patterns of these systems in various regions of the human lung [39].

The deposition pattern of the emitted dose at the stages of ACI is shown in Fig. 1. It was clear that the majority of RS-CS microspheres deposited in the lower stages of ACI, *viz.*, stages 5 (15.27 ± 2.66%), 6 (14.46 ± 2.14%), and 7 (12.71 ± 1.58%) and in the filter (8.99 ± 0.98%).

The FPF% of ideal systems should be > 50% of the total delivered dose [40]. Herein, the FPF percentages of the nebulizable RS-CS microspheres were higher: 60% at a flow rate of 15 L min^{-1} and 66% at a flow rate of 28.3 L min^{-1}. The particle size of inhalable systems must be in the range of 1–5 μm for effective deep lung delivery. Particles < 1 μm could be exhaled shortly after inhalation, while particles > 5 μm would be expected to deposit outside the lungs and/or in the oropharyngeal region, by impaction [11, 27, 41]. A droplet size of 1–3 μm is targeted to achieve deep deposition in the alveoli. Herein, the MMAD ± GSD of the microspheres was 2.069 ± 0.19 μm at a flow rate of 15 L min^{-1} and 1.506 ± 0.23 μm at a flow rate of 28.3 L min^{-1}. These results match with the observed size of RS-CS microspheres in TEM images, reported by Elkady *et al.* [18]. The potentiality of the developed system for alveolar targeting and attenuation of pulmonary emphysema could be inferred.

Cytotoxicity Evaluation

The US FDA had given the Commonly Recognized as Safe (GRAS) status to a large number of polymers and excipients. However, a limited number of them were approved for utilization in inhalation systems. Therefore, cell viability tests are urgently requested to check the system biocompatibility since these tests provide a valid gross estimate of the cell response to an insult [25]. Of the investigated cell lines, Calu-3 cells are usually employed as a respiratory model in drug development and preclinical applications to explore the toxicity of the inhaled aerosols delivered directly to the lung epithelium, either for immediate or prolonged therapeutic effects [42].

A Calu-3 cell viability of greater than 90% was revealed after incubation of RS-CS microspheres at concentrations up to 1000 μg/mL for 24h (Fig. 2). It could be inferred that the microspheres did not hamper the host cell viability following pulmonary administration. A good cytocompatibility was observed for the developed microspheres with no apparent cytotoxicity against Calu-3 cells [43].

The cytocompatibility of CS on Calu-3 cells was previously reported by Grenha *et al.* [44] who revealed the *in vitro* safety and biocompatiblity of CS nanoparticles with Calu-3 and A549 respiratory epithelial cells. In a parallel line, Sivadas *et al.* [45] reported the higher toxicity of PLGA on Calu-3 lung cells, compared to other polymers including CS. This high safety of CS for human use was declared by the US FDA and EU [46]. Interestingly, the cell viability% upon incubation with RS-CS microspheres were superior to those reported for pure RS and PEGylated RS forms, confirming the protective effect of CS [43, 47].

Cellular Uptake Efficiency

The results of HPLC analysis revealed the incorporation of RS at a concentration of 37.54 μg/mL inside the cells, with a cellular uptake percentage of 15% only. It could be assumed that the incorporation of CS and the subsequent development of RS-CS microspheres could enhance the

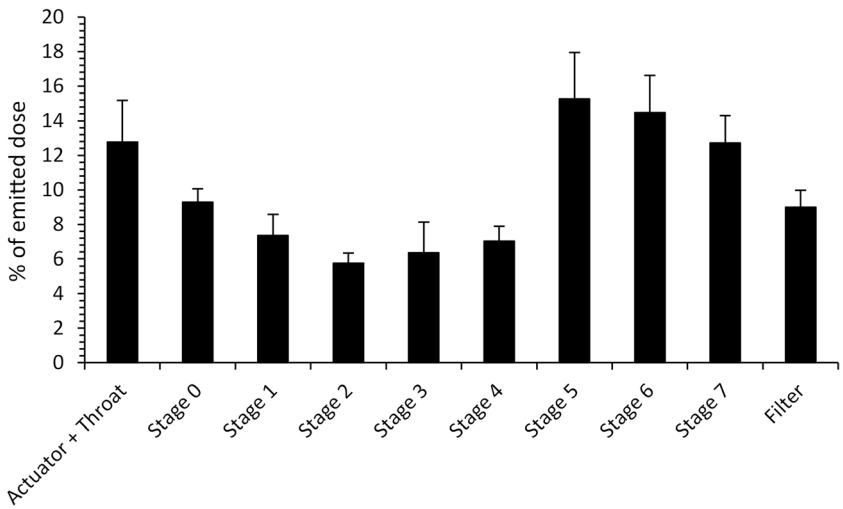

Fig. 1. Percent deposition of recovered RS from RS-CS microspheres in respective ACI components, n=3

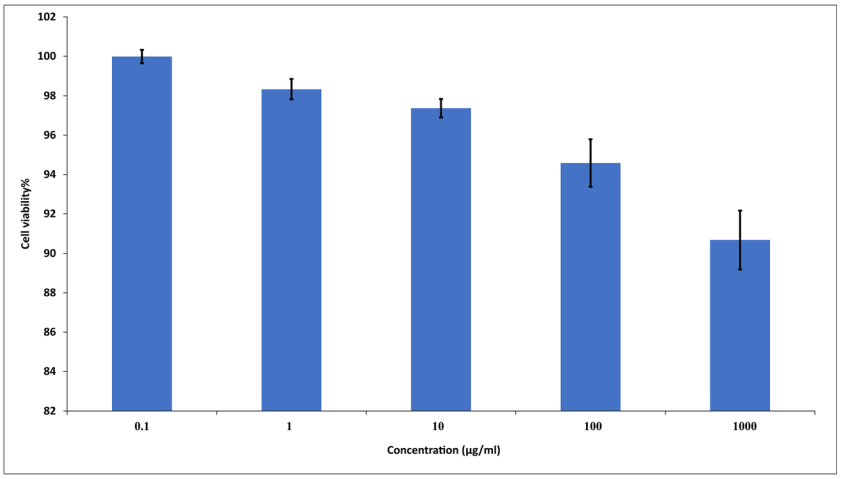

Fig. 2. Cell viability% of Calu-3 cells at different RS concentrations from RS-CS microspheres

uptake efficacy of RS via alveolar macrophages, rather than via Calu-3 cells. It is worth to note that the uptake of RS-CS microspheres by the alveolar macrophages is a particle size–dependent process [48]. Geiser *et al.* [2] found that particles ≥ 500 nm are present only in macrophages, demonstrating phagocytosis which allows the uptake of microparticles. The mechanism of particle uptake by alveolar macrophages is supposed to be mediated by phagocytosis (200–2000 nm) and endocytosis (100 nm). While the phagocytic ingestion of the deposited particles is the primary role of alveolar macrophages for keeping the lung surface clean, the alveolar epithelium uptake of the microspheres may be advantageous for the therapeutic targeting of the lung parenchyma by inhaled aerosols in COPD, including emphysema [2, 48].

In Vivo Characterization of RS-CS Microspheres

Histopathological Examination

The elastase-related acute lung damage is known for neutrophils and macrophages' rapid substantial infiltration in only 1 day, accompanied by the alveolar wall subsequent destruction and enlargement of airspace, resembling human pulmonary emphysema [49]. The H & E-stained sections of the negative control rats (group I) revealed a normal lung architecture, thin interalveolar septa, and folded columnar epithelial cells of the bronchioles. The alveolar sacs and the normal fibrous tissue distribution were clearly seen (Fig. 3a). On the other side, the lung tissues of group II were characterized with thick-walled bronchioles, moderately congested blood capillaries, infiltration of macrophages in the alveolar spaces along with degenerated and thickened interalveolar septa. Moreover, the lung tissues showed degeneration of the alveoli and interalveolar septum (Fig. 3b). The panacinar emphysema (Fig. 4) was clearly demonstrated with marked thinning of alveolar walls, which broke down and formed larger spaces than normal alveoli [35].

Immunohistochemical Analysis

The immunohistochemical staining of rat lung tissue using CD68 antibody was performed in order to investigate the role of the alveolar macrophages as a potential source of cytokines in pulmonary emphysema [50]. Noteworthy, CD68 is a highly expressed protein by tissue macrophages and is regarded as a phagocytic marker. Anti-CD68 is frequently used as a "pan-macrophage" marker in immunohistochemical studies in rats [37].

By spreading proteolyzed peptides from the extracellular matrix, the pulmonary elastase administration destroys the elastin framework of the lung alveolar structure and triggers the chemotactic reactions to inflammatory cells [35]. As shown in Fig. 5, PPE instillation markedly increased the accumulation of CD68-positive macrophage in alveolar parenchyma, which is considered a key feature of COPD [51]. Interestingly, RS administration diminished positive CD68 macrophage accumulation in the alveolar parenchyma, where the suppression of PPE-induced inflammatory cell accumulation along with RS administration was very consistent with CD68 immunostaining. According to the immunostaining results, the amount of CD68 macrophages detected in group III was markedly larger than in group IV. It could be inferred that the pulmonary administration of RS-CS microspheres was effective in the depletion of alveolar macrophages and, thus, diminished the elastolytic enzymes (proteases), including matrix metalloprotease (MMP-2, MMP-9, MMP-12), cathepsin K, L, and S that are normally secreted in response to irritants and infection. They are responsible for the destruction of lung parenchyma that commonly occurs in patients with emphysema [4]. As reported, the depletion of lung macrophages granted protection against emphysema development in experimental models of COPD [4, 5].

Flow Cytometric Characteristics

CD11b is highly expressed in active lung inflammation. It is considered a specific marker for inflammatory alveolar macrophages [52, 53]. The macrophage sub-populations were separated by flow cytometry using CD11b antibody. A significant increase (P <0.05) in the percentage of CD11b macrophages was revealed (2.7 ± 0.12% of the total alveolar cells) following PPE instillation, relative to normal saline instillation (0.46 ± 0.087% of the total alveolar cells) (Fig. 6).

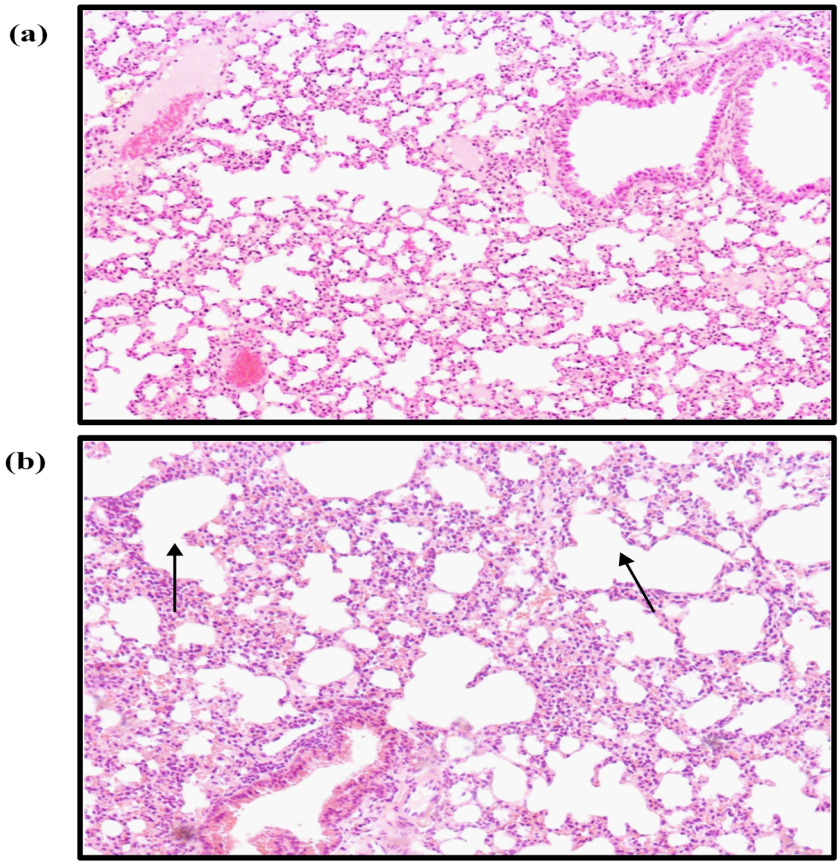

Fig. 3. A photomicrograph of lung tissue section in **a** the negative control group showing normal architecture of alveoli with thin interalveolar septa and normal interstitial tissues and **b** the positive control group showing loss of normal architecture marked thinning of the interalveolar septum and large emphysematous spaces (**arrows**) (magnification is 200 ×)

RS was reported to play a protective role during the early stages of PPE-induced pulmonary emphysema by suppressing the pro-inflammatory cytokine production including tumor necrosis factor-α (TNF-α) in resident cells such as alveolar macrophages, epithelial cells, lymphocytes, and neutrophils [35]. Herein, statistically significant ($P < 0.05$) dramatic depletions of the alveolar macrophages in the rat lung tissues were revealed following intratracheal or oral administration of RS (Fig. 6). It was clear that the number of intact alveolar macrophages following intratracheal administration of RS-CS microspheres (0.13 ± 0.011% of the total alveolar cells) was significantly ($P < 0.05$) lower than the number of intact alveolar macrophages following oral administration of RS (0.24 ± 0.029%).

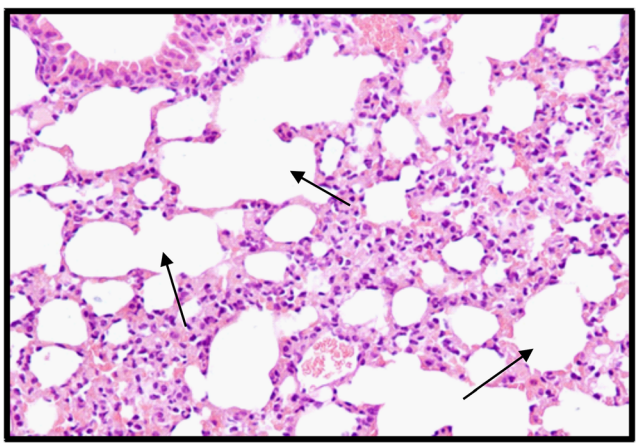

Fig. 4. A photomicrograph of lung tissue section in the positive control group showing Panacinar emphysema with marked destruction of the interalveolar septa (**arrows**) (magnification is 400 ×)

In view of the aforementioned findings, it could be postulated that the inhalation of RS-CS microspheres revealed high capability to induce alveolar macrophage apoptosis. On the other hand, since macrophages and osteoclasts are highly endocytic cells that share the same lineage, it would be logical for bisphosphonates to affect alveolar macrophages when administered orally [7]. It has been approved that highly phagocytic cells, such as macrophages, have the ability to internalize bisphosphonates, making them perfect targets for these drugs [54]. Nevertheless, the fast preferential accumulation of RS in bones following the oral gavage owing to their high affinity to hydroxyapatite would account for its limited role in the control of pulmonary emphysema [5, 7].

Further studies are required to discover the mechanism/s by which the inhaled RS-CS microspheres induce apoptosis in alveolar macrophages. There are, however, at least two alternatives that are not mutually exclusive. The first

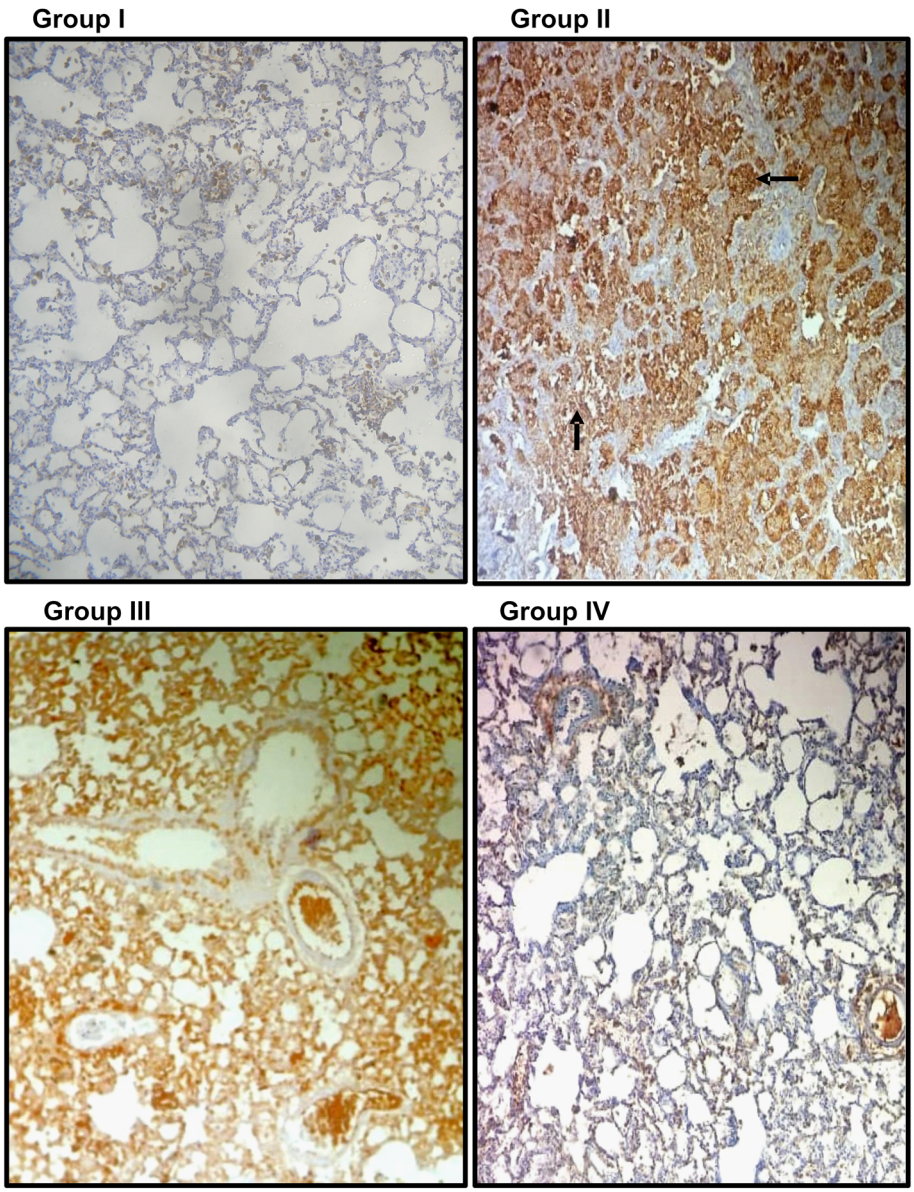

Fig. 5. Immunohistochemical staining of rat lung tissues with anti-CD68 antibody. Cells stained with anti-CD68 (representing macrophages) antibodies are visible in brown. All sections were counterstained with Mayer's hematoxylin. The arrows indicate positively stained cells

suggestion is related to the activated alveolar macrophages. The *in vivo* resident alveolar macrophages could be triggered by intratracheal elastase instillation. The activated alveolar macrophages have high potential to internalize RS through pinocytosis than the non-activated macrophages. The second possible explanation is related to the alveolar space that serves as a special environment, where precipitation of calcium phosphate occurs; thus, bisphosphonates can bind with high affinity through its P–C–P backbone structure [55].

CONCLUSIONS

RS-CS microspheres were developed via a crosslinker-free ionotropic gelation technique. The aerodynamic behavior of RS-CS microspheres following nebulization suggested deep alveolar as revealed from achieving high FPF% and suitable MMAD (60% and 2.069 μm, respectively, at a flow rate of 15 L min^{-1}, and 66% and 1.506 μm, respectively, at a flow rate of 28.3 L min^{-1}). Promising RS microsphere cytocompatibility was revealed on Calu-3 cells, with a cell viability > 90%. The pulmonary potential of RS-CS microspheres for treating elastase-induced emphysema in rats was elaborated by low CD68 and CD11b expression levels which could indicate alveolar macrophage apoptosis. The inhaled RS-CS microspheres could be considered a promising drug repurposing approach which could provide a promising therapeutic alternative for reprogramming of alveolar macrophages to help restoration of lung homeostasis. Further clinical studies are needed to assure their potential use in treating pulmonary emphysema.

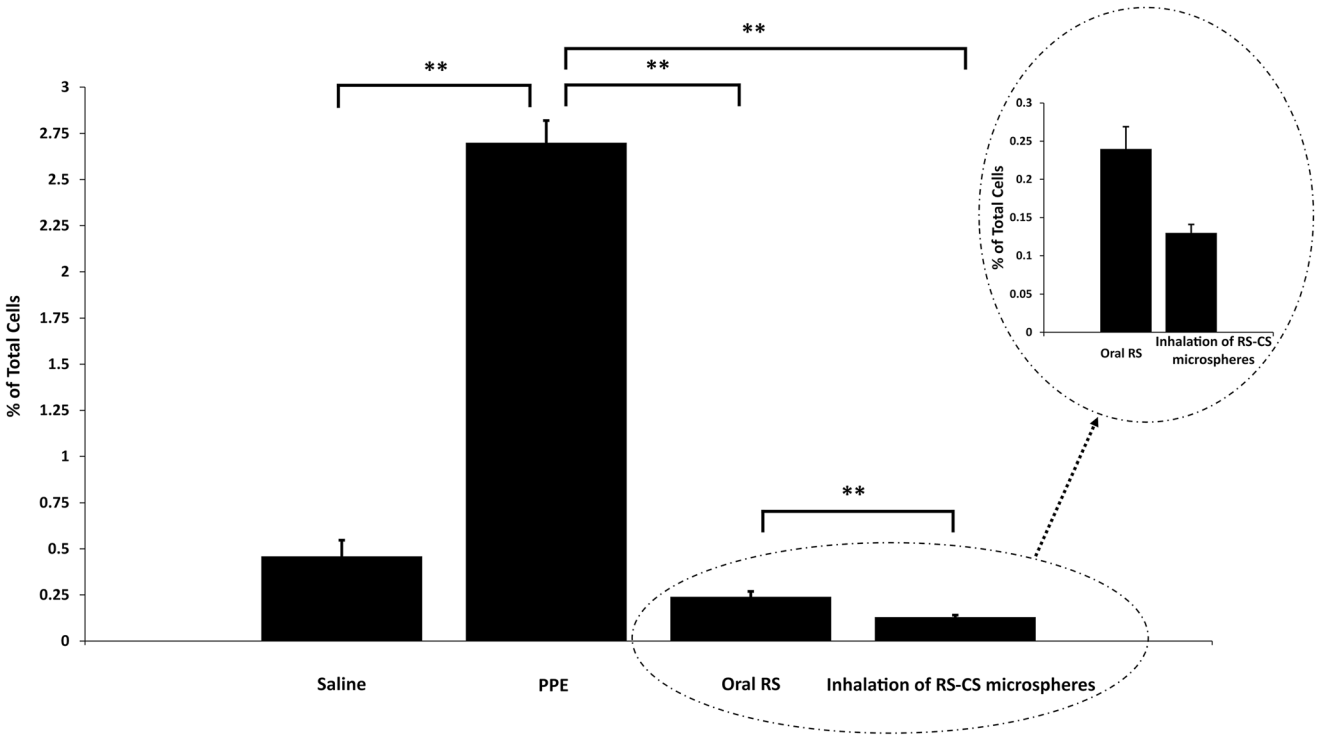

Fig. 6. Graphic representation of the number of CD11b macrophages as a percentage of total cells in all groups. Data represent mean values ± SD. $^{**}P < 0.05$, significantly different

AUTHOR CONTRIBUTION

The authors contributed equally to this work and participated in the following:

- The conception or design of the work; the acquisition, analysis, or interpretation of data
- Drafting the work and revising it critically for important intellectual content
- Final approval of the version to be published
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

DECLARATIONS

Conflict of Interest The authors declare no competing interests.

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