

Liposomes Containing Clodronate Attenuate Spleen Injury in the Rat Model with Severe Acute Pancreatitis

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Abstract

Background: Clodronate containing liposomes selectively deplete macrophages and Super Paramagnetic Iron Oxide (SPIO) nanoparticles facilitate cell labeling. In this research, we explored the protective role of clodronate-SPIO nanoparticle-liposomes in the rat model with Severe Acute Pancreatitis (SAP).

Methods: SPIO nanoparticles were synthesized by facile thermal decomposition method. SPIO and clodronate-SPIO nanoparticles containing liposomes were made by the process of the film method. The models of SAP were made using rat pancreatic subcapsular injection of sodium taurocholate. For the SAP model, rats were slowly injected with SPIO nanoparticle-containing liposomes via the tail vein (the P group). Alternatively, rats in the T and C groups were injected with clodronate-SPIO nanoparticles-containing liposomes and saline, respectively, in a volume of 2 mL/kg body weight. Serum Amylase (SAM), Interleukin (IL)-6, and Tumor Necrosis Factor (TNF)- α were analyzed by ELISA. Pathological alterations in the pancreas and spleen were detected by Hematoxylin and Eosin (HE) staining, and apoptosis was detected with TUNEL staining. Spleen injury was evaluated after exposure to either SPIO nanoparticle-containing liposomes or clodronate-SPIO nanoparticle-containing liposomes.

Results: Pathological alterations in the pancreas and spleen of SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes were slighter than those in SAP model rats administered SPIO-containing liposomes. The levels of serum amylase, IL-6, and TNF- α in SAP model rats administered SPIO nanoparticle-containing liposomes were higher than those in the C group, but were significantly lower in SAP model rats administered SPIO nanoparticle-containing liposomes than those in rats administered clodronate-SPIO nanoparticle-containing liposomes ($p < 0.01$). In spleens, the rate of apoptosis was higher in rats administered clodronate-SPIO nanoparticle-containing liposomes than in those administered SPIO nanoparticle-containing liposomes at 2 and 6 h.

Conclusions: Clodronate-containing liposomes protected against spleen injury in SAP rats, and SPIO nanoparticles could be used as a tracer for examination to detect spleen injury in SAP rats.

Keywords: Pancreatitis; Clodronate Disodium; Macrophage; Spleen Injury

Introduction

As with other multiple organ dysfunction syndromes, the major etiology of Severe Acute Pancreatitis (SAP) is hyper activation of systemic inflammatory mediators, such as inflammatory cytokines, which leads to distant organ damage and subsequent morbidity and mortality [1]. Indeed, Systemic Inflammatory Response Syndrome (SIRS) was involved in the pathogenesis of SAP because the majority of deaths that occur can be attributed to organ failure, particularly spleen injury, associated with SIRS [2,3]. During infection, the life cycle of Polymorphonuclear Neutrophils (PMNs) is prolonged, and apoptosis is delayed, resulting in various types of stresses, which stimulate inflammatory reactions and cause organ injury, such as spleen injury [4-6]. Macrophage accumulation and activation in the spleen have been shown to correlate with the progression of SAP [7,8]. Thus, monocyte/macrophage-induced inflammation may play a central role in the progressive loss of spleen function [9].

Clodronate is a first-generation bisphosphonate osteoporosis drug with potent osteoclast inhibitory activity. However, clodronate has poor cell membrane permeability. Therefore, researchers have sought to overcome this problem by encasing clodronate in liposomes; indeed, clodronate-containing liposomes selectively deplete macrophages from tissue by competing with Adenosine Triphosphate (ATP). When administered intravenously, clodronate-containing liposomes are phagocytosed by the mononuclear phagocyte system, and free clodronate is released from the liposomes to the intracellular region, resulting in destruction of the phagocytic cells. In addition, SPIO-nanoparticles have been used as cell labeling agents.

The purpose of this work was to detect the infiltration of macrophages into the spleen in SAP-induced spleen injury. Additionally, we evaluated the effects of clodronate, delivered via SPIO nanoparticle-containing liposomes, on splenic macrophages.

Materials and Methods

Reagents

Clodronate (clodronic acid) was purchased from Weijing Technology Enterprise (Shanghai, China). TUNEL assays (Roche,

Switzerland). TNF- α & IL-6 were obtained from Invitrogen Corp. (CA, USA). All other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO, USA).

Preparation of SPIO/clodronate-SPIO nanoparticle-containing liposomes

SPIO was prepared by facile thermal decomposition. The resulting black precipitate was washed several times with anhydrous alcohol, and the product was then dispersed in anhydrous alcohol. Reverse-phase evaporation was used to prepare SPIO nanoparticle-containing liposomes (empty liposomes) and clodronate-SPIO nanoparticle-containing liposomes. Briefly, 0.14 g lecithin and 0.028 g cholesterol were dissolved in 15 mL chloroform. The mixture was then evaporated to remove chloroform by revolving sanitization (150 rpm) at 37.1°C below the low-vacuity point. A single-molecule lipid membrane layer was formed on the flask walls. The flask was then dried in a 60°C thermostatic oven. Next, 0.1 g disodium clodronate and 2 drops of prepared SPIO were added into 15 mL Phosphate Buffered Saline (PBS), and samples were shaken vigorously, followed by 1 h continuous sonication to generate a phospholipid bilayer from the single-molecule phospholipid membranes. The resulting liposomes were washed with saline and PBS to remove unincorporated phospholipid molecules. Calculation of drug encapsulation efficiency was performed as follows:

Encapsulation efficiency = (liposome encapsulated drug/total liposomes) $\times$ 100%.

The drug encapsulation efficiency of prepared clodronate-SPIO nanoparticle-containing liposomes was 26.3%. The morphology and microstructure were evaluated with a Transmission Electron Microscope (TEM, Philips Tecnai-12).

Animal models and experimental grouping

The complete research procedure was performed following the standard guidelines of Jiangsu University (China). Forty eight Sprague Dawley rats weighing 350–400 g were maintained in a 12 h light-dark cycle under controlled temperature and humidity of the ambient atmosphere temperature of 21–23°C. The rats were provided food and water ad libitum and were fasted overnight before experiments. SAP model rats were prepared using Zhang JX's method [10,11]. After anesthesia, the abdominal cavity of the rats was opened. SAP models were prepared by a uniform injection of 3.5% sodium taurocholate (2 ml/kg body weight) into the subcapsular space of the pancreas.

SAP model rats were slowly injected with SPIO nanoparticle-containing liposomes (the P group), clodronate-SPIO nanoparticle-containing liposomes (the T group), or saline (the C group) via the tail vein at a volume of 2 mL/kg body weight, as used in previous studies. Suspensions were shaken before administration. At 2 or 6 h after injection initiation of the SAP model, the spleen and pancreas were collected.

Analysis of serum amylase, IL-6 and TNF- α level

Blood samples were collected from the Superior Mesenteric Vein (SMV) and centrifuged at 3000 rpm for 10 min. Supernatants were stored at 20°C until use. Serum amylase levels were detected using an automatic biochemical analyzer, IL-6 and TNF- α levels were detected using ELISA (Invitrogen Corp., CA, USA).

Histopathological examinations of the pancreas and spleen

Pathological alterations in the paraffin-embedded pancreas and spleen tissues (sectioned to 5 μ m thicknesses) were detected by HE staining. Each specimen was evaluated by an experienced histologist and assigned a score according to the scoring criteria pathological assessment of pancreatic tissue proposed by Kusser [12]. Histopathological analysis of spleen injury was performed according to a previously described scoring system [13].

Perrin's Prussian blue staining of spleen: The occurrence of ferric iron was identified by its combination with ferrocyanide, which resulted in Prussian blue staining in the paraffin-embedded spleen tissues (sectioned to 5 μ m thicknesses).

TUNEL assays of spleen sections: TUNEL assays were performed using the paraffin-embedded spleen tissues (sectioned to 5 μ m thickness) and were processed according to the manufacturer's protocol (Roche, Switzerland) [14].

Statistical analyses

Data are presented as means $\pm$ standard deviations. Statistical analyses were performed using PASW Statistics for Windows (version 18.0). Kruskal-Wallis tests and Mann-Whitney U tests were used for comparisons different groups. p values less than 0.05 were considered statistically significant.

Results

Characterization of SPIO and liposomes

SPIO nanoparticles prepared by the coprecipitation-hydrothermal method measured 20–50 nm in diameter. Liposomes entrapped SPIO, yielding a concentration of 5 μ mol Fe/mL. The appearance of the liposomes was dark brown, and the pH of the solution was 6.47. Liposomes had an average diameter of about 100–200 nm.

Changes in the levels of serum amylase (SAM), IL-6 and TNF- α

Compared to C group, P group exhibited significantly higher levels of SAM, TNF- α and IL-6 at each time point ($p < 0.01$). In contrast, T group rats exhibited markedly decreased levels of these targets at 2 and 6 h ($p < 0.01$). These data had been presented in a previous report from our laboratory (Figure 1) [15].

Gross changes, microscopic changes, and pathological scores of pancreases in all groups

The pancreas from C group rats did not show significant changes. In contrast, rats in the P group exhibited bloody ascites,

edema, and hemorrhagic necrosis. As well as in the T group, gross changes were similar, but less severe, than those in the P group. In terms of microscopic changes, rats in the C group did not show any abnormalities in pancreatic histology. In contrast, the pancreas of rats in the P group exhibited marginal edema with extensive infiltration by inflammatory cells, hemorrhagic necrosis were also detected. Schmidt's pathological scores were considerably higher in P and T group rats than in C group rats, and fewer pathological changes were detected in the T group rats than in the P group rats ($p < 0.01$; Figure 2).

Gross changes, microscopic changes, and pathological scores of spleens in all groups

The spleens of C group rats were morphologically and structurally normal. However, in the P group, many abnormalities including swelling, inflammatory cell infiltration and necrosis in the follicular center were detected at 2 h. In rats with mild symptoms and spotty necrosis in the germinal centers of lymphoid follicles of the spleen, expansion, congestion and local hemorrhage of the red pulp sinus were observed at 6 h in rats with severe symptoms. T group rats exhibited the same changes but to a milder degree. Pathological scores were significantly higher in the T and P groups than in the C group at 2 and 6 h. Pathological changes in the T group were less severe than those in the P group ($p < 0.05$; Figure 3).

Perl's Prussian blue staining

No positive staining was observed in rats in the C group. However, numerous blue stained macrophages could be found in the spleens of rats in the P group, and the number of positively stained macrophages was greatly diminished in rats in the T group (Figure 4).

Measurement of apoptosis by TUNEL assay

The rats in the T group shown many TUNEL-positive macrophages; however, the rats in the C and P groups, very few or no TUNEL-positive macrophages were found (Figure 5)

Discussion

In the current study, we examined whether liposomes containing clodronate prevent the development of SAP-induced spleen injury by modulating the inflammatory process. We developed an effective method for labeling of macrophages using SPIO nanoparticle-containing liposomes. Our study revealed important changes in spleen tissues in response to delivery of clodronate by SPIO nanoparticle- containing liposomes.

The major mechanism contributing to early and late mortality in patients with SAP is alteration of immune system function. Indeed, the primary cause of death during early SAP is SIRS

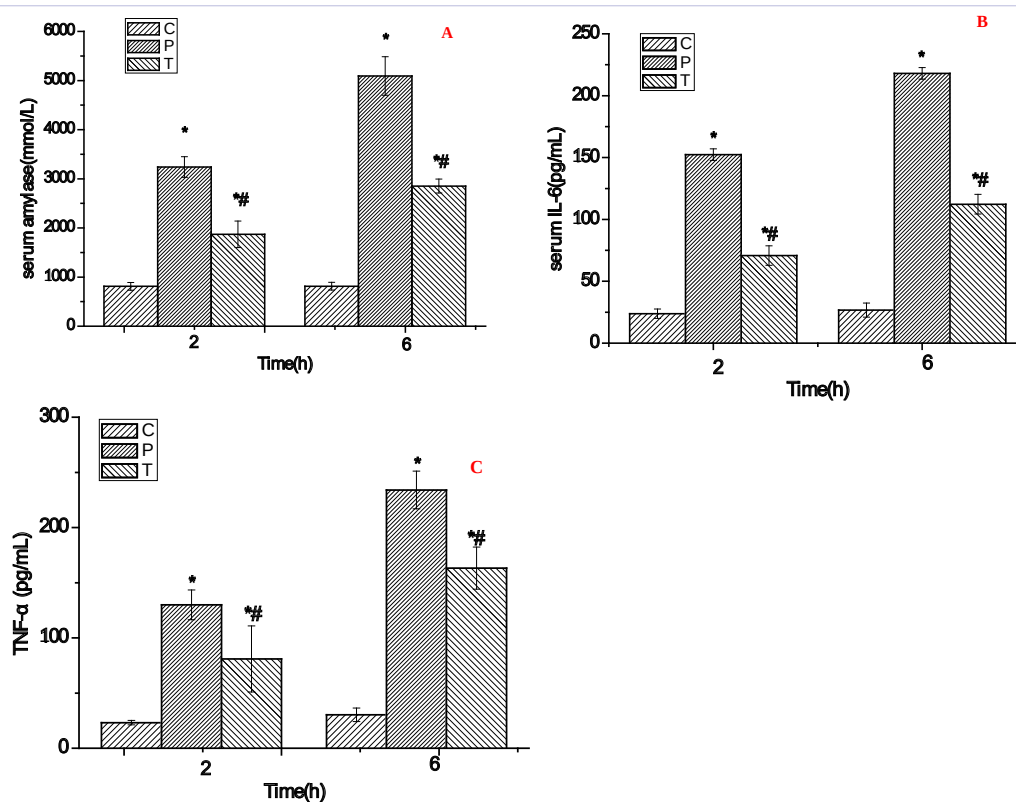


Figure 1: Serum levels of amylase (A), IL-6 (B), and TNF-α (C) at 2 and 6 h in each group. Data are means $\pm$ SDs ($n = 8$), * $p < 0.01$ vs. the C group; # $p < 0.01$ vs. the P group. P, SAP model rats administered SPIO nanoparticle-containing liposomes; T, SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes; C, control group administered saline only.

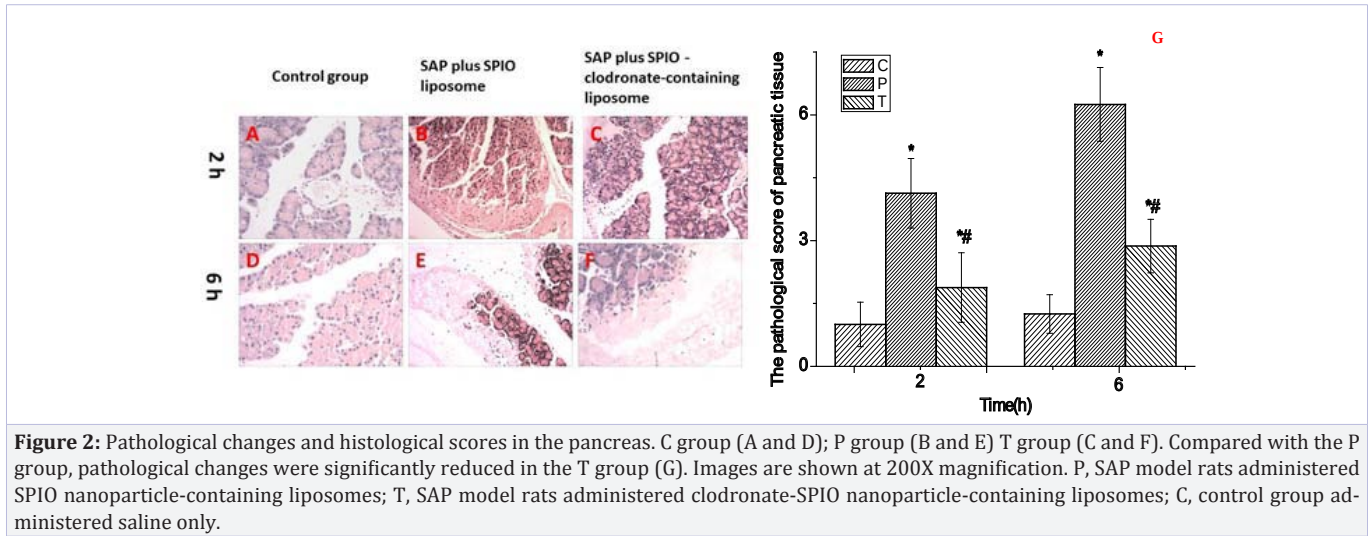


Figure 2: Pathological changes and histological scores in the pancreas. C group (A and D); P group (B and E) T group (C and F). Compared with the P group, pathological changes were significantly reduced in the T group (G). Images are shown at 200X magnification. P, SAP model rats administered SPIO nanoparticle-containing liposomes; T, SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes; C, control group administered saline only.

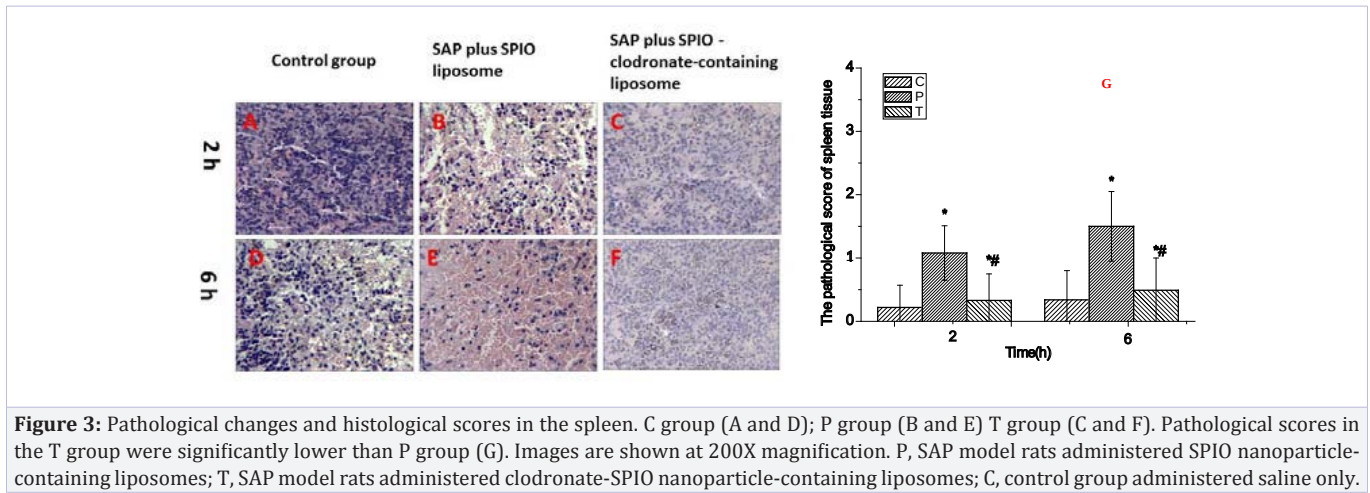


Figure 3: Pathological changes and histological scores in the spleen. C group (A and D); P group (B and E) T group (C and F). Pathological scores in the T group were significantly lower than P group (G). Images are shown at 200X magnification. P, SAP model rats administered SPIO nanoparticle-containing liposomes; T, SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes; C, control group administered saline only.

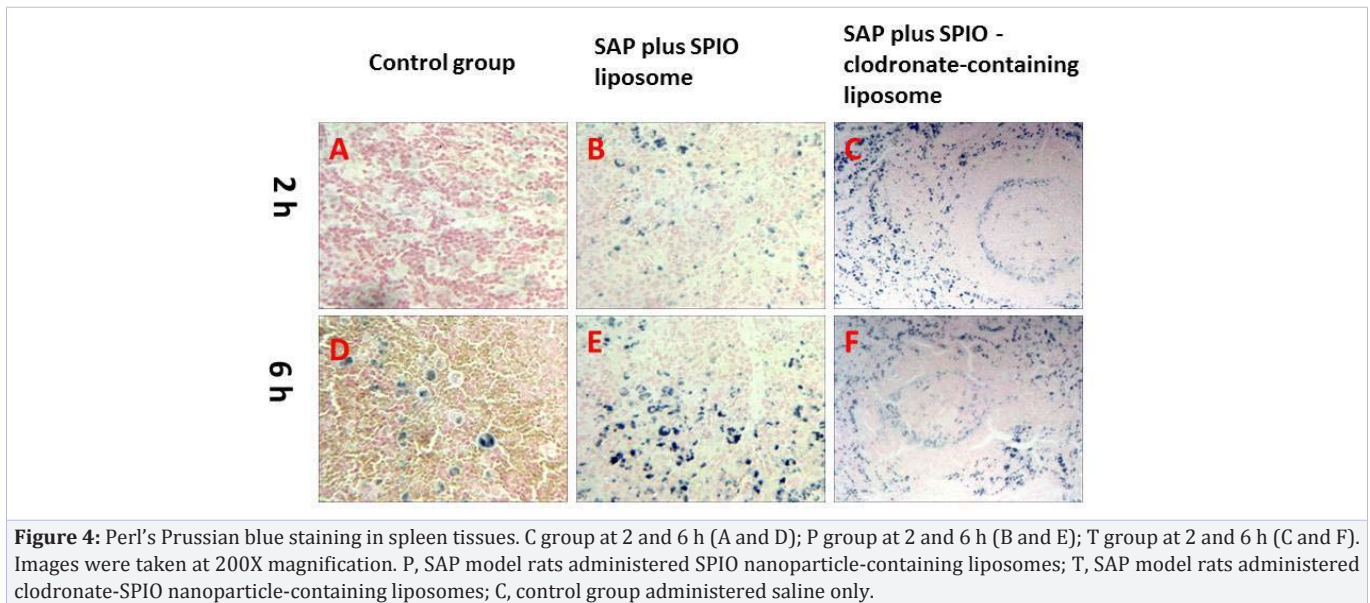


Figure 4: Perl's Prussian blue staining in spleen tissues. C group at 2 and 6 h (A and D); P group at 2 and 6 h (B and E); T group at 2 and 6 h (C and F). Images were taken at 200X magnification. P, SAP model rats administered SPIO nanoparticle-containing liposomes; T, SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes; C, control group administered saline only.

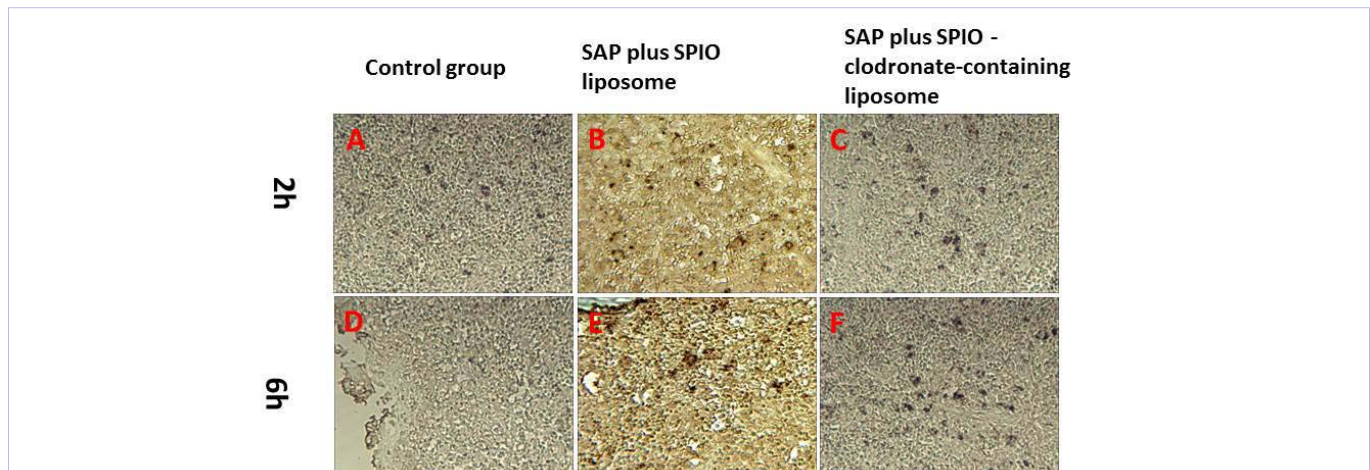


Figure 5: TUNEL assay for in situ detection of apoptosis (200X). Apoptosis of spleen cells in the C group (A and D), P group (B and E), and T group (C and F) are shown. P, SAP model rats administered SPIO nanoparticle-containing liposomes; T, SAP model rats administered clodronate-SPIO nanoparticle-containing liposomes; C, control group administered saline only.

[16]. Activation of macrophages and infiltration leads to initial pathological changes associated with SAP and is an essential pathophysiological step in MOF [15]. AP is generally associated with changes in the abdominal organs and approximately 20–30% AP patients develop to SAP [17], which is characterized by longer duration of disease, MOF, SIRS, and pancreatic necrosis. Mostly patients have a mild form of the disease, but the severe form develops in about 20% of the cases, and SAP is associated with a 7-15% mortality rate [18,19].

The spleen is the major site of blood filtration and therefore participates in both the innate and adaptive immune systems [20]. Thus, targeting of particular macrophage associated pathways is a potential therapeutic approach for suppressing macrophage-mediated spleen injury. Inflammation resulting from pathogens or tissue impairment triggers resident macrophages to increase the production of inflammatory mediators. Macrophages have several phenotypes and multifaceted functions within both the innate and adaptive immune responses. Activation of macrophages is important pathophysiological steps in multiple organ failure. Additionally, macrophages function is essential for controlling the expression levels of pro-inflammatory mediators (e.g., IL-1 and TNF- α). Our study revealed the possible contribution of macrophages located in the spleen injury in the severity of induced SAP. Serum TNF- α level was notably increased in the P group and the increase was suppressed by clodronate in T group rats ($p < 0.01$). Less damage in the spleen injury was observed in the T group than that in the P group ($p < 0.01$).

Macrophages are the most important pro/anti-inflammatory cells and are present in organs affected by SAP. Serum amylase and inflammatory mediators' levels increased dramatically in the group. Interestingly, injuries to the pancreas and spleen were milder in the T group rats than that in the P group.

In conclusion, clodronate-containing liposomes could prevent the progression of SAP-induced spleen injury by modulating

the inflammatory process. We also developed a new method for preparing SPIO nanoparticle-containing liposomes to label macrophages. Our results may provide a new approach for the assessment and treatment of spleen injury resulting from SAP.

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