MRI shows clodronate-liposomes attenuating liver injury in rats with severe acute pancreatitis

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BACKGROUND: Studies have revealed that macrophages play an important role in the development of severe acute pancreatitis (SAP). Activated macrophages can lead to a systemic inflammatory response, induce lipid peroxidation, impair membrane structure, result in injury to the liver and the other extrahaepatic organs, and eventually result in multiple organ dysfunction syndrome by promoting excessive secretion of cytokines. Liver injury can further aggravate the systemic inflammatory response and increase mortality by affecting the metabolism of toxins and the release of excessive inflammatory mediators. Clodronate is a synthetic bisphosphonate, which is often used for treating bone changes caused by osteoporosis and other factors. In the current study, we created liposomes containing superparamagnetic iron oxide particles (SPIOs) for macrophage labeling and magnetic resonance imaging, using a novel method that can bind the clodronate to induce apoptosis and deplete macrophages.

METHODS: Superparamagnetic Fe₃O₄ nanoparticles were prepared by chemical coprecipitation. SPIO-containing liposomes and SPIO-clodronate-containing liposomes were prepared by the thin film method. SAP models were prepared by injection of sodium taurocholate (2 ml/kg body weight) into the subcapsular space of the pancreas. Sprague-Dawley rats were randomly divided into a control group, a SAP plus SPIO-liposome group, and a SAP plus SPIO-clodronate-containing liposome group. Two and six hours after SAP models were available, T2-weighted MRI scans (in the same plane) of the livers of rats in each group were performed. At the end of the scans, 2 ml of blood was taken from the superior mesenteric vein to measure the levels of serum amylase, ALT, AST, TNF-α, and IL-6. Pathological changes in the liver and pancreas were assessed.

RESULTS: Transmission electron microscopy showed that the liposomes had a uniform size. No pathological changes in the pancreata of rats in the control group were noted. The pathological changes in the pancreata and livers of rats in the SAP plus SPIO-clodronate-containing liposome group were milder than those in the SAP plus SPIO-liposome group. The MRI signal intensity of the livers in the SAP plus SPIO-liposome and SAP plus SPIO-clodronate-containing groups was significantly lower than that in the control group. There were significant changes in the two experimental groups (P<0.01). In addition, the levels of serum amylase, ALT, AST, TNF-α, and IL-6 in rats in the SAP plus SPIO-liposome group were higher than those in the control group (P<0.01), while the corresponding levels in the SAP plus SPIO-clodronate-containing liposome group were significantly lower than those in the SAP plus SPIO-liposome group (P<0.01).

CONCLUSION: Clodronate-containing liposomes protect against liver injury in SAP rats, and SPIO can be used as a tracer for MRI examination following liver injury in SAP rats.

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KEY WORDS: pancreatitis, acute; clodronate disodium; liver injury; MR imaging; macrophage

Introduction

Despite intensive research efforts, the systemic morbidity of severe acute pancreatitis (SAP) has increased over time.¹ SAP involves a complex array of mediators that initiate and amplify the systemic inflammatory response, which leads to the failure of distant organ systems. SAP-induced liver injury is difficult to prevent and cure because of complicated pathogenesis. It frequently occurs in clinical practice, especially in the
early phase of SAP, and progresses rapidly. Though the mechanism underlying SAP-induced liver injury has not been fully elucidated, current studies suggest that it is potentially a complex pathophysiological process involving many influential factors such as inflammatory mediators, oxidative stress, and microcirculatory disturbance.\cite{2,3}

Recent studies have shown that Kupffer cells (KCs) not only act as phagocytes but also play a central role in various liver diseases as potent secretory cells. Accumulated evidence suggests that reduced numbers of KCs protect against liver injury, but no consensus on the protective mechanism has been reached.\cite{4,5} Activated KCs can lead to a systemic inflammatory response, induce lipid peroxidation, impair membrane structure, result in injury to the pancreas and the other extrapancreatic organs, and eventually result in multiple organ dysfunction syndrome (MODS) by enhancing excessive secretion of cytokines such as tumor necrosis factor-alpha (TNF-\(\alpha\)), interleukin (IL)-6, or IL-1.\cite{6,7}

Macrophage depletion can be achieved with the systemic injection of liposomes containing clodronate rather than free clodronate, which fails to permeate cellular membranes and has a short half-life in the systemic circulation.\cite{8} Clodronate belongs to the family of bisphosphonates, bone-seeking agents that are potent osteoclast inhibitors. Like other bisphosphonates, clodronate has poor cell membrane permeability.\cite{9,10} Liposomes are readily taken up by cells of the reticuloendothelial system, in particular macrophages. Liposome-mediated delivery of clodronate inactivates and kills macrophages after effective phagocytosis but has no toxicity to nonphagocytic cells.\cite{11} Superparamagnetic iron oxide nanoparticles (SPIO) have recently been shown to be an important tool for enhancing magnetic resonance contrast. Upon systemic application, SPIO particles are preferentially internalized by macrophages.\cite{12} In this study, we employed a novel method to make SPIO-containing liposomes for macrophage labeling and MRI, which were able to bind clodronate for macrophage apoptosis and depletion.

In this study, we employed liposomes as carriers to deliver clodronate into macrophages (including KCs) to induce apoptosis, reduce the release of inflammatory mediators, and deliver SPIO for MRI. Our investigation of the effect of SPIO-clodronate-containing liposomes on KCs in SAP provides a new basis for the treatment and MRI evaluation of liver injury with SAP.

**Methods**

**Animal models and experimental grouping**

Forty-eight healthy Sprague-Dawley rats weighing 350-400 g were provided by the Laboratory Animal Center at Jiangsu University School of Medicine, China. The rats were housed in a controlled environment with an ambient temperature of 21-23 °C and a 12 : 12-hour light-dark cycle. The rats were fed a standard laboratory diet, given water ad libitum, and fasted overnight before each experiment. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Jiangsu University, China. Rats were handled according to regulations stipulated by the Institutional Animal Care and Use Committee. Maximal effort was made to minimize animal suffering, and the number of animals used was the minimum number required to obtain significant data. All rats were randomly divided into three groups: control (group C), SAP plus SPIO-liposomes (group P), and SAP plus SPIO-clodronate-containing liposomes (group T). Each group was further divided into two subgroups with periods of 2 or 6 hours. After anesthesia, the abdominal cavities of the rats were opened. SAP models were prepared by injecting 5% sodium taurocholate (2 ml/kg body weight) into the subcapsular space of the pancreas.\cite{13} The rats were injected very slowly through the tail vein with either SAP plus SPIO-liposomes (2 ml/kg body weight) in the group P, SPIO-clodronate-containing liposomes (2 ml/kg body weight) in the group T, or normal saline (2 ml/kg body weight) in the group C. Dosing was performed as described above; all dosages had previously been proven to be effective. The suspension was shaken gently before administration. T2-weighted MRI scans (in the same plane) of the livers of rats in each group were performed. At 2 and 6 hours after SAP model creation, animals were sacrificed; the livers and pancreata were harvested.

**Materials**

Clodronate was from Shanghai Weijing Technology Enterprise Co., Ltd. (China), sodium taurocholate from Sigma (USA), and ferric trichloride hexahydrate (analytical reagent) and ferrous sulfate heptahydrate (analytical reagent) from Sinopharm Chemical Reagent (China) and Shanghai Second Chemical Reagent Factory (China), respectively. The JJ-1 electric stirrer, electronic balance, and Tecnai-12 transmission electron microscope were from Jintan Medical Instrument Factory (Jiangsu, China), Sartorius (Beijing, China), and Philips (The Netherlands), respectively. We also used an automatic biochemistry analyzer CL-7300 (Shimadzu, Japan), rotary evaporators R-200 (BUCHI Labortechnik AG), and MR (Siemens Magnetom Trio Tim 3.0T).
SPIO preparation

SPIO nanoparticles with a diameter of 20-50 nm were prepared using the coprecipitation-hydrothermal method. Briefly, concentrated hydrochloric acid (0.85 ml) was added to 25 ml of distilled water. Nitrogen gas was then added to the solution for 30 minutes to expel the oxygen. FeCl₃ · 6H₂O (5.2 g) and FeSO₄ · 7H₂O (2.68 g) were added successively while stirring. The mixture was then dropped into a four-neck flask containing 250 ml of 1.5 mol/L NaOH and 2 g of PEG-2000 while the solution was vigorously stirred and filled with nitrogen gas. The flask was placed in a water bath at 60 °C. The mixture was then stirred for another 60 minutes.

The pH value of the solution was maintained between 11 and 12 throughout the entire precipitation process. The final black solution was transferred to a 100 ml hydrothermal kettle and maintained at 180 °C for 8 hours. After magnetic separation of the resulting solution, black precipitates were washed with deionized water until neutral pH was achieved. Samples were then vacuum-dried at 50 °C for 8 hours to yield a black powder.

Preparation and use of SPIO-containing liposomes and SPIO-clodronate-containing liposomes

SPIO-containing liposomes and SPIO-clodronate-containing liposomes were prepared by the reverse phase evaporation method as described previously by van Rooijen. Briefly, phosphatidylcholine chloroform solution (100 mg/ml) was prepared and kept away from light at -20 °C for further use. In a 500 ml round-bottom flask, 8 mg of cholesterol was dissolved in 10 ml of chloroform. After that, 0.86 ml of the phosphatidylcholine chloroform solution containing 86 mg of phosphatidylcholine was added. Chloroform was then removed by rotary distillation (150 rpm) at 37 °C under low-vacuum conditions (gradually decreased from 200 mbar to 150 mbar). Finally, the mixture was then sonicated at 50 Hz for 3 minutes in a cold-water bath, and then kept under nitrogen for an additional 2 hours at room temperature to swell the liposomes. To remove the non-encapsulated clodronate and SPIO, the preparations were washed three times using sterilized PBS (centrifugation at 10 000 g for 30 minutes). Finally, the pellet was re-suspended by the addition of 4 ml sterilized PBS. The suspensions were stored in nitrogen until use (within 2 weeks) and shaken gently before administration to animals. This concentration represents an estimate, thus doses of liposomes are described as volumes rather than as concentrations.

MRI scan of liver

A Magnetom Trio Tim (3.0 T, Siemens) superconducting MRI scanner was used. A breast coil, self-made fixation tool for rats, water model, and image post-processing workstation (Leonardo) were utilized. The rats were put through the center of the coil along the vertical direction (that is, the long axis of the rat and the coil were mutually perpendicular). Physiological saline solution was placed below the coil for MRI. All rats were placed in the supine position. After rats were fixed by using the self-made fixation tool, they were wrapped through the coil and put on the scanning bed. All rats received coronal SE-T2WI (T2-weighted image) scans. Scan parameters were as follows: repetition time, 3000 ms; echo time, 107 ms; scan field, 180×100; slice number, 12; slice thickness, 3.0 mm; and slice spacing, 0.3 mm. Circular regions of interest (ROI) were used to determine the signal intensity of the liver after injection of contrast agent. To reduce error, the signal values of ROI in the right lower lobe of the liver were determined to calculate average values. The scanning sites before and after injection of contrast agent were consistently kept as distant as possible. The pancreata in rats were too thin to undergo MRI examination.

Analysis of serum amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), TNF-α and IL-6 levels

To assess serum amylase, ALT, AST, TNF-α, and IL-6 levels, blood was obtained from the superior mesenteric vein, placed on ice for 15 minutes, and centrifuged at 3000 rpm. The supernatant was retained and preserved at -20 °C. Serum amylase, ALT, and AST were measured with an automatic biochemical analyzer. Serum TNF-α and IL-6 were measured by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s protocol.

Immunohistochemical identification of macrophages in liver tissue

We detected CD68⁺ cells by means of a standard indirect three-step immunohistochemical method. A commercially acquired monoclonal anti-rat CD68...
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(macrophage-associated antigen) antibody was used to detect macrophages in formalin-fixed, paraffin-embedded tissues from rats.

Pathological examination

Paraffin-embedded pancreata and livers were sectioned (5 μm), then stained with hematoxylin and eosin. Experienced histologists, unaware of which treatment the animal was subjected to, scored each specimen histologically. This pathological assessment of pancreatic tissue was performed according to the scoring criteria proposed by Kaiser. Liver injury was scored on a scale of 0-3 for each criterion.

Statistical analysis

All data were expressed as mean±SD using SPSS statistical software (PASW Statistics for Windows, version 18.0). If equal variances were assumed, one-way ANOVA was used to evaluate the differences in serum amylase, ALT, AST, and T2WI; otherwise, the Kruskal-Wallis test was used. P<0.05 indicates a significant difference.

Results

Observation of Fe₃O₄ particles and liposomes with electron microscopy

As revealed by electron microscopy, bare Fe₃O₄ nanoparticles were nearly spherical. The majority of these nanoparticles aggregated, with an average diameter of 20-50 nm. The nanoparticles precipitated easily. After Fe₃O₄ particles were entrapped in the cores of liposomes, the resulting superparamagnetic Fe₃O₄ nanoparticles were dark brown in appearance, had a pH value of 6.47, contained 5 μmol Fe/ml, and showed stable suspension. The available liposomes had an average diameter of 100-200 nm. Under transmission electron microscopy, these liposomes displayed good shape and uniform size (Fig. 1).

Changes in MRI T2WI

The signal intensity of the liver on T2WI in the groups P and T was significantly lower than that in the group C at 2 and 6 hours (Fig. 2).

Changes in the levels of serum amylase, ALT, and AST

Levels of serum amylase, ALT, and AST at 2 and 6 hours in rats in the group P were higher than those in the group C (P<0.01). In contrast, the levels of serum amylase, ALT, and AST at both time points in the group T were lower than those in the group P (P<0.01) (Fig. 3).

Comparison of serum TNF-α and IL-6 levels

At both time points, higher levels of TNF-α and IL-6 were obtained in the group P than in the group C (P<0.01).

Fig. 1. A: Electron microscopy revealed that bare Fe₃O₄ nanoparticles were near-spherical and showed Fe₃O₄ electron diffraction patterns; B: SPIO-clodronate-containing liposomes were similar in size; iron particles were distributed uniformly.

Fig. 2. Signal intensity of the liver on T2WI in the group C (A, D), group P (B, E), and group T (C, F). The signal intensity in the groups P and T was significantly lower than that in the group C (G). *, P<0.01, vs. the group C; #: P<0.01, vs. the group P. Data are mean±SD (n=8) as below.
Compared with the group P, the levels of TNF-α and IL-6 decreased in the group T (P<0.01) (Fig. 4).

Morphological and pathological changes in the pancreas

Gross observation: In the group C, the pancreata showed no significant changes. In the group P, bloody ascites in the abdominal cavity as well as pancreatic congestion, edema, hemorrhage, and necrosis were noted. In the group T, pathological changes were milder than those in the group P. Light microscopy: In the group C, animals displayed normal pancreatic histology. In the group P, the pancreata were slightly edematous, with extensive infiltration by inflammatory cells (2 hours) and necrosis of the adjacent fat tissues, moderate hemorrhage, and more diffuse focal areas of nonviable pancreatic parenchyma; acinar cell necrosis was also observed (6 hours). The animals in the group T showed distinct signs of mild edematous pancreatitis characterized by interstitial edema, as well as infiltration of neutrophil and mononuclear cells, but without parenchyma necrosis and hemorrhage. These histological changes in the group T were less marked than those in the group P. According to Kaiser’s criteria, the histological scores showed that significant differences existed in the groups P and T as compared to the group C.

Less dramatic pathological changes were observed in the group T as compared to the group P (P<0.01) (Fig. 5).

Morphological and pathological changes in the liver

Gross observation: In the group C, the livers showed normal morphology. In the group P, varying degrees of congestion and swelling were seen at both time points; the liver became darker in color, and scattered necrotic foci were visible. In the group T, the above-mentioned changes were milder at 2 and 6 hours than those in the group P. Light microscopy: In the group C, the livers showed no morphological or structural abnormalities. In the group P, varying degrees of liver sinusoidal expansion, swelling and degeneration of liver cells, spotty or plaque hemorrhage, and necrosis in the parenchyma were seen at 2 and 6 hours. In the group T, pathological changes in the liver were significantly milder than those in the group P. The pathological severity score was higher in the groups P and T than in the group C (P<0.01), as well as lower in the group T than in the group P at 2 and 6 hours (P<0.01) (Fig. 6).

Immunohistochemistry for macrophage marker (CD68) in liver tissue

Immunostaining for the macrophage-specific marker CD68 in rat liver tissue sections can be seen in Fig. 7.
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Fig. 5. Pathological changes in the pancreas. In the group C, the pancreata of rats showed no morphological or structural abnormalities (A, D); in the group P, there were varying degrees of focal interlobular edema, necrotic areas without structure, and red blood cells in the tissue space, as well as massive inflammatory cell infiltration (B, E); in the group T, pancreatic edema, hemorrhage, and necrosis, as well as inflammatory cell infiltration were milder than in the group P (C, F). Histological scores showed significant differences between the groups P and T as compared to the group C; pathological changes in the group T were less severe than those in the group P (G).

Fig. 6. In the group C, rat livers showed no morphological or structural abnormalities, and liver sinusoidal expansion was occasionally noted (A, D); in the group P, varying degrees of liver sinusoidal expansion, swelling, and hepatocyte cell degeneration were seen at 2 hours; inflammatory cell infiltration, predominantly distributed in the portal area and necrotic areas, was noted at 6 hours (B, E); in the group T, pathological changes in the liver were significantly milder than those in the group P (C, F). Liver injury scores (0-3) were determined as described in the Methods section (G).

Fig. 7. Immunostaining for the macrophage-specific marker CD68 in rat liver tissue sections. Under homeostatic conditions (A, D); numerous KC clusters were observed in the group P; the tissue architecture was massively distorted (B, E); fewer KCs were observed in liver tissue sections from the group T (C, F).
Discussion

SAP is commonly known as "acute abdomen"; its underlying pathogenesis has not been fully elucidated. It is currently believed that, during the early phase of SAP, pancreatic acinar cells produce and release excessive inflammatory cytokines, which causes the inflammatory cascade to run out of control, induces or exacerbates the systemic inflammatory response syndromes, and results in MODS. Excessive release of systemic proinflammatory cytokines is a dominating factor for both local and systemic aggravation of SAP. Infiltration and activation of macrophages not only triggers the initial events of SAP, but also functions as an important pathophysiological step in multiple organ failure.

As the largest reservoir of macrophages in the body, the human liver plays a unique role in the systemic response to SAP. KCs in the liver account for approximately 50% of all macrophages, or 80%-90% of fixed macrophages in the body. KCs are the main effector cells in the production of inflammatory cytokines. Since liver injury is closely related to the development and prognosis of SAP, exploration of the mechanism underlying the development of liver injury is of great significance for preventing injury to extrapancreatic organs and reducing the occurrence of MODS. Experiments showed that cytokines generated by macrophages, such as TNF-α and IL-1β, directly cause liver injury and liver dysfunction. Therefore suppressing TNF-α and IL-1β produced by macrophages could reduce liver injury. Gloor et al. investigated the roles of cytokines secreted by KCs in SAP and found that, in untreated pancreatitis patients, the levels of cytokines were lowest in the portal vein, higher in the liver vein, and highest in peripheral blood. After blocking KCs with gadolinium chloride, the levels of cytokines in the liver vein and peripheral blood declined significantly, and the extent of liver injury was significantly lessened. This result suggests that cytokines that originate from KCs elevate cytokine levels in peripheral blood and play a role in related liver injury.

Bisphosphonate clodronate, clinically used in the treatment of osteoporosis, is known to deplete monocytic lineage cells. van Rooijen et al. found that intravenous injection of clodronate-containing liposomes selectively eliminates macrophages in vivo. Clodronate is a bisphosphonate drug that inhibits the viability of macrophages via the induction of apoptosis, possibly mediated by competing with ATP as a substrate for intracellular ATPase. After clodronate is incorporated within liposomes, its uptake by phagocytic cells such as macrophages is greatly enhanced, resulting in selective lethality that targets macrophage populations.

Liposomal clodronate inhibits the growth of cultured macrophages but has no effect on endothelial or smooth muscle cells.

Since SPIO nanoparticles are engulfed by KCs in vivo, they can be used for MR tracking and specific imaging in tissues containing high numbers of macrophages such as the liver, spleen, and lymph nodes. SPIO nanoparticles significantly reduce the signal values of T1WI and T2WI. The main effect of the SPIO particle in the MR image is a shortening of the T2 relaxation time. The signal intensity of tissues on T2WI decreases with an increase in SPIO content. As nanoscale particles, SPIO particles have uniform size, are water-soluble, and show no mutual aggregation. After being introduced into the liver, SPIO particles are recognized and engulfed by KCs that form part of the reticuloendothelial system. As a result, the signal intensity of the corresponding region is reduced. KCs in normal liver account for 80% of all reticuloendothelial cells. The vast majority of SPIO particles are engulfed by KCs after they are introduced into the body. The extent to which SPIO particles are engulfed in the liver depends on KC distribution. SPIO-enhanced T2WI MRI scans permit evaluation of the extent of liver injury in SAP. The signal intensity of the liver on T2WI was significantly lower in the group P than in the group C.

In the present study, we investigated the time-course of changes in KC expression of the macrophage-specific marker CD68 with SAP in rat liver using immunohistochemistry. Serum amylase, TNF-α, and IL-6 levels were notably increased after induction of SAP. Levels in the group T were lower than those in the group P. Pancreas and liver injuries in the group T were milder than those in the group P.

In our model, the degree of liver injury, which was assessed according to the standard scale of pathological examination, was closely paralleled by CD68 expression. In conclusion, KCs are involved in the pathogenesis of liver injury in SAP. The data in the present report indicate that macrophages play a crucial role in the development of SAP, and that activated macrophages contribute to the progression of liver injury. Moreover, clodronate-containing liposomes induce the apoptosis of macrophages in SAP rats. Thus, we can reduce the release of inflammatory mediators and lessen the inflammatory response by inducing the apoptosis of macrophages, and further improve the prognosis of SAP. This study shows that inactivation of macrophages by systemic administration of liposome-encapsulated clodronate inhibits intestinal macrophages in rats with SAP. These results validate our hypothesis that macrophages play a pivotal role in the pathogenesis of aggravated liver injury.
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Injury. In the study, liposomal clodronate was safely administered to macrophages. The doses were sufficient to relieve the inflammatory reaction by inhibiting macrophage function. These findings suggest that liposomal delivery of clodronate offers an effective novel approach to treat SAP in rats. This study also provides a new approach for clinical treatment of SAP and SAP-induced liver injury. Modulation of macrophage function. These findings suggest that clodronate-liposomes attenuating liver injury in rats with SAP. SPIO can be used as a tracer for MRI examination of liver injury.

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Ethical approval: Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Jiangsu University with the approval of the Institutional Animal Care and Use Committee. Efforts were made to minimize animal suffering, and the number of animals used was minimal to obtain significant data.

Contributors: ZJX proposed the study. DSC wrote the first draft and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. ZJX is the guarantor.

Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Correction

In the article entitled Clinical management of hepatitis B virus infection correlated with liver transplantation by Zhang et al (Hepatobiliary Pancreat Dis Int 2010;9:15-21.), the Unit of the dose in the Table should be μg.