

Protection Provided by a Gabexate Mesylate Thermo-Sensitive *In Situ* Gel for Rats with Grade III Pancreatic Trauma

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Background/Aims: This study investigated the protection provided by gabexate mesylate thermo-sensitive *in-situ* gel (GMTI) against grade III pancreatic trauma in rats. **Methods:** A grade III pancreatic trauma model with main pancreatic duct dividing was established, and the pancreas anatomical diagram, ascites, and serum biochemical indices, including amylase, lipase, C-reactive protein (CRP), interleukin 6 (IL-6), and tumor necrosis factor- α (TNF- α), were examined. The pancreas was sliced and stained with hematoxylin eosin and subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. **Results:** Ascites, serum amylase, lipase, CRP, IL-6, and TNF- α levels were significantly increased in the pancreas trauma (PT) groups with prolonged trauma time and were significantly decreased after GMTI treatment. The morphological structure of the pancreas was loose, the acinus was significantly damaged, the nuclei were irregular and hyperchromatic, and there was inflammatory cell invasion in the PT group compared to the control. After GMTI treatment, the morphological structure of the pancreas was restored, and the damaged acinus and inflammatory cell invasion were decreased compared to the PT group. Moreover, the cell apoptosis index was significantly increased in the PT group and restored to the same levels as the control group after GMTI treatment. **Conclusions:** GMTI, a novel formulation and drug delivery method, exhibited specific effective protection against PT with acute pancreatitis therapy and has potential value as a minimally invasive adjuvant therapy for PT with acute pancreatitis. (*Gut Liver* 2017;11:156-163)

Key Words: Grade III pancreatic trauma; Main pancreatic duct; Thermo-sensitivity; Apoptosis; Therapeutics

INTRODUCTION

The pancreas is a secretion gland with endocrine and exocrine functions. The pancreas is located deeply behind the costal arch and is not easily damaged, with the exception of severe blunt injury such as automobile accidents.^{1,2} Pancreatic trauma (PT) is one of the most troublesome consequences of abdominal trauma seen in the clinic, for which there is no specific effective treatment due to the complex nature of the injury, and is associated high mortality rate, especially when combined with main pancreatic duct injury.³⁻⁶ The clinical characteristics of PT include internal bleeding, pancreatic juice peritonitis and occasionally severe abdominal pain, especially for severe PT and/or cases where the main pancreatic duct is ruptured.^{1,2,4,7} Although the detailed mechanisms of PT and its high mortality have not been clearly elucidated, they may be associated with the deep location of the gland, adjacent tissue damage, and pancreatic enzyme activation that leads to autolysis of the pancreatic tissues and surrounding structures.^{4,8,9} Pancreatic enzyme activation is also considered to play an initial role in the pathogenesis of pancreatitis.^{8,10,11} In the clinic, protease inhibitors, such as such as gabexate mesylate (GM), are often used to remedy acute pancreatitis in many countries due to their ability to block premature trypsin activation.¹²⁻¹⁴

GM is a nonpeptide protease inhibitor that can inhibit various serine proteases to improve histology scores and reduce mortality, as described in the literature.^{12,15,16} However, the wide application of GM is limited due to the large dose needed and its complex infusion protocol. Specially, conventional infusion does not easily reach the damaged pancreas due to the impaired microcirculation. Therefore, present studies increasingly focus on how to raise the concentration of anti-proteases in pancre-

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Received on June 25, 2015. Revised on March 10, 2016. Accepted on March 11, 2016. Published online September 20, 2016

pISSN 1976-2283 eISSN 2005-1212 <https://doi.org/10.5009/gnl15281>

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atic tissue, such as through continuous regional arterial infusion and peritoneal lavage, both of which exhibit high efficiency and superiority to intravenous administration.^{17,18} Unfortunately, continuous regional arterial infusion must be performed under X-ray guidance and can cause complications. Additionally, peritoneal lavage offers poor positioning and carries a large risk for adverse reactions because of its long treatment duration and large dose. As an alternative delivery mechanism, temperature- and/or pH-sensitive block copolymer hydrogels have been extensively documented.¹⁹⁻²¹ When drugs are mixed with a polymer solution *in vitro*, they can form drug-hydrogel complexes *in situ* for *in vivo* applications. Stimuli-sensitive block copolymer hydrogels have several advantages, such as simple drug formulation and administration, no need for organic solvent, site specificity, sustained drug release, low toxicity, and the ability to deliver both hydrophilic and hydrophobic drugs. Therefore, a GM thermo-sensitive *in situ* gel (GMTI) was chosen for further study to assess the potential for GMTI-mediated protection in rats with grade III PT.

Due to the limitations of traditional approaches for PT therapy and the potential benefits of GMTI for disease treatment, we hypothesized that GMTI may ameliorate PT and represent a novel drug administration approach for the treatment of PT.

MATERIALS AND METHODS

1. Experimental animals and groups

A total of 42 Sprague-Dawley rats (male, 200 to 250 g) were provided by the National Institutes of Health for the Care of Laboratory Animals (license number, SYXK [Beijing] 2012-0004; housing temperature, 25°C±2°C; humidity, 40% to 60%, 12 hours light/dark cycle) and were randomly divided into three groups: control group (n=6), PT group (n=18, including three time points: 1, 6, and 24 hours), and GMTI group (n=18, including three time points: 1, 6, and 24 hours). The study was approved by the Chinese People's Liberation Army General Hospital Ethics and Experimental Committee.

2. Establishment of a grade III PT model

All of the aforementioned rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (3%; Sigma, St. Louis, MO, USA) at 3 mg/100 g before surgery. In the PT and GMTI groups, the rats were initially fixed in place, and an abdominal midline incision was made to expose the pancreas. Subsequently, according to the Organ Scaling Committee of the American Association for the Surgery of Trauma, a laceration (0.6 cm in length) with duct rupture was uniformly created using hemostatic scissors in a section of pancreas adjacent to the duodenum to mimic grade III PT. A novel GMTI was developed, and an optimum formulation of GMTI, consisting of 20.6% (w/w) P407 and 5.79% (w/w) P188 with different concentrations of GM, was used as a gelling solvent. Then, GMTI containing 0.1%

(w/v) GM (provided by the Department of Pharmaceutics, Beijing Institute of Pharmacology and Toxicology, Beijing, China) was directly injected into the body and head of the pancreas at a dose of 0.3 mL/100 g in the GMTI group; the same volume of 0.9% normal saline was injected into the pancreas in the PT group. At 1, 6, and 24 hours after treatment, ascites, serum and pancreatic tissue were collected for further analysis.

3. Measurement of ascites

Ascites was collected at 1, 6, and 24 hours after trauma in both groups. The total volume was calculated, and analysis was performed using Origin 9.5 software (OriginLab, Northampton, MA, USA; <http://www.originlab.com/>).

4. Measurement of serum biochemical markers using enzyme-linked immunosorbent assay

Whole blood was collected at the aforementioned time points and centrifuged at 3,000 to 5,000 rpm for 30 minutes at room temperature to separate serum for further measurements of serum amylase, lipase, CRP, IL-6, and TNF- α according to the manufacturers' instructions for the kits used. For the serum amylase assay, rat amylase enzyme-linked immunosorbent assay (ELISA) kits (cat. No. C016; Nanjing Jiancheng, Nanjing, China) were used, and the results were obtained at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Similarly, rat lipase ELISA kits (cat. No. A054; Nanjing Jiancheng), rat CRP ELISA kits (cat. No. H126; Nanjing Jiancheng), rat IL-6 ELISA kits (cat. No. H007; Nanjing Jiancheng) and rat TNF- α ELISA kits (cat. No. H052; Nanjing Jiancheng) were used, and results were obtained at 450 nm using a microplate reader. Analyses were carried out using Origin 9.5 software.

5. Hematoxylin and eosin staining

Slides were deparaffinized and rehydrated, and frozen or vibratome sections were mounted on slides and then rehydrated. The sections were slightly over-stained with hematoxylin for approximately 3 minutes to 5 minutes, depending on the thickness of the section and fixative used (up to 20 minutes if the solution was not fully ripened), and excess stain was removed in tap water. The sections were destained for a few seconds in acidic alcohol until the sections appeared red, usually requiring four to five dips. Then, the sections were briefly rinsed in tap water to remove the acid. Bicarbonate was applied for approximately 2 minutes until the nuclei stood out sharply in blue. The hematoxylin-stained slides from the last tap water rinse were then placed in 70% ethanol for 3 minutes, followed by eosin for 2 minutes. Then, the slides underwent a clearing series of three washes in 95% ethanol for 5 minutes followed by immersion in absolute ethanol. Images were then captured using a microscope connected to a charge-coupled device (CCD) camera at $\times 40$ magnification.

6. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining

Slides were deparaffinized and rehydrated, followed by two washes with phosphate-buffered saline (PBS; 5 minutes each). The slides were then incubated with proteinase K solution at 37°C for 20 minutes, followed by two washes with PBS (5 minutes each). Blocking buffer was then added at room temperature for 10 minutes, followed by two washes with PBS (5 minutes each). Slides were then incubated with 50 μ L of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture for 1 hour in a humid chamber in the dark, and then the slides were rinsed three times (5 minutes each) with PBS. Then, 50 μ L streptavidin-HRP solution (Beijing Zhongshanjin-qiao Co., LTD, Beijing, China) was added and incubated at 37°C for 30 minutes, followed by three washes with PBS (5 minutes each). Next, 100 μ L DAB solution was added for 10 minutes at room temperature to develop the slides, followed by three washes with PBS (5 minutes each) and hematoxylin redyeing. Images were captured using a microscope connected to a CCD camera, and the apoptosis index was counted, followed by analysis using Origin 9.5 software (OriginLab).

7. Statistical analyses

All data are expressed as the mean \pm standard deviation. Statistical analyses was performed with one-way analysis of variance (ANOVA) using SPSS software version 21.0 (IBM Corp., Chicago, IL, USA), and the Student t-tests were performed for comparisons of two groups of samples. Values of $p < 0.05$ and $p < 0.01$ were considered to indicate significant differences and highly significant differences, respectively.

RESULTS

1. Establishing a grade III PT model

To establish a model of grade III PT, the main pancreatic duct was exposed, as indicated by the yellow arrows in Fig. 1A, and then divided by hemostatic scissors, as indicated by the yellow arrows in Fig. 1B. The results indicated that a grade III PT model was correctly established.

2. The volume of ascites significantly decreased with GMTI treatment

With increasing time after the initial injury, the volume of ascites increased. However, this volume was decreased in the GMTI group at 1 hour (2.42 ± 1.24 mL), 6 hours (4.88 ± 1.29 mL), and 24 hours (6.17 ± 2.52 mL) after treatment compared to the volume in the PT group at 1 hour (7.92 ± 2.13 mL), 6 hours (14.58 ± 1.77 mL), and 24 hours (18.42 ± 2.75 mL). This difference between groups was statistically significant ($p < 0.01$) (Table 1, Fig. 2A). At 24 hours posttrauma, a large volume of ascites accumulated in the abdominal cavity, which was significantly reduced with GMTI treatment (Fig. 2B).

3. The serum amylase, lipase, CRP, IL-6 and TNF- α levels in the PT group all significantly increased with increasing time posttrauma, but were significantly decreased with GMTI treatment

In contrast to the control group, serum biochemical markers, including amylase, lipase, CRP, IL-6 and TNF- α , were all increased in the PT group. However, after GMTI treatment, serum amylase, lipase, CRP, IL-6 and TNF- α were all clearly decreased in comparison to the PT group. The enzymatic activity of amylase was significantly increased at 1 hour posttrauma ($p < 0.01$)

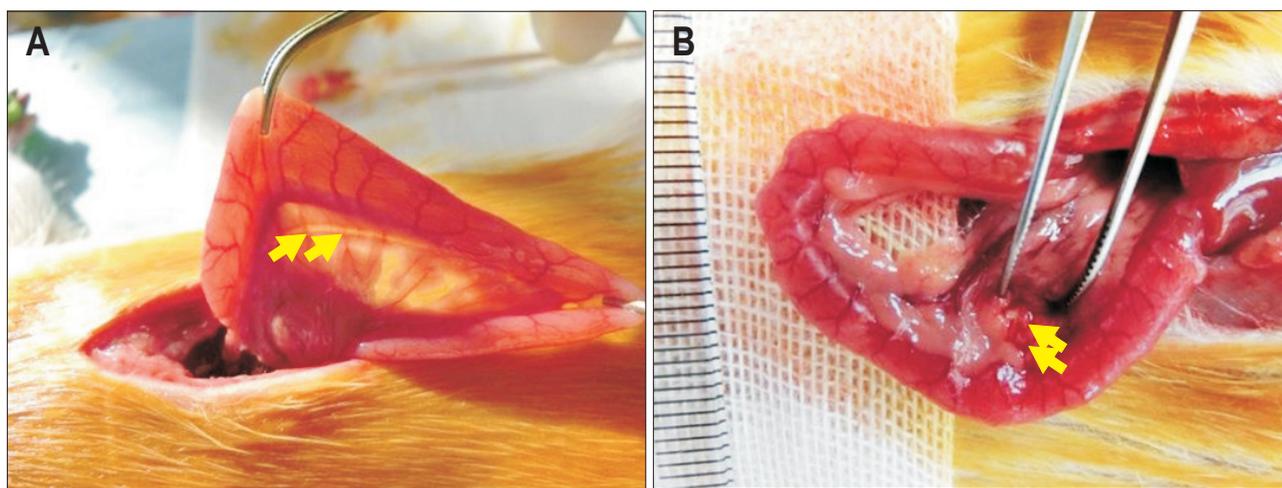


Fig. 1. Anatomical diagram illustrating the main pancreatic duct before and after grade III pancreatic trauma. (A) Anatomical diagram illustrating the main pancreatic duct before grade III pancreatic trauma. (B) Anatomical diagram illustrating the main pancreatic duct after grade III pancreatic trauma. The yellow arrows indicates the main pancreatic duct before and after grade III pancreatic trauma and shows that the main pancreatic duct is divided after grade III pancreatic trauma.

(Fig. 3A) in the PT group but slightly decreased compared to those of the PT group at 1 hour posttrauma ($p<0.05$) (Fig. 3A) in the GMTI group. At 6 hours and 24 hours posttrauma, the enzymatic activity of amylase was significantly increased ($p<0.01$) (Fig. 3A) in the PT group and significantly decreased ($p<0.01$) (Fig. 3A) in the GMTI group. The values for serum lipase ($p<0.01$) (Fig. 3B), CRP ($p<0.01$) (Fig. 3C), IL-6 ($p<0.01$) (Fig. 3D) and TNF- α ($p<0.01$) (Fig. 3E) all exhibited the same trend.

4. Pancreatic edema, yellowing and severe adhesions in the PT group and recovery with GMTI treatment

As shown in the pathological images from the control group, the pancreas appeared bright red with no edema or adhesions (Fig. 4A). However, in the PT group, the pancreas was edematous, yellowed, and showed severe adhesions with the stomach, spleen and intestine at 24 hours posttrauma (Fig. 4B). After GMTI treatment, the pancreas reverted to a bright red color, and the edema and adhesions were not visible at 24 hours post-trauma (Fig. 4C).

Table 1. Comparison of the Ascites Volume in the PT and GMTI Groups (n=6)

Group	Ascites, mL		p-value
	PT	GMTI	
1 Hour	7.92 \pm 2.13	2.42 \pm 1.24	<0.01*
6 Hours	14.58 \pm 1.77	4.88 \pm 1.29	<0.0001*
24 Hours	18.42 \pm 2.75	6.17 \pm 2.52	<0.0001*

Data are presented as mean \pm SD.

PT, pancreas trauma; GMTI, gabexate mesilate thermo-sensitive *in-situ* gel.

* $p<0.05$.

5. The morphological structure of the traumatized pancreas was reversed with GMTI treatment

In the control group, hematoxylin and eosin staining showed pancreatic cells that were tightly distributed, with regular acini, no hyperchromatic nuclei and no inflammatory cell invasion (Fig. 5A). In contrast, in the PT group, the pancreatic cells were loosely distributed, with edematous acini, hemorrhage, necrosis, distorted septal architecture, hyperchromatic nuclei and severe inflammatory cell invasion at 24 hours posttrauma (Fig. 5B). With GMTI treatment, the pancreatic cells showed a restored tight distribution, regular acini and alleviation of inflammatory cell infiltration at the 24 hours time point (Fig. 5C).

6. Cellular apoptosis levels were significantly increased in the PT group and were restored to those of the control with GMTI treatment

Based on TUNEL staining of the pancreatic tissues, the cellular apoptosis level and apoptosis index were both significantly increased in the PT group compared to those in the control group. With GMTI treatment, however, the cellular apoptosis level and apoptosis index were restored to those of the control group ($p<0.01$) (Fig. 6A and B).

DISCUSSION

Here, we demonstrated that GMTI was effective for the amelioration of PT, with clear reversal of the serological changes that occurred with PT, including the levels of amylase, lipase, CRP, IL-6 and TNF- α . With GMTI treatment, the pancreas reverted to a bright red color, and the edema and adhesions disappeared; histologically, the pancreatic cells recovered a tight distribution with regular acini and reduced inflammatory cell invasion.

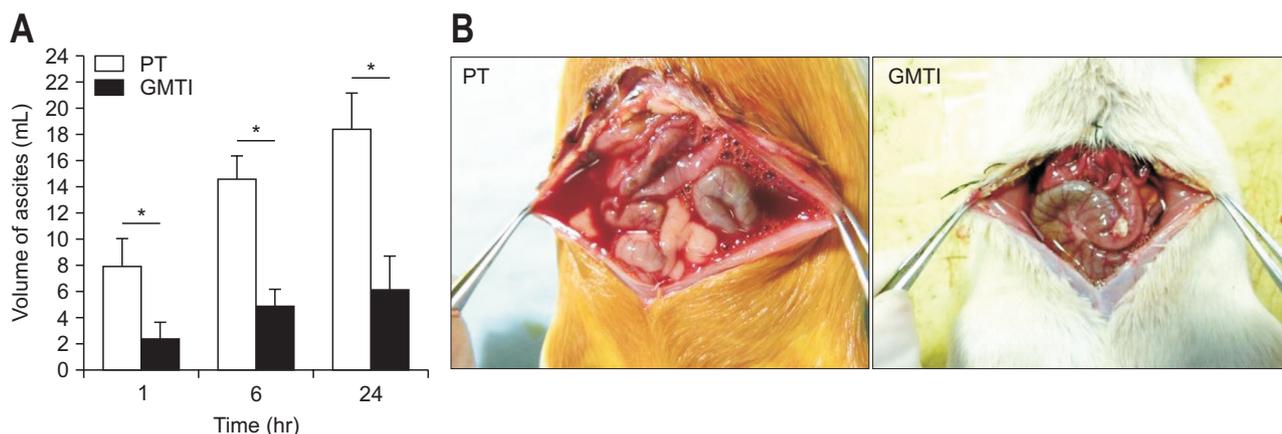


Fig. 2. Comparison of ascites volume in the pancreas trauma (PT) and gabexate mesylate thermo-sensitive *in-situ* gel (GMTI) groups and an anatomical diagram illustrating accumulated ascites in the abdominal cavity. (A) Histogram analysis of the ascites volume in PT and GMTI groups with prolonged time (n=6). (B) Anatomical diagram illustrating the accumulated ascites in the abdominal cavity. The images show that the ascites volume is significantly decreased in the GMTI group (* $p<0.01$, compared to the PT group) and is significantly reduced after 24 hours GMTI treatment.

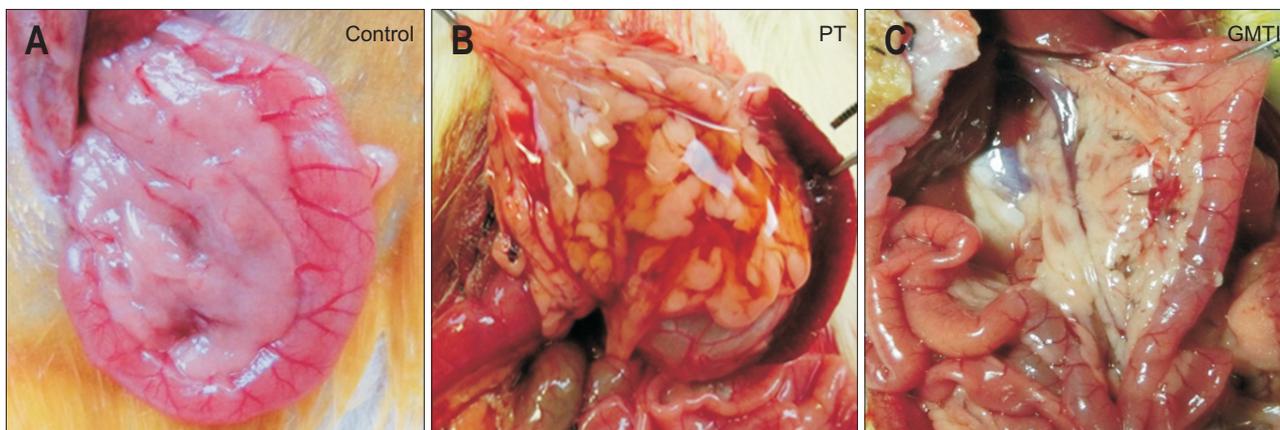
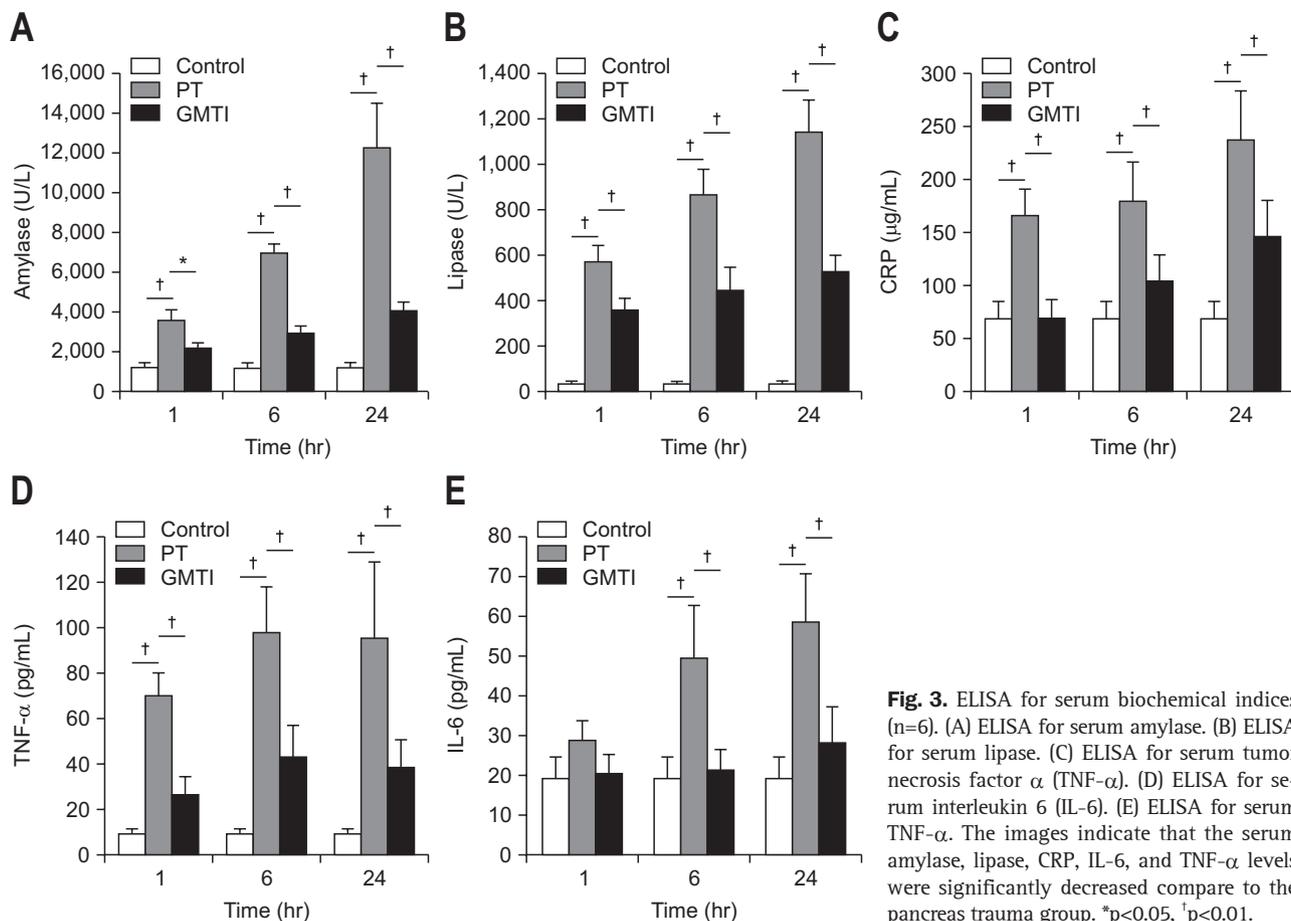


Fig. 4. Anatomical diagram of the pancreas in the control, pancreas trauma (PT) and gabexate mesylate thermo-sensitive *in-situ* gel (GMTI) groups after 24 hours trauma. (A) Anatomical diagram of the pancreas in the control group after 24 hours trauma. (B) Anatomical diagram of the pancreas in the PT group after 24 hours trauma. (C) Anatomical diagram of the pancreas in the GMTI group after 24 hours trauma. The images indicate that the pancreas has edema, yellowing and severe adhesion with the stomach, spleen and intestine after 24 hours trauma in the PT group, while it became bright red with no edema or adhesion in the GMTI group.

According to the Organ Scaling Committee of the American Association for the Surgery of Trauma,²² the division of the main pancreatic duct is one of the key determinant factors that dictates the invalidism and mortality rates in PT,²³⁻²⁵ which is thought to be partially due to large volumes of pancreatic juice

being released with injury, leading to the onset of autolysis and infection. The effective and timely inhibition of trypsin activity may have many potential benefits for patients. When the main pancreatic duct is divided after PT, there is a significant increase in the incidence of critical complications, including pancreatic

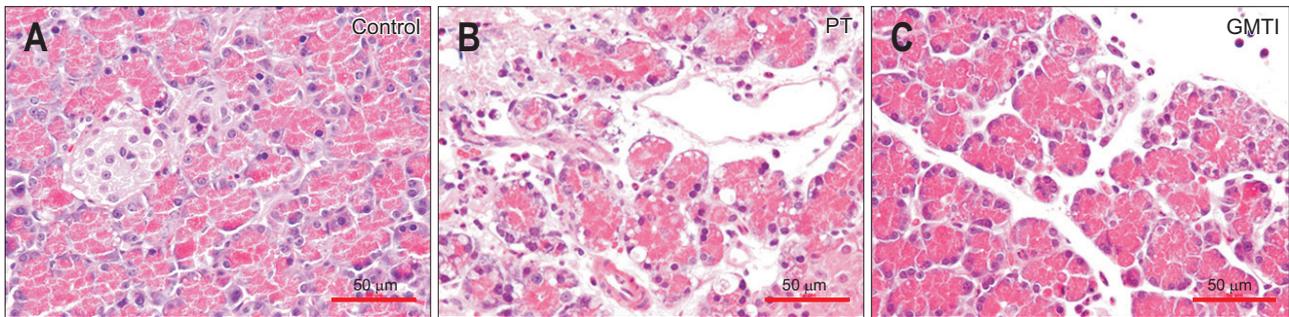


Fig. 5. The morphological structure of the pancreas in the control, pancreas trauma (PT) and gabexate mesylate thermo-sensitive *in-situ* gel (GMTI) groups after 24 hours trauma based on H&E staining ($\times 200$). (A) The morphological structure of the pancreas in the control group after 24 hours trauma. (B) The morphological structure of the pancreas in the PT group after 24 hours trauma. (C) The morphological structure of the pancreas in the GMTI group after 24 hours trauma. The images indicate that the pancreatic cells are loosely distributed, the acinus is damaged, nuclei are hyperchromatic and there is severe inflammatory cell invasion after 24 hours trauma. The pancreatic cells are restored to a tight distribution, regular acinus and no inflammatory cell invasion in the GMTI group.

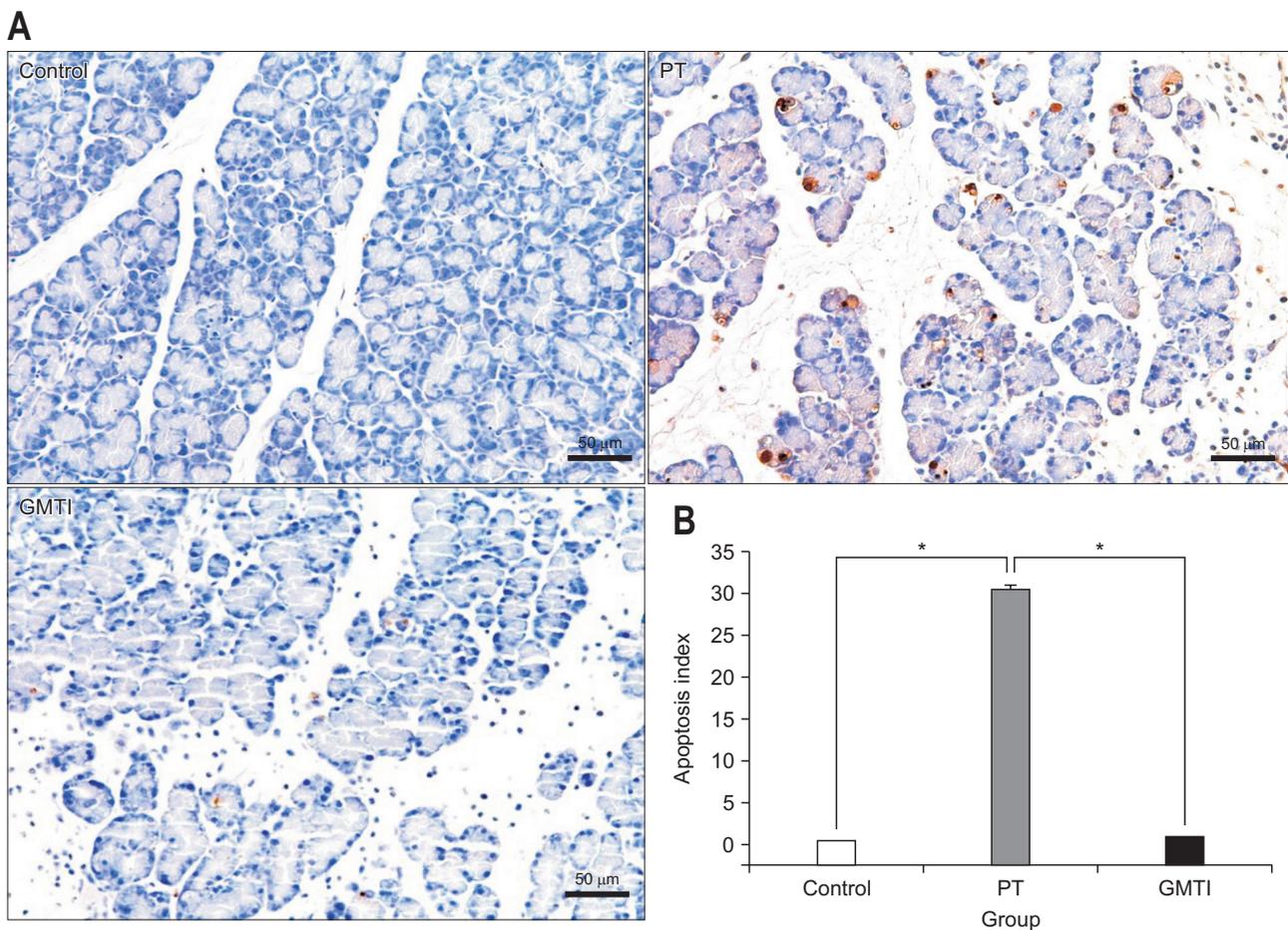


Fig. 6. Cell apoptosis assay using terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) staining and histogram analysis of the cell apoptosis index. (A) Cell apoptosis assay using TUNEL staining ($\times 200$). (B) Histogram analysis of the cell apoptosis index ($n=6$). The images indicate that cell apoptosis level and apoptosis index are significantly increased in the PT group and restored to that of the control group after GMTI treatment ($*p<0.01$).

fistula, chemical peritonitis, bleeding, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome. In a sense, division of the main pancreatic duct is the deciding factor in PT treatment protocols. Grade I and II PT without main

pancreatic duct division can be managed nonoperatively with conservative management. However, grade III PT, with main pancreatic duct division, carries a higher mortality rate regardless of patient age, lacks a standardized therapeutic algorithm,

and requires a personalized treatment plan.^{26,27} Because PT is often combined with vascular injury, traditional intravenous administration of medication is likely to be ineffective. Therefore, a new delivery system using thermo-sensitive *in situ* gels, with reverse gel properties, was developed. Such an approach has been widely adopted in ophthalmic, nasal, rectal and injectable formulations due to advantages¹⁹⁻²¹ such as an improved local drug concentration, accurate positioning and sustained release.

Recently, injectable sustained-release preparations have become a hotspot of pharmaceuticals, as they can be used for the injection of local tissues and target organs and for implantation. After local injection administration, sustained-release preparations can directly release drug at the therapeutic position and slowly enter into the bloodstream, thereby extending treatment time, enhancing treatment effects, and reducing systemic toxicity. Thus, this technology has the potential to reduce administration time, increase patient medication adherence and reduce therapeutic expenses.^{28,29} Several injectable sustained-release preparations have been developed, including for analgesic and endomyocardial applications, the steroid amcinonide, polypeptides, vaccines and gene-targeting drugs.^{30,31}

Poloxamer 407 is a triblock copolymer that exhibits concentration-dependent, reverse thermal gelation.^{32,33} This preparation been widely used in pharmaceutical formulations due to its surfactant and protein-stabilizing properties, and its use has been documented in antitumor and anti-infection studies.^{34,35} However, no study has reported its application for PT treatment. In this study, we first established a grade III PT model in rats using main pancreatic duct division and then explored the treatment potential of GMTI in the model. In the PT group, the serum amylase, lipase, CRP, IL-6, and TNF- α levels all increased compared to those in the control group, and this effect was significantly diminished with GMTI treatment. In particular, the volume of ascites decreased and histopathological injury was alleviated with GMTI treatment, and the cellular apoptosis levels were also significantly decreased with treatment. These results indicate that a thermo-sensitive gel can extend the drug delivery time *in vivo*, which has significant clinical application value. There are also some limitations to this study, including the temperature dependency of GMTI, the lack of a traditional PT model, an injury etiology different from clinical pancreatic injury, the short time course of our model, and the fact that the pancreas is different in rats and humans.

Nevertheless, this study highlights GMTI as a novel formulation and drug delivery modality that served as an effective remedy for PT in rats, and our results provide a significant reference point for the development of adjuvant clinical therapies for PT in humans.

Indeed, consistent with our hypothesis, the ascites volume and serum levels of amylase, lipase, C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor α (TNF- α) in the PT groups increased with time after the traumatic injury, and

these levels were decreased after GMTI treatment. In addition, the morphological structure of the pancreas was restored, and the number of damaged acinus cells and invasive inflammatory cells were decreased in the GMTI group compared to those in the control group; the cellular apoptosis index was also significantly decreased with GMTI treatment. These results indicate that GMTI, as a novel formulation and drug delivery mechanism, may serve as an effective remedy for PT, especially PT in combination with main pancreatic duct trauma.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

This research project was supported by the National Natural Science Foundation of China.

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