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Original Article

Diabetes aggravates acute pancreatitis possibly via activation of NLRP3 inflammasome in db/db mice

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Abstract: Clinical studies have confirmed that patients with diabetes had an elevated risk of acute pancreatitis (AP) and diabetes was associated with increased severity and mortality in patients with AP. However, these studies failed to prove a cause-and-effect relationship between diabetes and AP. In the present study, we for the first time have evaluated the effects of diabetes on AP by adopting a type 2 diabetes animal model db/db mice and investigated the possible underlying mechanisms. The results showed that in comparison to wide type (WT) mice, db/db mice showed exacerbated pancreatic and pulmonary injuries, elevated serum amylase and lipase levels, increased myeloperoxidase (MPO) expressions in pancreatic and pulmonary tissues as well as increased apoptotic acinar cells after AP induction. Furthermore, we observed that NLRP3 inflammasome in pancreatic tissues was remarkably activated in db/db mice compared with WT mice. In addition, we also found that diabetes could increase the susceptibility of mice to AP. Taken together, our results indicated that diabetes could predispose and aggravate the disease severity of AP potentially via promoting the activation of NLRP3 inflammasome pathway.

Keywords: Type 2 diabetes, acute pancreatitis, NLRP3 inflammasome, db/db mice, susceptibility

Introduction

Acute pancreatitis (AP) is a fatal disease of the exocrine pancreas with variable severities ranging from mild with self-limited disease course to severe with high mortality [1]. Severe AP (SAP) accounts for about 20% of patients with AP, who develop necrosis of the (peri) pancreatic tissue and/or (multiple-) organ failure [2]. The overall mortality of AP was found to be 2.6% and in severe AP, 10.1% [3]. Various factors such as gallstones, alcohol, hyperlipidemia and comorbidity affect the pathogenesis and development of AP [4].

Diabetes mellitus (DM), a metabolic disease involving pancreatic exocrine function has globally increasing prevalence and concern. Among adults in China, the estimated overall prevalence of diabetes was 10.9%, and that for prediabetes was 35.7% [5]. From the health and economic perspectives, individuals with DM require at least 2-3 times the healthcare resources compared with individuals without, and diabetes care accounts for up to 15% of national healthcare budgets [6]. Given the close anatomical and physiological relationship between exocrine and endocrine tissue of pancreas, a large number of studies have focused on the interaction between pancreatitis and diabetes [7].

Previous studies indicated that transient hyperglycemia could be observed in up to 50% of patients with AP, persistent diabetes may occur in 1-15% of patients with AP, and the prevalence of diabetes in chronic pancreatitis varied between 30% and 83% [8, 9]. Patients with type-2 DM had an elevated risk of AP compared with patients without diabetes and type-2 DM was associated with increased severity and mortality in patients with AP [10-12]. It has

been well-recognized that pancreatitis could cause the development of DM and vice versa [13]. Furthermore, the presence of hyperglycemia itself has been included in scoring systems such as the Ranson criteria to predict severity in AP [14]. A large number of clinical studies have verified the hypothesis that diabetes may aggravate pancreatitis [10-12, 15]. However, these correlative studies cannot prove a cause-and-effect relationship between them and possible underlying mechanism is needed to be explored.

The purpose of this present study was to verify the hypothesis of whether diabetes could predispose and influence the development of AP, as well as exploring the possible underlying mechanism.

Materials and methods

Animals

Eight-week male wild-type (WT) mice and db/db mice were purchased from Model Animal Research Center of Nanjing University. The blood glucose levels of db/db mice were > 11.1 mM. The animals were housed under specific pathogen-free (SPF) conditions in an air-conditioned animal facility at 24°C on a twelve hours light/dark cycle. Animals were allowed access to water and standard laboratory chow ad libitum. The Principles of Laboratory Animal Care (NIH publication no. 85Y23, revised 1996) was followed, and all experimental protocols were approved by the experimental animal ethics committee of Jinling Hospital affiliated to medical school of Nanjing university (No. 20160905).

Induction of experimental pancreatitis

Mice were injected with 7 doses of 50 μg/kg Caerulein (Cae, AnaSpec, Inc. San Jose, CA, USA) at 1-h intervals intraperitoneally to induce AP model. WT mice and db/db mice were randomly assigned to 4 groups: WT+PBS (phosphate-buffered saline, PH=7.4) group, WT+Cae group, db/db+PBS group, db/db+Cae group (n=8 for each group). The WT+PBS and db/db+PBS groups were given PBS solution intraperitoneally instead of Cae.

Sample collection and preparation

Blood samples were obtained from the tail veins of isoflurane-anesthetized mice at 0, 12, 24 hours after the first Cae injection. Animals were anaesthetized with an intraperitoneal administration of sodium pentobarbital (50 mg/kg) and sacrificed at 24 h after the first Cae injection. A portion of the pancreatic and pulmonary tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin for histological analysis and the rest were stored at -80°C for further investigation.

Analysis of plasma parameters

Serum amylase and lipase activities were determined by amylase kits (Zhongsheng Beikong BioTechnology, Beijing, China.) and lipase kits (Nanjing Jiancheng Corp., Nanjing, China). Total cholesterol (TC), triglyceride (TG) and fasting blood glucose levels were determined with a commercially available kit (Beijing Zhongsheng Beikong Biochemistry Company, Beijing, China.) according to the manufacturer's protocol.

Histological examination

The paraffin sections of pancreas and lung tissue were stained with hematoxylin and eosin (H&E). Two investigators who were blind to the experiment grouping scored the degree of pancreatic injury by light microscopy, evaluating the severity of edema, inflammation and necrosis, as we described in previous study [16]. The degree of pulmonary injury was scored by evaluating the severity of neutrophil infiltration, thickness of alveolar and alveolar congestion, and the scoring standards were described previously [16].

Immunohistochemical examination and TUNEL staining

The slices from paraffin-embedded pancreatic tissues were subjected to immunohistochemical (IHC) staining for myeloperoxidase (MPO) and NLRP3 protein detection. According to our previously described methods [17], slides were incubated overnight at 4°C in a humid chamber with an antibody against MPO (1:200 dilution, ab9535, Abcam, Cambridge, UK) and NLRP3 (1:200 dilution, ab4207, Abcam, Cambridge, UK) then incubated by goat anti-rabbit secondary antibody (1:500 dilution, ab150079, Abcam, Cambridge, UK) for 60 minutes. Images were acquired by using microscope (IX73, Olympus, Tokyo, Japan). In the same way, the slices from paraffin-embedded lung tissues were subjected to IHC staining for MPO examination.

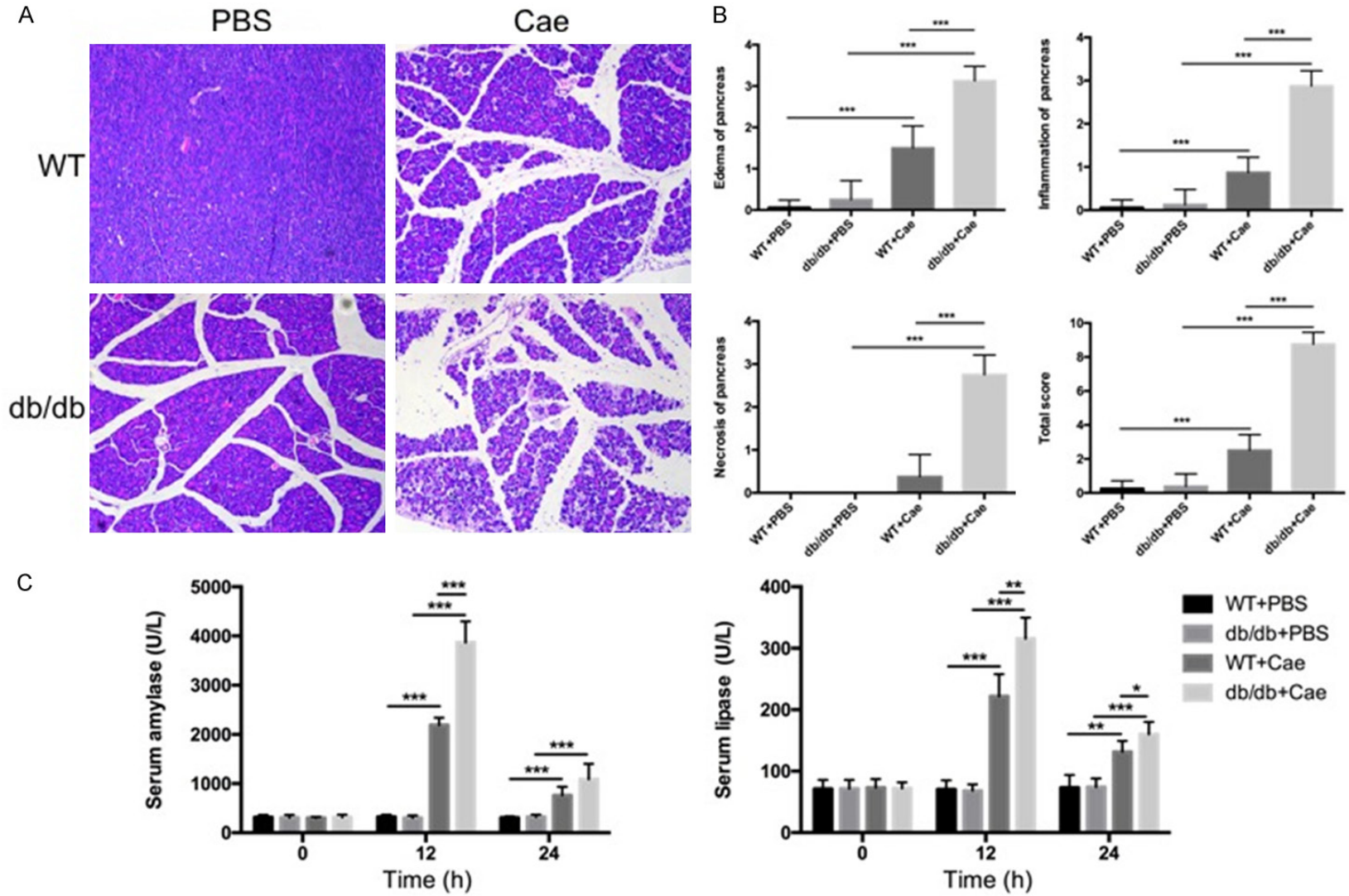

Figure 1. Diabetes aggravated pancreatic injuries in AP in mice. (A) Representative pathological changes in pancreas. HE stained sections of pancreas in magnification 100X. (B) Histological scores of pancreatic tissues and (C) Serum levels of amylase and lipase. *P < 0.05, **P < 0.01, ***P < 0.001.

Apoptosis was quantified on pancreatic tissue by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. The TUNEL staining was performed with a commercial cell death detection kit (Roche Diagnostics, Indianapolis, USA) according to the manufacturer's protocol. The stained slices were observed by microscopy (IX73, Olympus, Tokyo, Japan) and images were recorded. The apoptosis index was calculated as the percentage of stained cells, as described previously [16].

Western blotting (WB)

According to our previously described methods, the membranes were incubated overnight at 4°C with primary antibodies against NLRP3 (1:1000 dilution, 15101, CST, MA, USA.) and IL-1β (1:1000 dilution, ab9722, Abcam, Hong Kong, China) in blocking buffer. On the next day, membranes were washed with TBST (3 × 10 min) and incubated with a secondary goat anti-rabbit IgG horseradish peroxidase (HRP) antibody (1:10000 dilution, Sigma-Aldrich Co., St. Louis. MO, USA) diluted in 5% (w/v) dry non-fat milk in TBST for 1 h at room temperature. Finally, membranes were washed with TBST (3 × 10 min), developed by using the ECL detection system (Santa Cruz Biotechnology), quickly dried, and exposed to ECL flm [18].

Statistical analysis

Results were presented as the mean ± standard deviation (SD). The Kruskal-Wallis test followed by the Mann-Whitney U test was used to evaluate the difference in histopathologic scores. Statistical analysis was performed using one-way ANOVA followed by the Student-Newman-Keuls test as a post hoc test. Statistical analyses were performed using SPSS statistical software (version 22.0, IBM Analytics, Armonk, NY).

Results

Fasting blood glucose was remarkably elevated in db/db mice

As expected, all the db/db mice exhibited significantly higher fasting blood glucose levels and were more obese (higher body weight) than the WT mice on the day before Cae administration. Similarly, serum TC and TG levels of db/db mice were significantly higher than that in WT mice (Supplementary Figure 1).

Diabetes aggravated disease severity in AP mice

Histological examination of edema, inflammatory cell infiltration and acinar cell necrosis were generally adopted to evaluate the severity of AP in vivo [16]. There was no abnormal histological manifestation of AP in mice of WT+PBS and db/db+PBS groups. After AP induction, the WT mice exhibited a classical edematous pancreatitis manifestation, which was mainly characterized as edema, inflammatory cells infiltration without obvious acinar cell necrosis. In contrast, db/db mice showed remarkably aggravated histological manifestations and a large number of acinar cells became necrotic apart from edema, substantial inflammatory cells infiltration (**Figure 1A**, **1B**). Acute lung injury (ALI) is one of the most common complications of AP and evaluating lung injury could reflect the systemic inflammatory state in AP mice [19]. Results showed that the histological features of lung injury in db/db+Cae mice were markedly exacerbated in comparison with the WT+Cae mice, characteristic as higher thickness of alveolar, more neutrophils infiltration, and severer alveolar congestion, as shown in **Figure 2**.

The increased serum level of amylase and lipase is one of the most specific serological indicators of pancreatic injury in AP. As shown in **Figure 1C**, the levels of serum amylase and lipase in WT+Cae group were remarkably raised compared to the WT+PBS group. Unsurprisingly, the serum amylase and lipase levels turned out to be much higher in db/db+Cae group than in WT+Cae group, which was in consistence with the results of histological examination. The increased serum levels of amylase and lipase further confirmed the aggravating effect of diabetes on pancreatic injury.

It has been recognized that inflammatory response plays an important role in the pathogenesis and development of AP. Myeloperoxidase (MPO) is mainly expressed in neutrophils and could be used as a biomarker of activated neutrophils. Therefore, immunohistochemistry (IHC) examinations for MPO of pancreatic and pulmonary tissues were adopted to assess the condition of tissue neutrophil infiltration. The results of IHC of MPO indicated that the db/db mice had obviously more neutrophils infiltration in pancreatic and pulmonary tissues compared

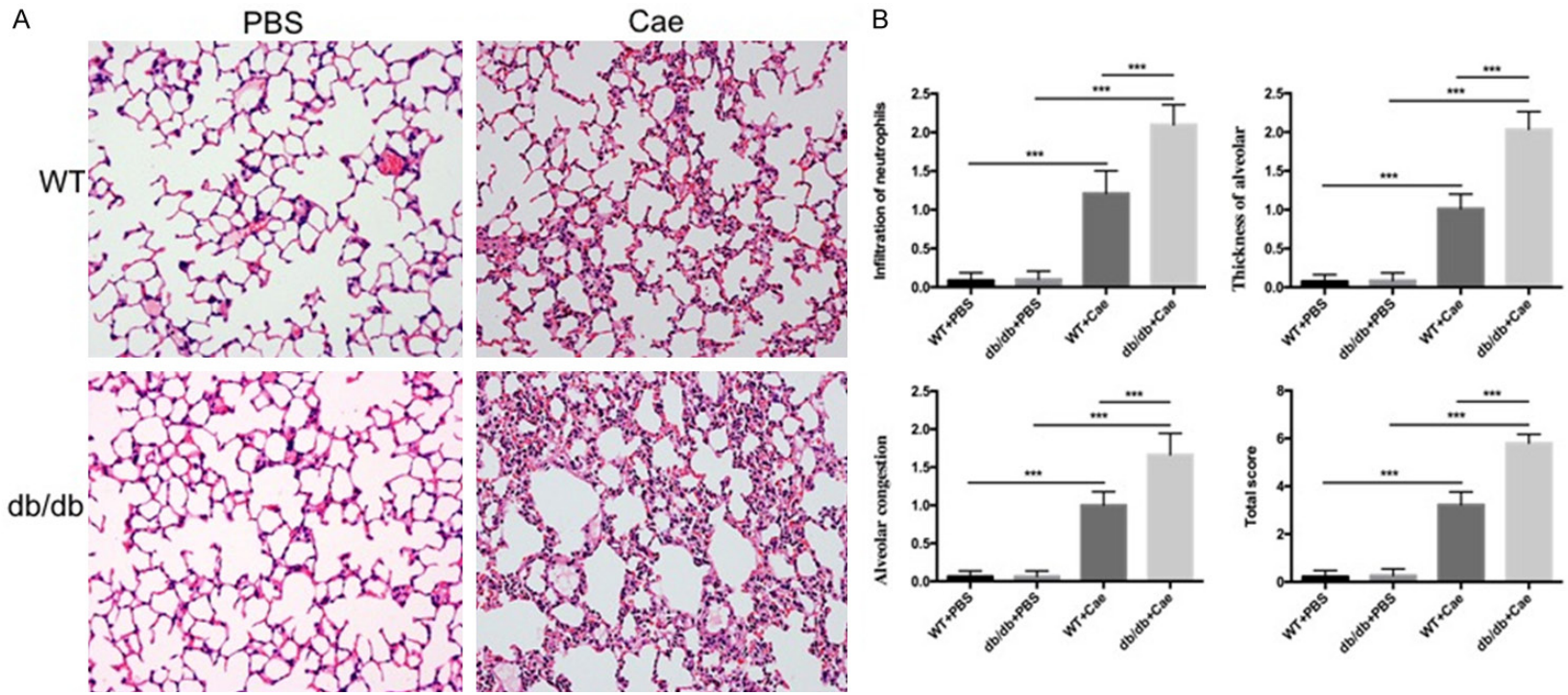

Figure 2. Diabetes exacerbated the severity of acute lung injury in AP in mice. A. Representative pathological changes in lung tissues. H&E stained sections of lung in magnification 200X. B. Histological scores of pulmonary tissues. *P < 0.05, **P < 0.01, ***P < 0.001.

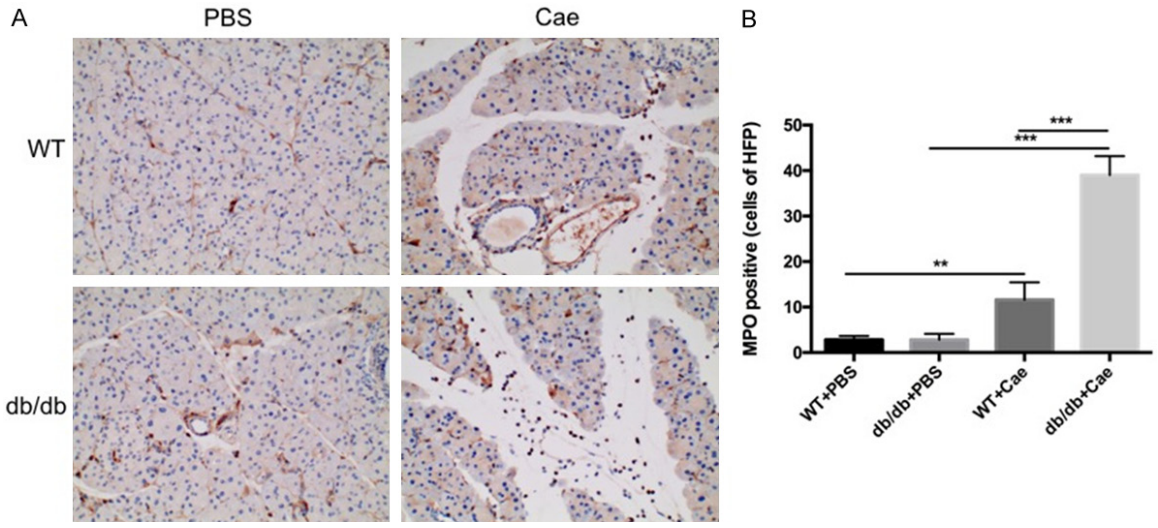

Figure 3. The effect of diabetes on neutrophil infiltration in pancreatic tissues in AP in mice. A. Representative immunohistochemistry images for myeloperoxidase (MPO) in the pancreas. IHC stained sections of pancreas in magnification 200X. B. MPO activity of pancreas. *P < 0.05, **P < 0.01, ***P < 0.001.

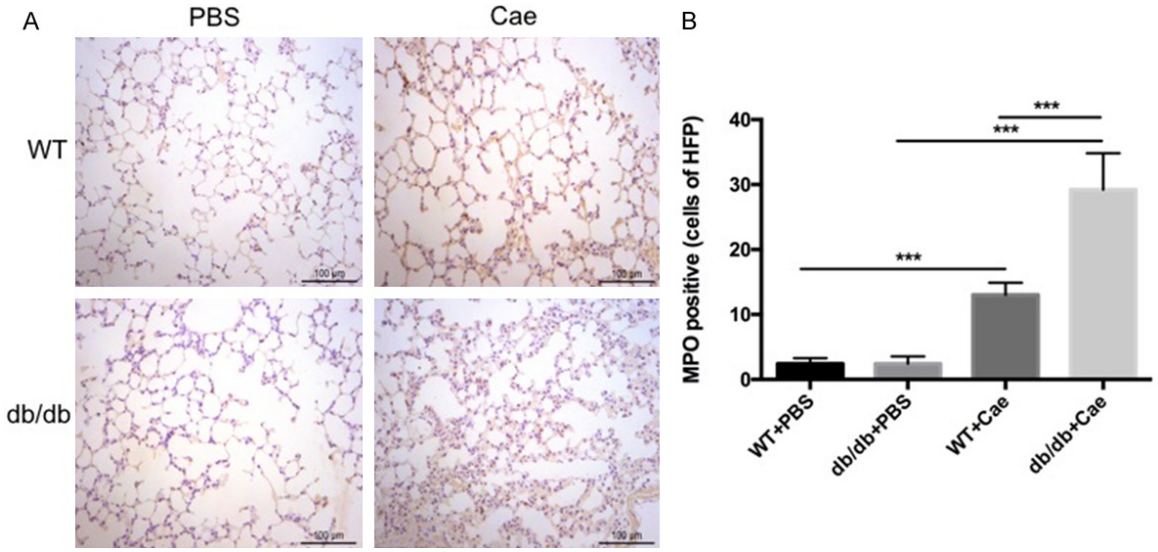

Figure 4. The effect of diabetes on neutrophil infiltration in pulmonary tissues in AP in mice. A. Representative immunohistochemistry images for myeloperoxidase (MPO) in the lungs. IHC stained sections of lungs in magnification 200X. B. MPO activity of lungs. *P < 0.05, **P < 0.01, ***P < 0.001.

with WT mice after AP induction (**Figures 3** and **4**).

Diabetes promoted acinar cells apoptosis

Pancreatic acinar cell apoptosis is the predominant cause of cell death in mild AP, while pancreatic acinar cell necrosis is the main cause of cell death in severe AP. Caerulein-induced mild pancreatitis is characterized by more apoptosis and less necrosis of acinar cells [20]. Therefore, we adopted TUNEL staining method to analyze the apoptosis of acinar cells in db/db mice after AP induction. As shown in **Figure 5**, in comparison with WT mice, the number of TUNEL-positive cells was significantly increased in db/db mice after Cae administration, which revealed that pancreatic acinar cells in db/db mice were involved in much more extensive apoptosis process.

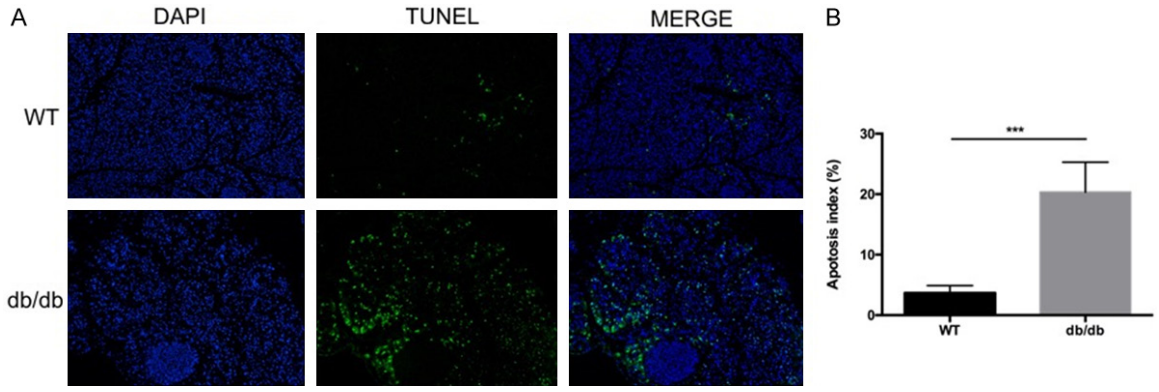

Figure 5. Diabetes promoted acinar cells apoptosis in AP in mice. (A) Representative images for TUNEL staining in pancreatic tissue and (B) The apoptotic cells counting of TUNEL staining. *P < 0.05, **P < 0.01, *** < 0.001.

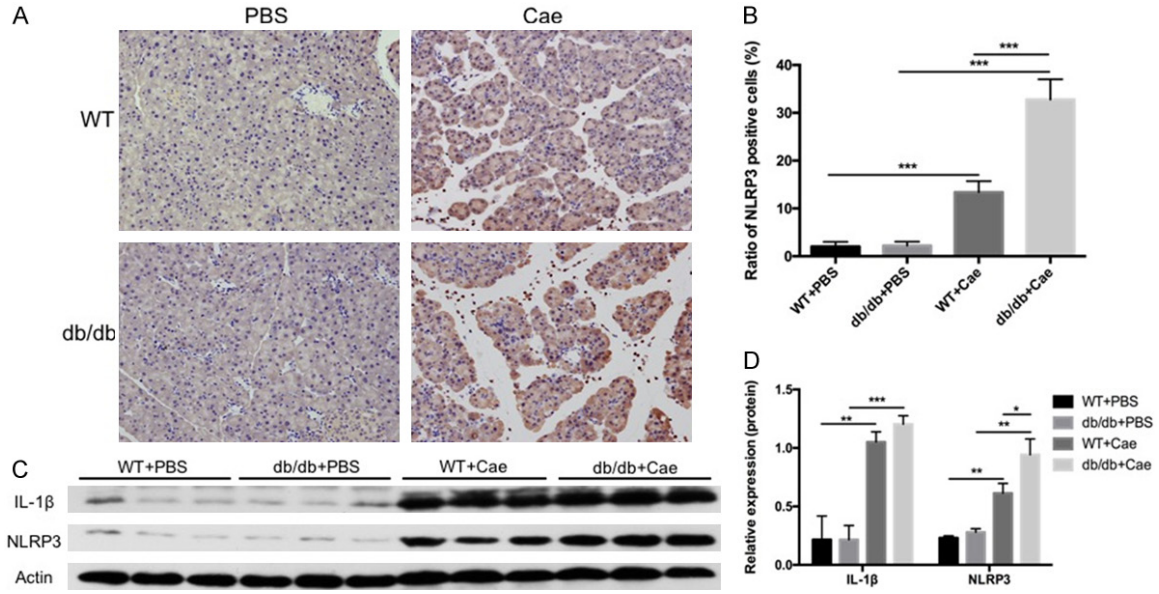

Figure 6. Diabetes activated NLRP3 inflammasome pathway in AP in mice. A. Representative immunohistochemistry images for NLRP3 stained sections in pancreatic tissue. IHC stained sections of pancreas in magnification 200X. B. The positive cells counting of NLRP3 staining. C. NLRP3, IL-1β protein expression were detected by Western blot analysis. Actin was used as a control for protein loading; D. The relative protein expression of NLRP3, IL-1β. *P < 0.05, **P < 0.01, ***P < 0.001.

Diabetes activated NLRP3 inflammasome pathway in AP mice

Previous studies suggested that disruption of NLRP3 inflammasome had a major role in the inflammatory response as well as metabolic disorders and NLRP3 inflammasome activation has been definitely observed in type 2 diabetes [21]. In this study, we examined the effect of diabetes on the activation of NLRP3 inflammasome in AP mice. Firstly, we detected the NLRP3 activity of pancreatic tissues by IHC staining, as expected, the activation of NLRP3 inflammasome in db/db+Cae group was significantly enhanced in contrast to WT+Cae group. Furthermore, by adopting western blotting method, we detected that the expressions of NLRP3 and IL-1β proteins were significantly elevated in db/db mice when contrasted with WT mice after AP induction (**Figure 6**).

Diabetes increased mice susceptibility to AP

Clinical studies showed that the incidence of AP increased in diabetic patients, suggesting that diabetes may predispose diabetic patients to AP [10]. Hence, we planned to explore whether diabetes could increase the susceptibility

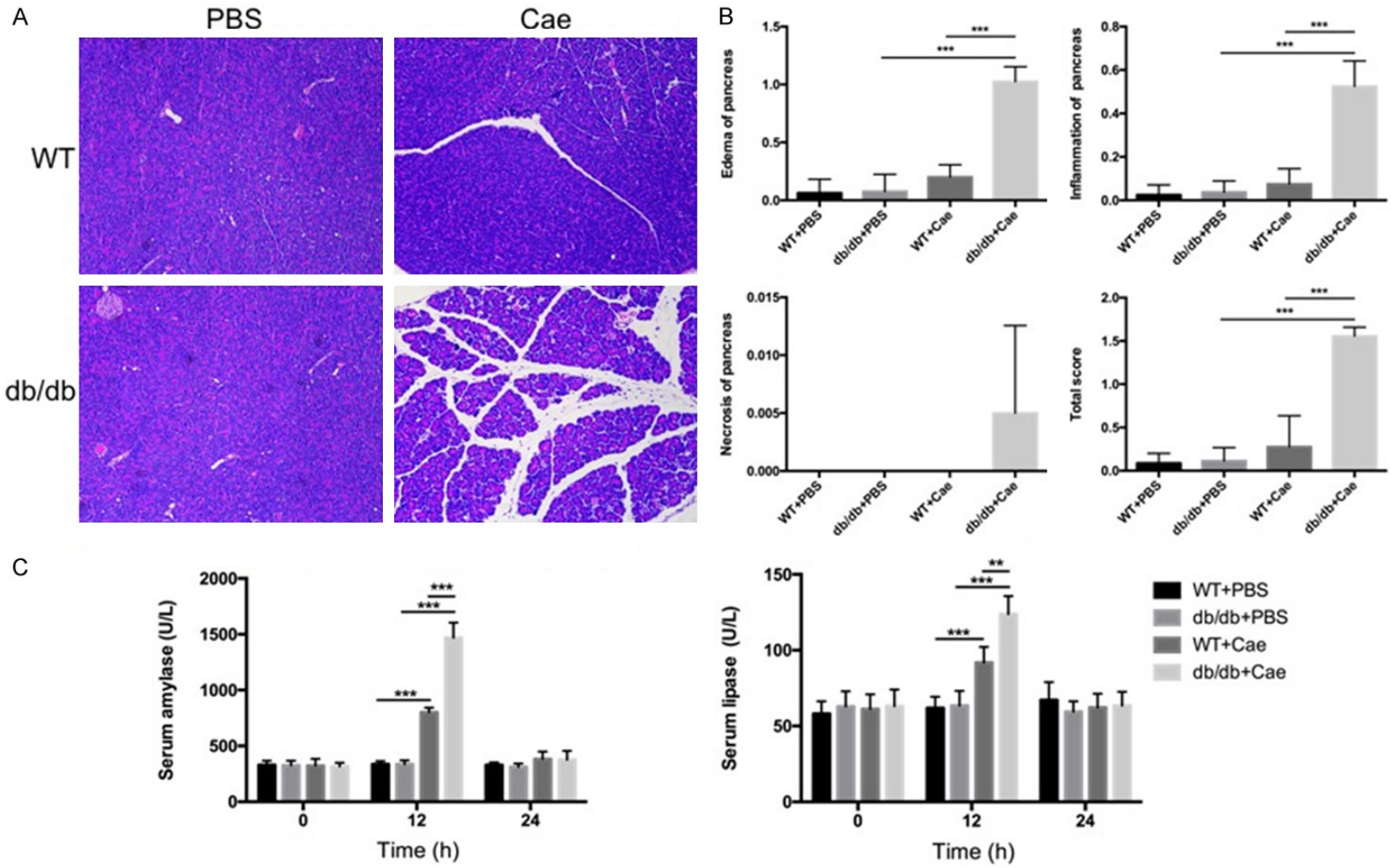

Figure 7. Diabetes increased susceptibility to AP in mice. (A) Representative pathological changes in pancreas. H&E stained sections of pancreas in magnification 100X. (B) Histological scores of pancreatic tissues and (C) Serum levels of amylase and lipase. *P < 0.05, **P < 0.01, ***P < 0.001.

of mice to AP. The low-dose 10 ug/kg Cae with 7 intraperitoneal injections at hourly intervals was adopted to induce AP. As the results showed, we failed to observe the typical pathological changes of AP in WT mice and there was no significant difference in pathological manifestations between Cae-treated and PBS-treated WT mice. While in 10 ug/kg Cae treated db/db mice, obvious edema and inflammatory cells infiltration were observed in sections of pancreatic tissue (**Figure 7**) along with the remarkable elevations in serum amylase and lipase levels, which demonstrated that diabetes increased the susceptibility of mice to AP.

Discussion

The incidence of AP is increasing and diabetes is much more common nowadays [22]. Type 2 diabetes is a main part of the metabolic syndrome, which brings a subsequent increased risk of cardiovascular diseases. Major components of the metabolic syndrome have been reported to be tightly associated with the presence of AP, including type 2 diabetes, obesity, dyslipidemia, and hypertension [23-25]. Given the close anatomical and physiological relationship of these two parts, the interactions between the exocrine and endocrine pancreas has been studied for many years [7]. For example, the incidence of AP was elevated in patients with diabetes; the use of incretin-based drugs, such as dipeptidyl peptidase 4 inhibitors and glucagon-like peptide1 agonists was associated with an increased risk of AP, while which was controversial until now [26]; it has been the accepted fact that pancreatic disease could deteriorate the pancreatic exocrine functions and pancreatogenic DM is classified as a form of secondary (named as type 3c) DM by the American diabetes association [27, 28]. However, clinical studies could not prove a cause-and-effect relationship between AP and diabetes because of the limitations of clinical research. In basic research studies, D. Zechner and his colleagues adopted the streptozotocin induced type 1 diabetes mouse model and they found that diabetes aggravated AP and suppressed regeneration of the exocrine tissue [29]. In the clinical setting, in comparison to type 1 diabetes, AP patients are more likely to be complicated with type 2 diabetes. Therefore, in our study, we adopt the classic type 2 diabetes animal model db/db mice to carry out our experiment.

As far as we know, this study for the first time has evaluated the effects of diabetes on AP by adopting a type 2 diabetes animal model and investigated the possible underlying mechanisms. The results showed that diabetes could exacerbate the disease severity of AP, as evidenced by aggravation of pancreatic and pulmonary injuries, elevated serum amylase and lipase levels, increased neutrophil infiltrations and increased apoptotic acinar cells possibly via the activation of NLRP3 inflammasome pathway. In addition, we also found that diabetes could increase the susceptibility of mice to AP.

Inflammasome is a multiprotein oligomer that is assembled in the cytoplasm by cytosolic NOD-like receptors (NLRs) [30]. A well-studied NLR inflammasome is the NLRP3 inflammasome, which senses many pathogen-derived, environmental and host-derived factors. Upon activation, NLRP3 binds to apoptosis-associated speck-like protein containing a CARD (ASC). ASC in turn interacts with the cysteine protease caspase-1 to form a complex termed the NLRP3 inflammasome. This results in the activation of caspase-1, which cleaves the pro-inflammatory cytokines IL-1β and IL-18 to their active forms. NLRP3 has been implicated in the pathogenesis of a number of complex diseases, notably including metabolic disorders such as type 2 diabetes, atherosclerosis and obesity [31]. Moreover, NLRP3 inflammasome pathway activation has been proven to play an increasingly important role in the inflammatory disease [32], such as AP [33-35]. Our study has further confirmed that NLRP3 inflammasome has exerted critical effects in the pathogenesis of AP and NLRP3 inflammasome may be an important molecular link between AP and diabetes.

In conclusion, our study adopted the classic type 2 diabetes animal model db/db mice to compare the progression of AP in non-diabetic and diabetic mice. Moreover, we confirmed that experimental diabetes caused an aggravation of caerulein-induced AP potentially via promoting the activation of NLRP3 inflammasome pathway.

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Disclosure of conflict of interest

None.

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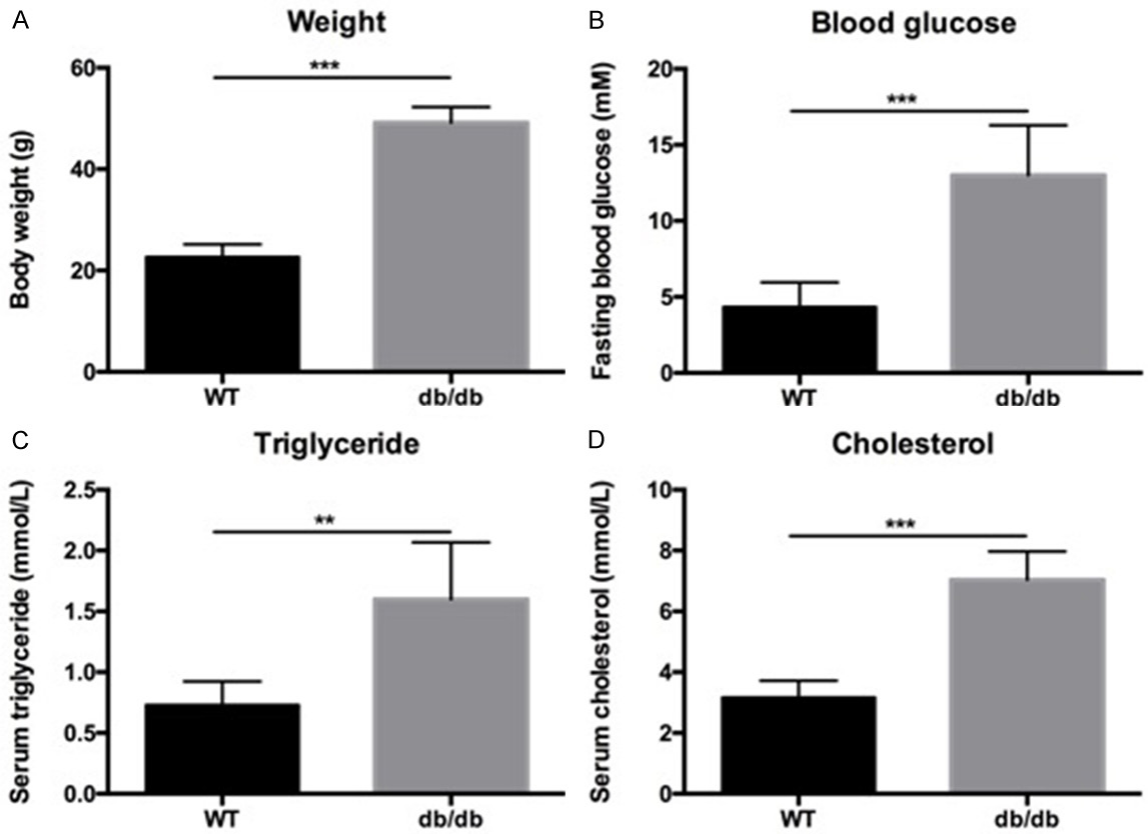

Supplementary Figure 1. Fasting blood glucose was elevated in db/db mice. (A) The body weight of WT mice and db/db mice on the day before Cae administration. (B) Fasting blood glucose levels, (C) Serum triglyceride and (D) Total cholesterol levels of WT mice and db/db mice. *P < 0.05, **P < 0.01, ***P < 0.001.