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Long-term high-fat diet induces pancreatic injuries via pancreatic microcirculatory disturbances and oxidative stress in rats with hyperlipidemia

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Abstract

Relations between hyperlipidemia and chronic pancreatitis remain unclear. Microcirculatory disturbances and oxidative stress are involved in pathogeneses of a high numbers of diseases. The objective of this study was to induce hyperlipidemia in rats by long-term high-fat diet intake, then investigate the biochemical, microcirculatory, and histological alterations in blood and pancreatic tissues of these animals, and discuss their potential significances. Pancreatic blood flow was detected by intravital microscope; malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured in pancreatic tissues for assessment of oxidative stress and α-smooth muscle actin (α-SMA) expression was determined by immunohistochemical staining and RT-PCR. The results showed that the velocity of pancreatic microvascular blood flow of rats with hyperlipidemia decreased significantly as compared to control value ($p = 0.008$). Pancreatic MDA content increased whereas SOD activity decreased in these rats ($p = 0.022$; $p = 0.039$, respectively). Histologically, microvesicles in acinar and islet cells, dilated rough endoplasmic reticulum, swollen mitochondrion and modified vascular endothelial cells were observed under light microscope and transmission electron microscope. In addition, α-SMA expression was up-regulated significantly ($p < 0.05$). These results suggest that long-term high-fat diet can induce chronic pancreatic injuries which could be considered as "nonalcoholic fatty pancreatic disease", and pancreatic microcirculatory disturbances and oxidative stress may play an important part in the underlying pathogenesis.

Keywords: High-fat diet; Hyperlipidemia; Pancreatic injury; Microcirculation; Oxidative stress; Malondialdehyde (MDA); Superoxide dismutase (SOD); α-Smooth muscle actin (α-SMA)

Studies on etiology and pathogenesis of chronic pancreatitis are highlighted at present [1–3]. Alcohol is considered the main cause of chronic pancreatitis [4]. Relations between high-fat diet-induced hyperlipidemia and chronic pancreatitis remain unclear. Severe hyperlipidemia, especially genetic hypertriglyceridemia, is only considered a potential cause of acute pancreatitis [4,5]. On the other-hand, experimental studies have found that high-fat intake can induce hyperlipidemia and lead to pancreatic endocrine and exocrine alterations [6–8]. In addition, high-fat diet may also be a risk factor of pancreatic cancer [9]. These findings demonstrate a potential connection between high-fat intake and chronic pancreatic injuries. But, the underlying pathogenesis has not been investigated and clarified till now.

Additionally, there is an abundant evidence that high-fat diet-induced hyperlipidemia has tight relations with vascular damage and oxidative stress. Hyperlipidemia incites endothelial cell activation, along with lipid deposit and oxidative stress, throughout the microvasculature

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[10,11]. In addition, hyperlipidemia induces defective endothelium-dependent vasodilator responses, promotes neutrophils binding to endothelia cells, and results in microcirculatory blood flow disturbances [12,13]. Therefore, there are confirmable relationships between high-fat-induced hyperlipidemia, microcirculation, and oxidative stress.

On the other hand, clinical and experimental studies suggest that oxidative stress is involved in pathogeneses of a high numbers of diseases [11,14–18]. Also, previous studies demonstrate that high-fat diets lead to liver injury and insulin resistance through oxidative stress [15,19,20], and the increased production of reactive oxygen species (ROS) may cause lipid peroxidation followed by an inflammatory response, even activate stellate cells and lead to fibrogenesis [21,22].

Thus, in view of the above considerations, we hypothesize that long-term high-fat diet would induce deleterious effect on pancreas on bases of endothelial damage, microcirculatory disturbances, and oxidative stress within pancreas. In the present study, we induced hyperlipidemia in rats by long-term feeding of high-fat diets, and investigated the biochemical, microcirculatory, and histological alterations in blood and pancreatic tissues of these animals, and discussed their underlying significances.

Materials and methods

Animal models. Male Wistar rats, weighing 170–190 g, were obtained from the Laboratory Animal Center of Shandong University. Experiments were performed in accordance with the Laboratory Animal Care and Use Regulations of Shandong University. The rats were housed in a temperature-controlled (22 ± 2 °C) and humidity-controlled (55 ± 5%) room on a 12-h light/dark cycle. They received standard rat chow (carbohydrate 68.58%, protein 22.7%, fat 3.91%, raw fiber 3.18%, calcium 1.03%, and phosphonium 0.6%, finally analyzed by the Laboratory Animal Centre, Shandong University) and tap water *ad libitum* for 1 week to acclimatize to their environment before being divided into different research dietary groups. The rats were then divided into two dietary groups on the basis of comparable mean body weight. One group (group control) received a standard chow; another group (group HFD) was fed a high-fat diet (2% cholesterol, 10% lard, and 88% standard chow as control group). All the rats were fed for 20 weeks from the beginning of the experiment.

Blood biochemical detections. Blood samples from the portal vena of anesthetized rats were immediately centrifuged at 2000*g* for 5 min for the separation of serum. Serum was stored at −20 °C until assayed. The serum triglycerides (TG), total cholesterol (TCH), amylase, lipase, and glucose were detected using commercial kits by an autoanalyzer, Beckman Coulter synchron LX20 (Beckman Coulter Inc., USA).

Pancreatic microcirculation. Being anesthetized with pentobarbital sodium (50 mg/kg), spontaneously breathing animals were placed in a supine position on a heating pad by continuous circulating water (38.5 °C) providing constant body temperature (37 °C±). The abdomen was opened by midline laparotomy, and part of the pancreas was gently pulled out and positioned on an adjustable microscopy stage under microscope. The exposed pancreas was permanently superfused with 37 °C warm saline to avoid drying. The pancreatic microcirculatory parameters, especially the velocity of centreline blood flow, were observed using an intravital microscope in the pancreatic capillary fields according to the method described elsewhere [23,24].

Pancreatic MDA content and SOD activity determinations. The thiobarbituric acid method was used to quantify lipid peroxidation in tissues, measured as thiobarbituric acid-reactive substances (TBARS) according to previous study [25]. The quantity of lipid peroxides is reported as nanomolar malondialdehyde (MDA) equivalents/mg protein of pancreatic tissue. The superoxide dismutase (SOD) content was measured using the xanthine oxidase technique based on the spectrophotometric monitoring of SOD-mediated reduction of DTNB at 550 nm as previously described [26,27]. SOD activity was expressed as U/mg protein of pancreatic tissue.

RT-PCR assay for α-smooth muscle actin mRNA. Pancreatic α-smooth muscle actin (α-SMA) mRNA expression was determined by reverse transcription polymerase chain reaction (RT-PCR). Pancreatic samples were rapidly immersed in RNAlater (Sigma, USA) for RNA protection, and stored at −20 °C before assay. Total RNA was extracted from pancreatic tissues using TRIzol reagent (Invitrogen, USA) and was reverse-transcribed using oligo(dT) as a primer. The sequences used as β-actin, an internal standard control housekeeping gene, and α-SMA specific primers for PCR were: β-actin (GenBank Accession No. NM_031144.2):forward: 5′-AAG,ATC,CTG,ACC,GAG,CGT,GG-3′(20 bp); reverse: 5′-CAG,CAC,TGT,GTT,GGC,ATA,GAG,G-3′(22 bp), product: 327 bp; α-SMA (GenBank Accession No. X06801): forward: 5′-AGT,CGC,CAT,CAG,GAA,CCT,CGA,G-3′(22 bp); reverse: 5′-ATC,TTT,TCC,ATG,TCG,TCC,CAG,TTG-3′ (24 bp), product: 296 bp. PCR amplification cycles were carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, and then with a extension at 72 °C for 7 min. PCR products were separated by agarose gel electrophoresis and then visualized by ethidium bromide staining. Specific bands were quantitated by scanning densitometry and normalized to the signal of β-actin.

Histologic studies. Each formalin-fixed and paraffin-embedded pancreas specimen was cut into 5 μm thick sections. Hematoxylin and eosin (H&E) staining was performed for routine histologic observations.

Immunohistochemistry staining for α-SMA was performed as follows: The sections were deparaffinized, immersed in 3% H_2O_2 (v/v) to quench endogenous peroxidase activity, and microwaved in 10 mM sodium citrate (pH 6.0) for 15 min for antigen retrieval. Then, the avidin and biotin were applied to eliminate endogenous biotin-related background staining. The sections were then incubated with primary antibodies (1:150) (Santa Cruz, USA) at 4 °C overnight and incubated, respectively, with biotinylated goat anti-mouse antibodies and HRP-conjugated streptavidin (Santa Cruz, USA) for 15 min at room temperature. The slides were washed and the chromogen was developed for 5 min with liquid 3,3′-diaminobenzidine before observation. Distilled water with 0.4% Tween 20 was used as a rinsing solution. All samples were evaluated blindly by the same pathologist.

For observations of pancreatic ultramicrostructure, the pancreatic samples (fragments of about 1 mm^3) were quickly excised and immersed in a freshly prepared solution of 2.5% glutaraldehyde in 15 mM cacodylate buffer, pH 7.2, at 4 °C for 90 min, then fixed with 1% osmium tetroxide, and dehydrated in a series of graded ethanols. After immersion in propylene oxide, the samples were immersed in a mixture (1:1) of propylene oxide and Epon618 resin, overnight, and embedded in Epon618. The regions to be studied were sectioned using ultramicrotomy into ultrathin sections (50 nm). The sections were mounted on formvar-coated copper slot grids, stained with uranylacetate and lead citrate, and then observed in a transmission electron microscopy (JEM-1200EX, JEOL Ltd., Japan) at an acceleration voltage of 80 kV.

Statistical analysis. Data are presented as means ± SD. The significance of differences between the two experimental groups was analyzed by means of independent-samples *t* test using SPSS for windows. Probability values less than 0.05 were considered significant.

Results

General information

At the start of the experiment, the mean body weights of rats in HFD and control group were at a same baseline.

During the fist several weeks after the beginning of the experiment, all rats in both groups gained weight steadily at nearly a same speed. Four weeks later, rats in HFD group increased their body weight more quickly than controls. But during the last several weeks, the acceleration of body weight increase tended to decrease in both groups. By the end of 20 weeks, the body weight of HFD rats was significantly higher than controls (Table 1).

During the experimental period, no rat died, and no rat had diarrhea, polyuria, and emaciation. All rats in both groups showed a increased body weight at the end of the experiment. In addition, a large amount of fatty tissues were observed around the pancreas in HFD rat when laparotomy was performed.

Table 1
Body weight and serum biochemical analysis (means ± SD)

Parameters	Control	HFD
Body weight (g)		
Baseline	180.20 ± 7.56	184.80 ± 9.93
Week 20	434.60 ± 25.72	524.40 ± 51.51 ($p = 0.013$)
TG (mmol/L)		
Baseline	0.41 ± 0.12	0.39 ± 0.08
Week 20	0.46 ± 0.18	1.79 ± 0.42 ($p = 0.001$)
TCH (mmol/L)		
Baseline	1.08 ± 0.41	1.02 ± 0.11
Week 20	1.11 ± 0.38	3.32 ± 0.70 ($p = 0.001$)
Amylase (U/L)		
Baseline	895.39 ± 138.12	872.00 ± 149.53
Week 20	890.92 ± 150.76	940.83 ± 139.48
Lipase (U/L)		
Baseline	238.40 ± 74.11	268.41 ± 71.30
Week 20	236.41 ± 78.33	303.11 ± 44.68
Glucose (mmol/L)		
Baseline	3.36 ± 0.94	3.55 ± 0.54
Week 20	3.52 ± 1.05	4.09 ± 1.11

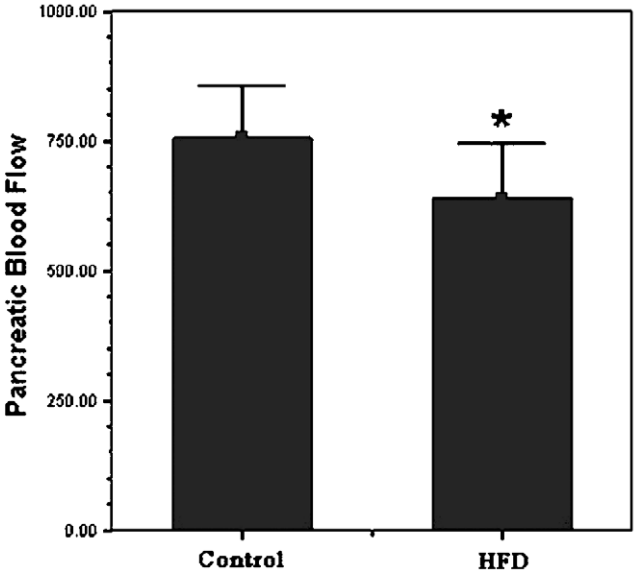

Fig. 1. Measurement of pancreatic blood flow (PBF) (μm/s) in rats of control and HFD group. The PBF of HFD rats decreased significantly in comparison to controls ($^*p = 0.008$). Data presented as means ± SD.

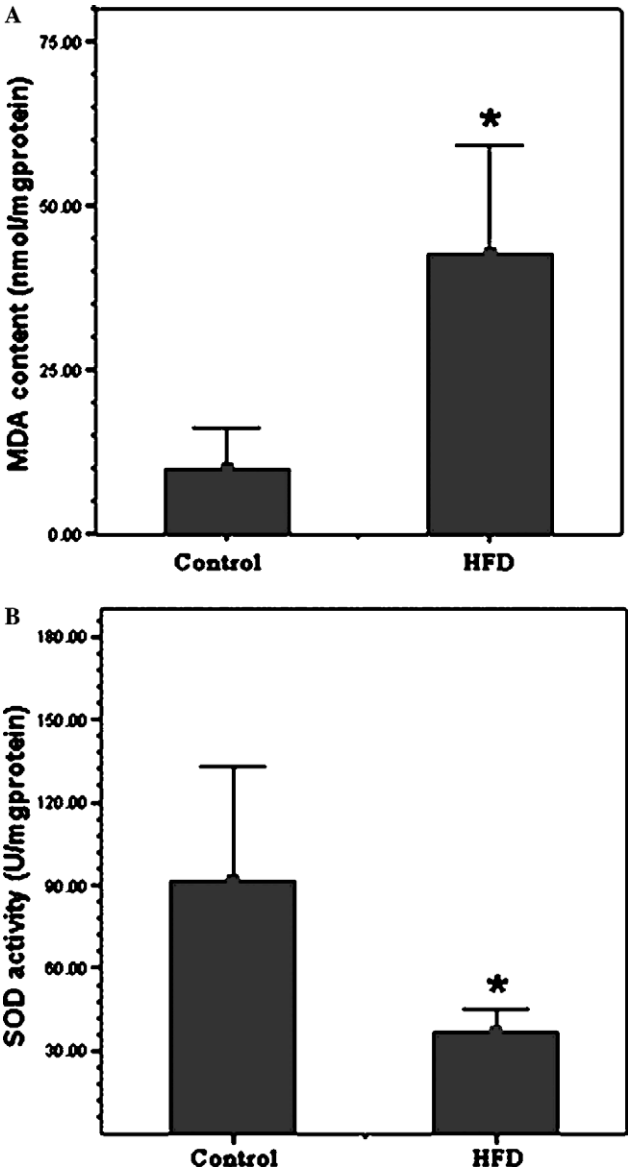

Fig. 2. (A, B) Pancreatic MDA content and SOD activity in rats of control and HFD group. There was a significant increase of MDA content ((A) $^*p = 0.022$) whereas a significant decrease of SOD activity ((B) $^*p = 0.039$) in HFD rats than in controls. Data presented as means ± SD.

Effect of high-fat diet on blood biochemical assay

Basal values of serum TG and TCH levels of rats in both HFD and control groups were at a same level at the beginning of the experiment. But two weeks later, rats in HFD group had a tendency to develop serum lipid disorders, and elevated levels of serum TG and TCH were detected in comparison to control rats (data not shown). By the end of 20 weeks, serum was obtained from all rats in both groups for entire biochemical analysis. Most of the sera samples

were limpid, while some samples from HFD group seemed to be chyllform. In HFD group, biochemical analysis showed that the serum TG and TCH levels increased by 289.13% and 199.10%, respectively, above the control values. The serum levels of amylase, lipase and blood glucose were also elevated in HFD group than in control group, but the differences were not statistically significant (Table 1).

Effect of high-fat diet on pancreatic microcirculation

Intravital microscopic examinations were performed in the rats in order to investigate the alterations of pancreatic microcirculation. As a result, slower pancreatic blood flow (PBF) was observed in pancreata of HFD rats. Especially, irregular intermittent perfusion, even stagnant blood flow, was observed in some capillaries. Hemodiapedesis was also found in some HFD rats. Quantitatively, the velocity of PBF of these animals significantly decreased by 15.39% to 639.70 ± 104.63 μm/s as compared to control value (756.08 ± 98.88 μm/s, $p = 0.008$) (Fig. 1).

Effect of high-fat diet on MDA content and SOD activity in pancreatic tissues

In order to investigate whether oxidative stress occurred in pancreas in HFD rats, MDA content and SOD activity in pancreatic tissues were detected in these animals. As a result, there was a 332.46% increase in MDA content (42.77 ± 16.53 nmol/mg protein versus 9.89 ± 6.15 nmol/mg protein, $p = 0.022$, Fig. 2A) whereas there was a 59.82% decrease in SOD activity (36.91 ± 8.49 U/mg protein versus 91.87 ± 41.35 U/mg protein, $p = 0.039$, Fig. 2B) in the pancreas of HFD-fed rats compared with control rats.

Effect of high-fat diet on pancreatic histology

H&E stained sections of the pancreas of HFD group showed frequent microvesicles in acinar and islet cells. In some local areas, the vacuolization was extensive (Fig. 3A, right). In addition, acinar cells atrophy was common; pyknotic cells and enlarged interlobular interspaces were also observed in some pancreatic specimens. Inflammatory cell infiltration was not obvious and only a few lymphocytes were observed in inter- or intra-lobular areas. Sparse interlobular and intralobular fibrosis was observed. Hemorrhage was not found in these studied pancreatic samples.

Under transmission electron microscope, we found that high-fat diet-induced dilated rough endoplasmic reticulum and swollen mitochondrium, and the nuclear membrane was also modified by tumidity. In addition, the vascular

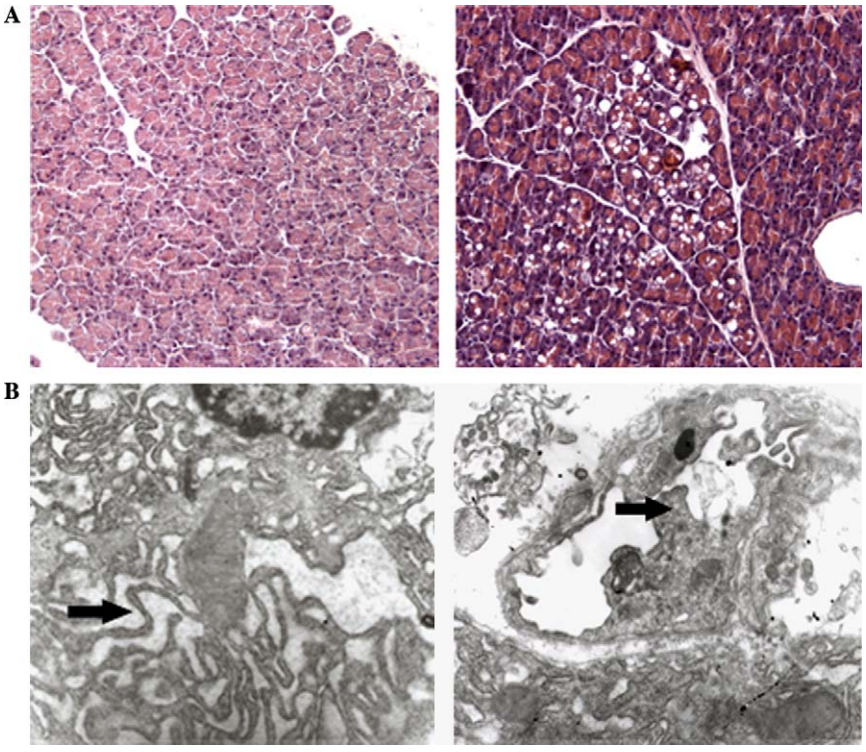

Fig. 3. (A, B) Representative histological observations of pancreatic tissues from control and HFD group by H&E staining and transmission electron microscope (TEM). (A) No histopathological changes were noted in control group (left, H&E, original magnification 200×), but in HFD group, cytoplasma vacuolization was obvious in acinar cells (right, H&E, original magnification 200×). (B) For HFD rats, the pancreatic acinar cells showed dilated rough endoplasmic reticulum (arrow) and swollen mitochondrion (left, TEM, original magnification 10,000×), and the vascular endothelium showed focal discontinuity and abnormal projections in the narrowed vascular lumens (arrow) (right, TEM, original magnification 10,000×).

endothelial cells showed abnormal projections inside the narrowed vascular lumens, and some endothelia were discontinuous (Fig. 3B).

Effect of high-fat diet on α-SMA immunohistological staining and mRNA expression

In order to ascertain weather some pro-fibrogenesis events occurred in the pancreas of rats after long-term high-fat diet feeding, α-SMA was detected by immunochemistry staining. Interestingly, although obvious fibrosis was not found in H&E section, α-SMA-positive cells were observed easily in the pancreatic tissues of rats in HFD group. The α-SMA-positive stained cells were mainly presented in periacinar space (Fig. 4A, right). Quantitated by image analysis, the amount of α-SMA-positive stained cells increased significantly in pancreata of HFD rats in comparison to controls (Fig. 4B, $p = 0.001$). Likewise, in rats fed high-fat diets for 20 weeks, α-SMA mRNA expression was up-regulated significantly (Fig. 5, $p = 0.000$).

Discussion

Chronic pancreatitis is characterized by inflammation, fibrogenesis, and impairment of pancreatic exocrine and endocrine functions [4]. High-fat diet can induce hyperlipidemia and abnormal pancreatic secretary alterations in animals [6–8]. But whether high-fat diet can lead to chronic pancreatitis and the mechanisms by which high-fat diet induces deleterious effect on pancreas are currently unknown.

In our study, long-term high-fat diet led to a significant increase in body weight and serum concentrations of triglycerides and cholesterol. The weight differences between

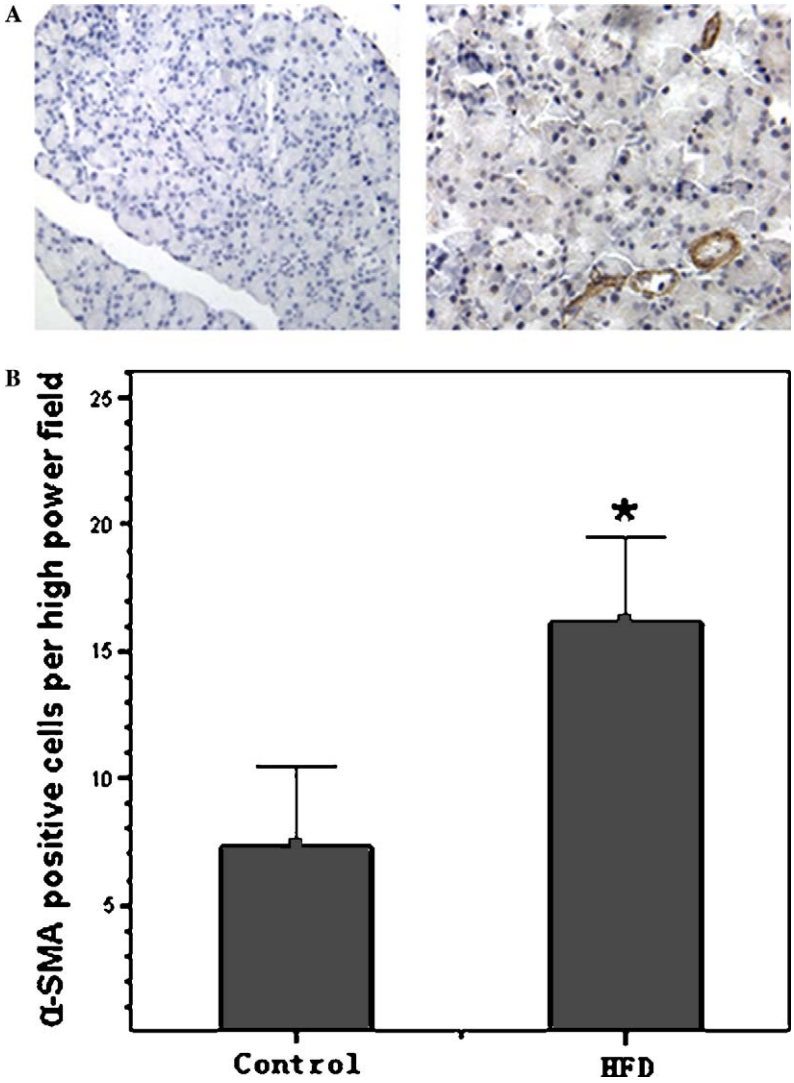

Fig. 4. (A, B) Immunohistochemical staining for α-SMA in pancreatic tissues. (A) Representative observations of α-SMA staining in control group (left, original magnification 200×) and HFD group (right, original magnification 400×). α-SMA-positive stained cells (positive as brown) were observed in pancreas from HFD rat. (B) α-SMA-positive stained cells per high power field in pancreas from both groups (*$p = 0.001$, compared with control group). Data presented as means ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

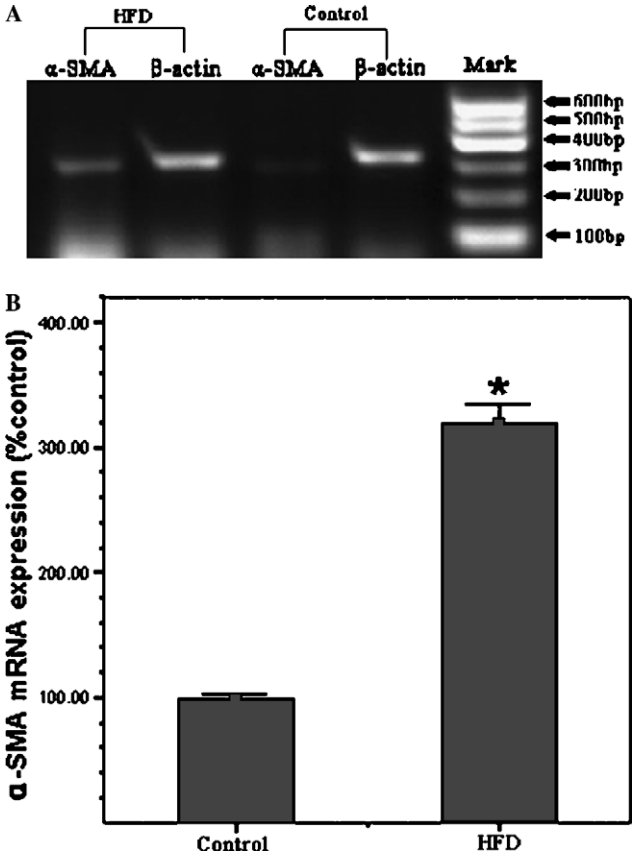

Fig. 5. (A, B) α-SMA mRNA expression in the pancreas of rats of control and HFD group. Pancreatic tissues were assayed for α-SMA mRNA using RT-PCR with β-actin as the housekeeping gene as described in Materials and methods. (A) Agarose gel electrophoresis shows over-expressed α-SMA mRNA in HFD rats than in controls. (B) Specific bands were quantitated by scanning densitometry and normalized to the signal of β-actin ($^{*}p = 0.000$, compared with control group). Data presented as means ± SD.

the two experimental groups might be due to significant visceral and subcutaneous fat accumulations in rats fed high-fat diet according to our observations and previous study [19]. Although the levels of glucose did not increase significantly in the present study, glucose intolerance could not be excluded [6]. Since serum amylase and lipase are sensitive markers for acute pancreatitis, the results in the current study indicate that high-fat diet can induce hyperlipidemia, but cannot induce acute pancreatitis.

Histologically, vacuolization was observed within pancreatic acinar and islet cells under light microscope in rats fed high-fat diets. These vacuoles indicate that exclusive fat tissues were accumulated inside pancreatic cells. Together with the large amounts of fat tissues accumulated around the pancreas that we observed macro- and microscopically, we may consider that the normal pancreas has turned to be a "fatty pancreas," which is surrounded and infiltrated by fat tissues after long-term high-fat diet intake [28]. In addition, we also observed acinar cells atrophy, which indicated a loss in pancreatic exocrine function [29,30]. Besides ultrastructural observations, these histological alterations would most certainly impede normal secretory functions of pancreatic cells if these structural changes persist and develop further.

Although we did not find obvious inflammatory cells' infiltration and fibrogenesis under light microscope, we found up-regulated expression of α-SMA, a marker of activated pancreatic stellate cells (PSCs) [31], in pancreatic tissues of rats fed high-fat diets. Since PSCs have a strong connection with fibrogenesis [32,33], these findings indicate that high-fat diet may lead to pancreatic fibrosis ultimately if this effective factor exists persistently.

The involved pathogenesis of these alterations related to high-fat diet is not clarified. As we know, elevated serum lipid can induce hemodynamic disturbances in many organs, and result in a large number of related diseases [11,12,15]. Being an important digestive organ, pancreas should be involved in these pathophysiological abnormalities associated with hyperlipidemia. When hyperlipidemia was incited, it seemed to be logical that the systemic hemodynamic, including pancreatic perfusion, would be influenced. Accordingly, decreased pancreatic blood flow was observed in rats fed high-fat diets in the current study. Hyperlipidemia incited by high-fat diet could promote activation of endothelial cells, adhesion of leucocytes, and disorders of endothelium-dependent vasodilator responses through lipid deposit and metabolic disorders [6,11,12]. These alterations may contribute to the pancreatic microcirculatory disturbances. On the other hand, the decreased blood perfusion in pancreas would certainly induce further deleterious effect on pancreatic endothelial, acinar, and islet cells.

Since increased lipid peroxidation results in increased production of MDA, determining MDA level provides a measurement of oxidative stress [34], while the activity of pancreatic SOD reflects the antioxidant defensive ability of the pancreas [35]. In the current study, the elevated levels of MDA content and decreased levels of SOD activity in pancreatic tissues demonstrated an increased lipid peroxidation and a decreased anti-oxidative ability in pancreas of rats with hyperlipidemia.

The pancreatic microcirculatory disturbances and lipid metabolic disorders may contribute to the elevated oxidative stress in these animals [10–12]. It is known that oxidative stress is involved in pathogeneses of many diseases including acute pancreatitis [36] and chronic pancreatic acinar and islet cell injuries [18,37–40]. Increased lipid peroxidation not only damages cell membrane, but also induces secondary intracellular responses including alterations of nucleus, endoplasmic reticulum, and mitochondria [15,35,41]. Since mitochondria are an important source of ROS [42], mitochondrial dysfunction would promote oxidative stress in turn. On the other hand, the pathogenesis by which α-SMA is up-regulated may be that lipid peroxidation products activate nuclear factor κB (NF-κB) [43], and then NF-κB promotes overexpression of TGF-β_1, which can result in PSCs activation and contribute to pancreatic fibrogenesis consequently [29,44,45].

Taken together our results suggest that long-term high-fat diet can induce chronic pancreatic injuries, and microcirculatory disturbances and oxidative stress may play an important part in this course. Although these abnormal alterations may not be obvious from clinical point of view, but it really occurs from biochemical, pathophysiological, and pathological points of view. These permanent occult alterations may lead to exocrine and endocrine dysfunction and pancreatic fibrosis as permanent and irreversible damages, and develop to typical chronic pancreatitis. Since high-fat intake can induce nonalcoholic fatty liver disease [15], these pancreatic abnormal alterations related to high-fat diet may be considered as "nonalcoholic fatty pancreatic disease." Considering that there are lots of people being fond of high-fat diets and suffering from obesity with hyperlipidemia, further clinical and experimental studies on relations between high-fat diet and pancreatic injuries should be encouraged to clarify the profound clinical significances and underlying pathogenesis.

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