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Original Article

Docosahexaenoic acid has an anti-diabetic effect in streptozotocin-induced diabetic mice

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Abstract: Consumption of fish oil-rich foods containing docosahexaenoic acid (DHA) can result in a low incidence of diabetes. The underlying mechanisms of these anti-hyperglycemic effects are ambiguous. This study aims to investigate the role of DHA in the prevention and treatment of type 1 diabetes in a murine model. Forty streptozotocin-induced diabetic mice were divided into control with diabetes, diabetes prevention (500 μg/kg DHA orally for 5 days) or diabetes treatment groups (DHA solvent in DMSO into the colon for 5 days). The groups were observed for 25 days after administration of DHA. Mice in the prevention and treatment group had shinier fur, increased body weight, significantly lower food and water intake and were more active compared with the control group with diabetes. Elevated insulin and liver SOD and T-AOC levels were also observed. Furthermore, islet cell apoptosis was reduced and islet cell GLP-1R expression increased.

Keywords: Docosahexaenoic acid (DHA), diabetes, glucagon-like peptide-1 receptor

Introduction

Docosahexaenoic acid (DHA) is a long-chain polyunsaturated fatty acid with activities in both infants and adults. DHA plays important roles in infant development and in the maintenance of human health [1, 2]. The biological activity of DHA has the potential to produce pronounced effects, including effects on platelet function, lipid levels, oxidation, glycemic control, and immune function. Rodent toxicology and *in vitro* mutagenicity studies provide the basis of general safety from a pre-clinical perspective, and an understanding of the effect and safety of DHA is important in the consideration of the essential activities of this fatty acid [2].

Diabetes mellitus (DM) is one of the most important global health problems. The worldwide incidence of DM continues to grow [3]. DM is a chronic heterogeneous metabolic disorder, characterized by absolute insulinopenia (T1DM) or insufficient production of insulin or inadequate peripheral tissue response to physiological levels of insulin (T2DM). Hyperglycemia is the main clinical symptom of DM, which causes glycation of body proteins that in turn leads to secondary complications affecting the eyes, kidneys, nerves and blood vessels [4]. Admittedly, different types of oral anti-diabetic agents, such as amylin analogues, alpha glycosidase inhibitors, are available. Contrary to expectations, these drugs also have certain adverse effects such as hypoglycemia at higher doses, liver problems and lactic acidosis [5]. Previous studies showed that fish oil-rich foods containing DHA resulted in a low incidence of diabetes [6]. However, the mechanisms of how DHA exerts its anti-hyperglycemic effect are ambiguous. Hence, this study aims to investigate the possible mechanisms of the anti-hyperglycemic effect of DHA, using a streptozotocin (STZ)-induced diabetic animal model.

Materials and methods

Materials and chemicals

STZ was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The glucose analyzer and strips were purchased from Roche Diagnostic Co. (Indianapolis, IN, USA). Mouse insulin enzyme-linked immunosorbent assay (ELISA)

Table 1. Effects of 5-day treatment with DHA on body weight, food intake and water intake in STZ-induced diabetic mice

Treatment	Body weights (g)		Food intake (g/kg)		Water intake (g/kg)	
	Day 1	Day 25	Day 1	Day 25	Day 1	Day 25
Normal	23.8±2.689	35.68±3.84	143.67±15.64	165.33±15.16	190.21±32,41	122.43±32.83
Diabetic control	22.16± 3.02	26.08±3.72*	147.28±26.95	257.26±17.33**	204.40±33.04	758.28±81.55**
Prevention group	24.56±2.78	29.66 ±3.83**	142.95±21.90	198.78±51.50**	219.01±20.33	678.64±41.30**
Therapy group	24.24±2.19	31.27±5.02*	145.35±16.61	199.74±6.74**	195.50±20.12	498.39±65.03**

Note: All values represent the mean±standard deviation (n = 10). *: Significant difference compared with normal group on day 25 (P < 0.05). **: Significant difference compared with normal group on day 25 (P < 0.01).

kits were purchased from R&D Systems (Minneapolis, MN, USA). Total-anti oxygen capacity (T-AOC) and total-superoxide dismutase (T-SOD) kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade from Peking Chemical Co. (Peking, China).

Experimental diabetic animals and treatments

Male Kunming mice averaging 4 weeks old were provided by the Experimental Animal Breeding Centre affiliated to Jilin University. All mice were housed in an air-conditioned room at 20±2 °C, with humidity of 55±15%, and a constant 12-h light and dark cycle. They were fed with a standard laboratory diet and given tap water ad libitum. All experimental animals were overseen and approved by the Animal Care and Use Committee of Jilin University before and during experiments. After 1 week of adaptation, the 40 animals were randomly divided into four groups (n = 10 per group). Diabetes was induced in overnight fasted mice by intra-peritoneal injection of 60 mg/kg of STZ freshly diluted in citrate buffer (0.1 M, pH 4.5) for 5 consecutive days. Normal control mice received the same volume of citrate buffer intra-peritoneally. To confirm diabetic state, tail vein blood glucose concentration in all STZ-injected mice was confirmed 7 days post STZ injection following a 12-h fast. Mice having fasting plasma glucose levels greater than 13.3 mmol/L were considered diabetic. The diabetes prevention group was given 60 mg/kg STZ intra-peritoneal injection and at the same time was orally administered 500 μg/kg DHA in DMSO with a mice gavage needle for 5 consecutive days. The diabetes treatment group was given DHA solvent in DMSO into the colon for 5 consecutive days after the confirmation of the diabetes model. The diabetes control group and normal control mice were given DMSO 0.1 mL into the colon at the same time. The four groups were observed for 25 days after administration of DHA and then sacrificed to collect blood and tissue samples.

Determination of blood glucose and serum insulin

Before acquiring blood samples, animals were fasted for 8 h with free access to water. Prior to the experiment, animals were fasted for 8 h and then blood was taken from tail veins for fasting blood glucose (FBG) and fasting serum insulin (FINS) measurement. The mouse serum insulin level was measured according to the usage instructions at an interval of 7 days. Meanwhile, the body weight of experimental mice was recorded using a balance every week until they were sacrificed. Animals were deprived of food overnight prior to being sacrificed by decapitation. Blood was collected from the dorsal aorta and serum was separated by centrifugation for 5 min and kept at -70 °C. The pancreas and liver were promptly removed. The pancreas was fixed in 10% buffered formalin (Sigma) for 1 week and the liver was stored at -70 °C until required.

Determining T-AOC and SOD activity in liver tissue

After blood was collected, experimental animals were sacrificed. Livers were then removed rapidly, washed with physiological saline and weighed. Hepatic homogenates were centrifuged at 3000 rpm at 4 °C for 15 min. Supernatant homogenates were transferred to clean microcentrifuge tubes and stored at -80 °C. The SOD and T-AOC levels in liver tissue were measured by chemochromatometry (as described by the manufacturer).

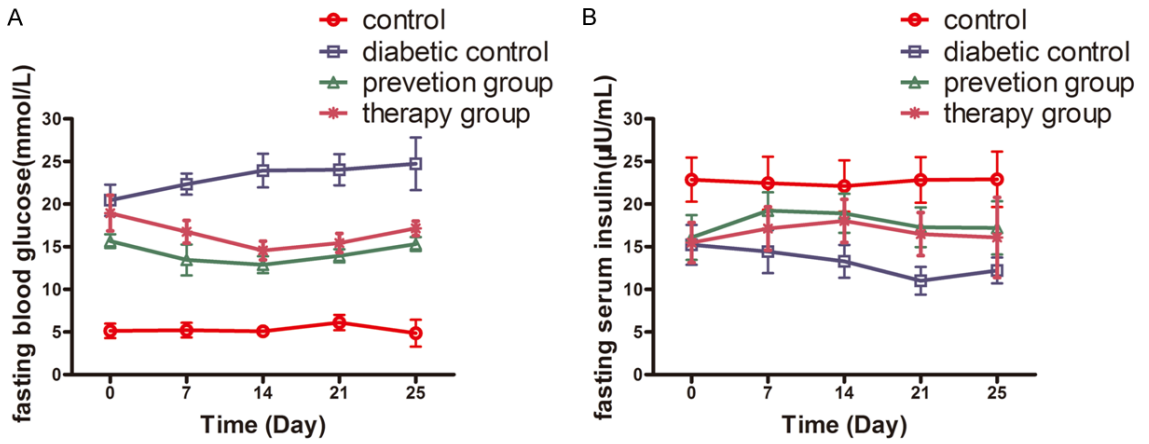

Figure 1. Effect of intracolonic DHA administration on plasma insulin and blood glucose levels. Data were collected from the first day of STZ administration. Changes in plasma insulin and blood glucose levels in response to intracolonic DHA administration are shown for the normal control (○), diabetic control (□), prevention group (▲) and therapy group (*). Each data point represents the mean±SD from n = 10 mice.

Pancreatic histology

Sections of pancreatic tissue (4 μm thick) were stained with hematoxylin and eosin for normal histological assessment, where cytoplasm stained pink, nuclei stained deep purple and pancreatic islets appeared pale in color. Halmi modified Gomori's aldehyde fuchsin stain was used to stain granulated pancreatic β-cells.

Immunohistochemical procedures

The endogenous peroxidase activity of sectioned formalin-fixed pancreatic tissues was inhibited by incubating sections in 3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min. Nonspecific binding of antibodies was blocked by their incubation with a 1% horse serum (Gibco Invitrogen, Carlsbad, CA, USA) for 10 min. Sections were incubated with monoclonal rabbit anti-sera against mouse pancreatic glucagon-like peptide-1 receptor (GLP-1R) protein (1:1000, Santa Cruz Biotech Inc., Santa Cruz, CA, USA) for 1 h at 4 °C, and then incubated with the biotinylated secondary antibody. Binding of the antibody was visualized using a diaminobenzidine (DAB) solution and counterstained with hematoxylin.

TUNEL assay in pancreatic tissues

Formalin-fixed pancreas tissues were embedded in paraffin and sectioned. The Dead End™ Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) was used to detect apoptosis according to the manufacturer's protocol. Briefly, the equilibration buffer was added to slides and incubated for 10 min followed by 10 min incubation in 20 μg/mL proteinase K solution. Sections were washed in PBS and incubated with TdT enzyme at 37 °C for 1 h in a humidified chamber, for incorporation of biotinylated nucleotides at the 3'-OH ends of DNA. The slides were incubated in horseradish peroxidase-labeled streptavidin to bind the biotinylated nucleotides followed by detection with stable chromogen DAB. Slides were visualized with a light microscope equipped with a computer-controlled digital camera. Three slides per group were stained and apoptotic cells were identified by dark brown cytoplasmic staining.

Statistical analysis

All results were expressed as means±standard deviations (SD) for each group. Statistical calculations were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Data were statistically analyzed by one-way analysis of variance, followed by post-Tukey test for statistical analysis to compare control and treated groups. P values of less than 0.05 were considered significant.

Results

Effect of DHA on body weight, fasting blood glucose and serum insulin levels in normal and STZ-induced diabetic mice

No difference between groups in body weight, food intake and water intake was seen at the

Table 2. Effect of DHA on liver T-AOC and SOD activity in STZ-induced diabetic mice

Group	T-AOC	SOD
control	19.98±4.13	17.42±6.43
diabetic control	28.27±7.13*	31.45±10.23*
prevention group	25.11±6.99*	26.39±6.98*
therapy group	23.36±4.72*	27.48±7.55*

Note: All values represent mean±standard deviation (n = 10). *: Significant difference compared with normal group ($P < 0.05$).

beginning of the experiment (**Table 1**). The mice induced with STZ in the diabetic control group had a greater loss of body weight ($P < 0.01$) than that in the normal control group, replicating clinical symptoms seen in patients with DM. However, the body weight of the two groups administered with DHA was significantly increased compared with the diabetic control group ($P < 0.05$ or $P < 0.01$), but decreased compared with the normal control group ($P < 0.05$ or $P < 0.01$). Opposite changes were seen with food and water intake. Therefore, DHA could improve weight loss and alleviate excess food and water intake.

The effect of DHA on FBG and FINS in normal and STZ-induced diabetic mice is shown in **Figure 1**. We can see that mice in the normal control group had a normal FBG and FINS level when compared with diabetic mice treated with STZ ($P < 0.01$). After 7 days, the FBG level in the prevention group and therapy group in STZ-induced diabetic mice was significantly inhibited by DHA (**Figure 1A**). Compared with vehicle treatment, DHA significantly increased the plasma insulin level on the 7th day ($P < 0.05$; **Figure 1B**). Notably, the glucose level in the DHA treatment and prevention groups was far below that of the normal control group at the end of the experiment ($P < 0.05$). Based on this finding, the use of DHA as a prevention or therapy had a positive effect on both FBG and FINS level in STZ-induced diabetic mice.

Effects of DHA on liver SOD and T-AOC

As shown in **Table 2**, there was a significant ($P < 0.01$) rise in liver SOD and T-AOC levels in diabetic mice compared with the normal control group. When treated with DHA, diabetic mice showed remarkably higher hepatic SOD activities compared with those in the diabetic control group. These results indicate an obvious antioxidant effect of DHA in STZ-induced diabetic mice.

Effect of DHA on pancreatic islet histology

Figure 2 shows the histology of mice pancreatic islets stained with hematoxylin and eosin. **Figure 2A** shows a normal, large and round pancreatic islet, with clusters of islet cells surrounded by exocrine acini. The pancreatic islets from STZ-diabetic mice were atrophic and vacuolated in the diabetic control (**Figure 2B**). Prevention therapy with DHA protected islets from the destructive effect of STZ (**Figure 2C**). Histology from this group was similar to features seen in the DHA therapy group (**Figure 2D**), which included absence of vacuolization. A decrease in the size of the islet, however, was observed. Less degranulation was observed in the β-cells (**Figure 2C** and **2D**).

Effect of DHA on expression of GLP-1R in pancreatic tissue

A decrease in total expression of GLP-1R (~40%) was seen in the diabetic control group when compared with normal controls. DHA prevention and DHA therapy caused a significant increase in GLP-1R expression by 24% and 26%, respectively, compared with vehicle in the diabetic control group (**Figure 3**). A higher portion of GLP-1R was observed to be retained in the light microsomal pool in the diabetic control group compared with the normal control group.

Effect of DHA on apoptosis in pancreatic tissue

To further investigate the effect of DHA on apoptosis in pancreatic tissue, sections of pancreas were stained with TUNEL for detection of DNA fragmentation (**Figure 4**). STZ induced DNA fragmentation (brown staining). STZ treatment led to apoptosis in 26.21±8.21% of the pancreatic cells, whereas DHA prevention and treatment induced apoptosis in 12.23±5.04% and 10.34±6.22% of the pancreatic cells, respectively. All treatments were significantly different from the control (*$P < 0.01$).

Discussion

Previous studies have shown the beneficial role of DHA for diabetic patients [7, 8]. In this study,

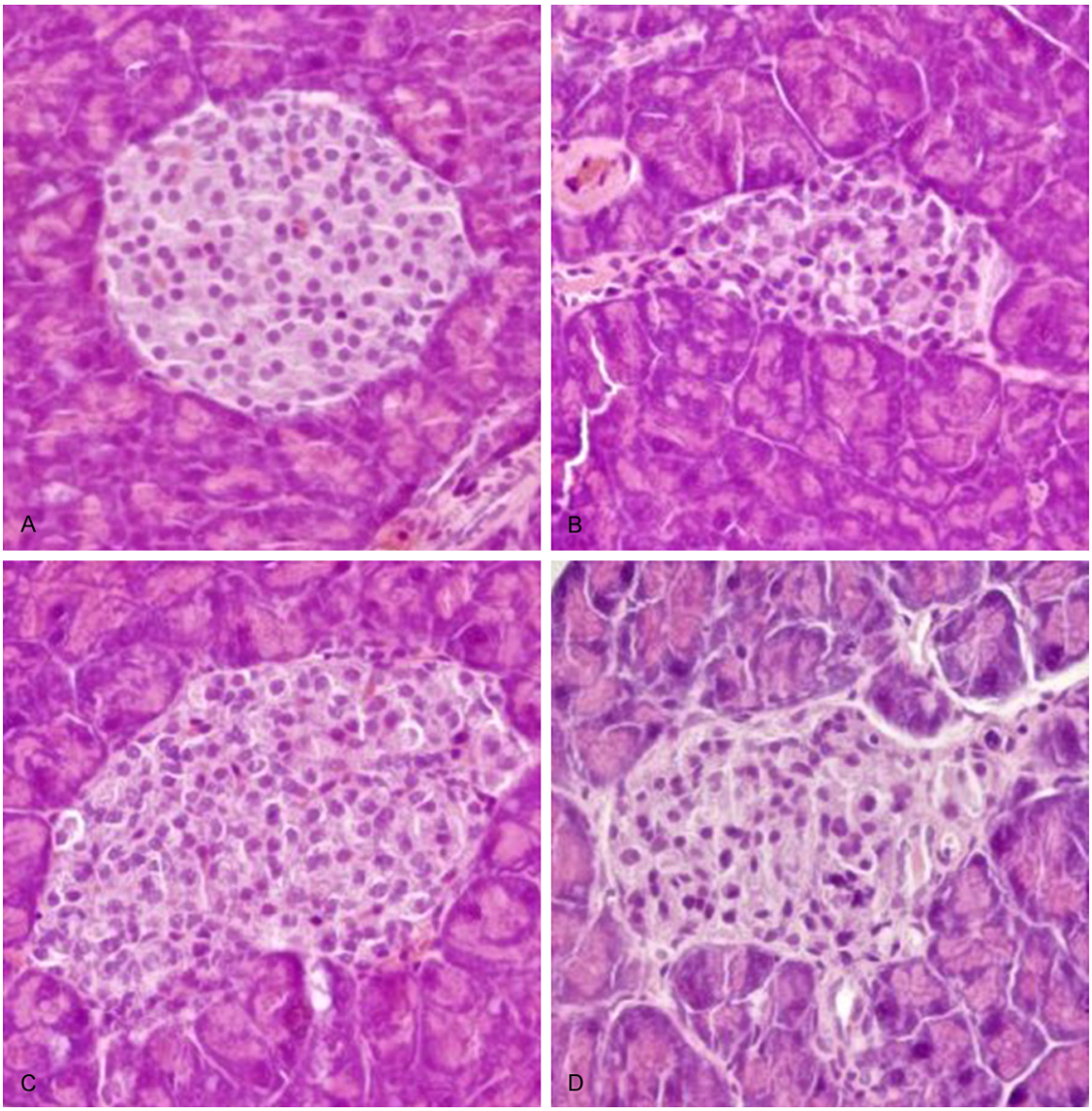

Figure 2. Hematoxylin & eosin staining of pancreatic islets from STZ-induced diabetic mice. Treatments (500 μg/kg DHA and vehicle for both normal control and diabetic control) were given daily through gavage for 5 days. Pancreas was isolated at the end of day 25 and fixed in 10% buffered formalin for 1 week before processing, sectioning and staining. Images shown (400×) are representative of the corresponding treatment group. A. Normal control, B. Diabetic control, C. Prevention group, D. Therapy group.

we have revealed that DHA significantly decreased fasting blood glucose. This is likely responsible for the anti-diabetic effect of DHA and its possible mechanism of action in STZ-induced diabetic mice.

STZ, an antibiotic produced by *Streptomyces chromogenes*, is the most commonly used agent in experimental diabetes [9]. It was demonstrated that STZ induced damage to pancreatic cell membranes and evoked oxidative stress [generation of reactive oxygen species (ROS)] in islet cells [10]. In addition, STZ has been shown to induce DNA strand breaks and DNA alkylation in pancreatic islet cells [11]. STZ induces T1DM or T2DM, depending on the dose administered [10, 12]. Multiple low-doses of STZ in this study resulted in diabetic mice that closely resemble T1DM in humans, which is characterized by insulitis with accumulation of inflammatory cells and degranulation of pancreatic β-cells [13].

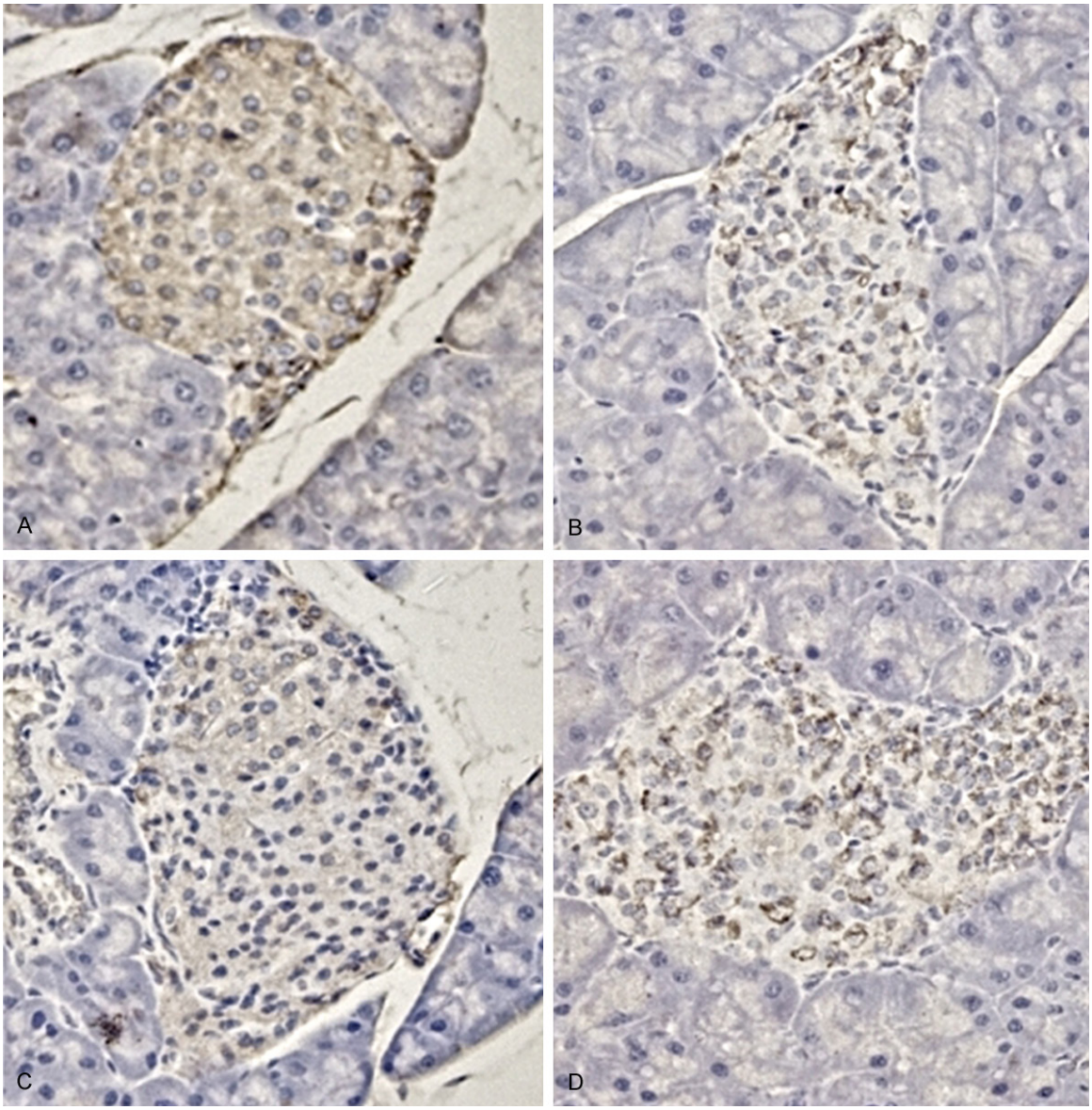

Figure 3. Insulin immunohistochemical staining for GLP-1R in the pancreas. Treatments (500 μg/kg DHA and vehicles for both normal control and diabetic control) were given daily through gavage for 5 days. Pancreas was isolated at the end of day 25 and fixed in 10% buffered formalin for 1 week before processing, sectioning and staining. Images shown (400×) are representative of the corresponding treatment group. A. Normal control, B. Diabetic control, C. Prevention group, D. Therapy group.

STZ-induced diabetes is characterized by hypoinsulinemia, polydipsia, polyuria and decreased body weight. Treatment with DHA greatly improved polyphagia and polydipsia (**Table 1**), indicating improvement in diabetic conditions. In this study, DHA caused significant improvements in body weight, suggesting another benefit for the treatment of diabetes, especially T1DM. In a previous study, treatment with omega-3 fatty acids (EPA and DHA) along with anti-diabetic drugs for a period of 2 months in non-insulin-dependent diabetic patients had beneficial effects on serum triglycerides, HDL-cholesterol, lipid peroxidation and antioxidant enzymes. These effects may lead to a decreased rate of occurrence of vascular complications in diabetes [7].

Several previous studies have proposed that DHA might improve glucose tolerance and insulin sensitivity [7, 14]. Previous studies have shown that DHA has different site-specific

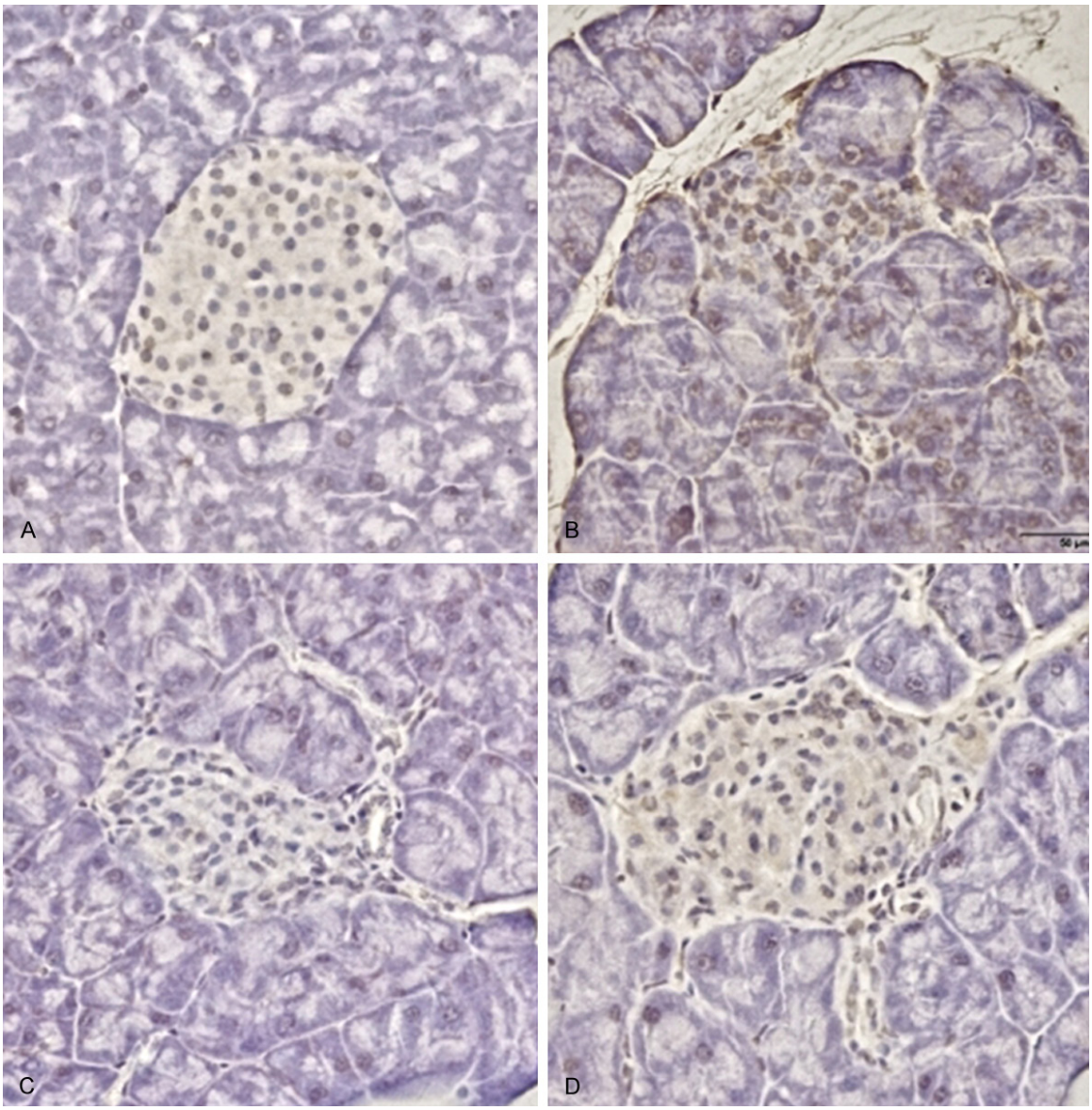

Figure 4. TUNEL staining of pancreatic tissue for induction of apoptosis. Quantification of apoptotic cells from TUNEL staining and quantitation of positive apoptotic cells. Apoptotic tumor cells stained brown. Percentages of TUNEL-positive cells were quantified by counting 100 cells from six random microscopic fields. Images shown (400×) are representative of the corresponding treatment groups: A. Normal control, B. Diabetic control, C. Prevention group, D. Therapy group.

effects [15-17]. Colonic administration of DHA most strongly enhances plasma insulin secretion and reduces blood glucose levels compared with stomach or jejunum administration [17]. We also found that, compared with the vehicle in diabetic controls, the colonic administration of DHA caused a significant increase in insulin level in the prevention and therapy groups. Histological assessment of the pancreatic sections revealed that pancreatic cells from the DHA-treated mice were less damaged (no vacuolization and less degranulation) compared with those in the diabetic control, suggesting that there were more functional pancreatic cells in the DHA-treated group. This may explain the higher level of insulin that was found.

The incretin hormone GLP-1 is secreted by enteroendocrine L cells in response to digestion of food and exhibits insulinotropic and pancreatic β cell-proliferating effects [18]. GLP-1

activates the pancreatic β cell through the GLP-1R, and stimulates insulin secretion. We assessed GLP-1R in pancreatic tissue and found that mice treated with DHA have more GLP-1R expression in the cytoplasm of pancreatic islet cells when compared with diabetic control mice. Further investigations are needed to characterize the pathway by which DHA increased GLP-1R expression.

An abundance of clinical evidence has demonstrated that diabetes correlates closely with oxidative stress, resulting in increased ROS production or a reduction in the antioxidant defense system [19]. Both increased production of oxidants and decreased actions of antioxidants play roles in the increased oxidative stress in experimental diabetes [20]. There are many enzymatic and nonenzymatic antioxidants *in vivo*, such as SOD and T-AOC. SOD is the only substrate used as the superoxide anion scavenger enzyme, and as such it constitutes the first line of defense against ROS. T-AOC is an important marker of oxidation, which mainly reflects the nonenzymatic pathway but includes the activity of a minority of small molecular enzymatic systems. Animal and clinical studies have confirmed that antioxidant treatment plays an effective role in diabetes [21, 22]. Our results demonstrated that liver SOD and T-AOC in the prevention and therapy groups were lower than that of diabetic control subjects.

A reduction in β cell mass due to increased β cell apoptosis is an important event in the pathogenesis of diabetes mellitus. In this study, DHA therapy and prevention reduced β cell apoptosis compared with the diabetic control group. Many factors contribute to β cell apoptosis, including oxidative stress and the GLP-1R. Previous reports have demonstrated that GLP-1 stimulates β cell proliferation and protects pancreatic β-cells from apoptosis, in addition to its insulin biosynthesis and secretagogue action through GLP-1R [23, 24].

Conclusions

DHA may have therapeutic potential to modulate and regulate oxidative stress, and upregulate the GLP-1R, thus exerting favorable effects on STZ-reduced T1DM. Thus, it may be worth further studying DHA as a potential clinical treatment.

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Disclosure of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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