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Research

Changes of Pancreatic Protein Profile Upon High Fat Diet Intake In Diabetic Rat Model

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ABSTRACT

The impact of a high-fat diet (HFD) on changes in pancreatic protein expression of the T2DM rat model was investigated. This is important as proteins are the functional components that regulate an organ's activity. Adult male Sprague-Dawley rats were induced to diabetes using streptozotocin (STZ) and nicotinamide (NA). The diabetic rats and control rats were respectively fed with either a normal diet (ND) or a high-fat diet (HFD) that contained 39% fat for 6 consecutive weeks. The pancreases were harvested from the rats for proteomic analysis upon completion of the treatment period. Two-dimensional electrophoresis (2-DE) analysis was applied for protein separation and the significantly (p<0.05) upregulated protein spots in pair-wise comparison between different groups of rats were subjected to LC-MS/MS analysis for protein identification. The results showed that upregulation of anionic trypsin 1 and 2 in diabetic rats fed with ND compared to control rats fed with the same diet. The upregulation of these two proteins in diabetic rats acts as the defense mechanism against pancreatic tissue inflammation upon HFD intake. Moreover, upregulation of chymotrypsinogen B and transgelin-3 were detected in diabetic rats fed with HFD compared to those fed with ND, suggesting that these proteins may be induced by HFD intake, where upregulation of chymotrypsinogen B is a sign of pancreatic inflammation. Thus, HFD may influence specific pancreatic processes based on the observed protein changes.

Key words: 2-D electrophoresis, high-fat diet, LC-MS/MS, pancreas, type 2 diabetes mellitus

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INTRODUCTION

The pancreas is located in the left upper abdominal cavity, between the duodenum and spleen (Rela & Reddy, 2015). The pancreas is comprised of exocrine and endocrine systems, the two systems are involved in the regulation of glucose homeostasis and nutrient digestion, respectively (Röder *et al*., 2016; Paniccia & Schulick, 2017). Type 2 diabetes mellitus (T2DM) can be caused by insulin deficiency or peripheral insulin resistance (Rachdaoui, 2020). Reduced insulin sensitivity is usually related to T2DM and it is mainly caused by fat accumulation in body cells (Zaccardi *et al*., 2016). This leads to metabolic disorders, generating excess glucose and fatty acids that progressively cause pancreas inflammation and β-cells death (Acosta-Montaño & García-González, 2018). A 30-40% reduction of β-cells was observed in T2DM patients (Durruty *et al*., 2019). The role of the pancreas in the regulation of blood glucose makes it a critical organ to be studied to understand the development of T2DM. Jiang *et al*., (2011) reported the upregulation of oxidative stress proteins, heat shock protein 60 kDa, and peroxiredoxin 4 in the pancreas of T2DM rats. Furthermore, proteolysis proteins (carboxypeptidase A2, PRSS3 protein, and chymotrypsinogen B) and carbohydrate metabolism proteins (pancreatic alpha-amylase and phosphoglycerate

mutase 1) were upregulated in T2DM mice (Pérez-Vázquez *et al*., 2014). A recent study that combines both transcriptomics and proteomics profiling techniques revealed the regulation of aldolase B and glucose-6-phosphate in T2DM pancreatic islets was associated with a reduction of glycolysis and insulin secretion (Haythorne *et al*., 2019). These studies reveal the mechanisms involves in deterioration of pancreatic function associated with T2DM pathology which are useful in discovery of therapeutic treatments for diabetes.

Excess energy or calories due to the high intake of fat is stored in adipocytes as triglycerides, the accumulation of triglycerides can lead to hypertrophy and hyperplasia of adipocytes (Dixon, 2010) that reduces insulin responsiveness, decreased glucose uptake, and increased secretion of proinflammatory adipokines. Therefore, it is reasonable to assume that a diet high in fat aggravates diabetes disease. High-fat diet (HFD) is a risk factor for obesity and T2DM (Marshall & Bessesen, 2002; Risérus *et al*., 2009). HFD induces metabolic syndrome in important organs such as the pancreas, liver, kidney, and heart (Panchal *et al*., 2011), and promotes lipotoxicity in these organs (Heydemann, 2016). Consumption of a diet high in fat content generates excessive adipose tissues in the body. This will in turn increase endoplasmic reticulum stress in organs' cells and deteriorate insulin secretion (Hariri & Thibauly, 2010). Besides, HFD reduces the function of insulin receptors as well as energy storage function in the liver and muscles and disrupts glucose metabolism (Hariri & Thibauly, 2010). As HFD contributes to the risk of diabetes, eating of balanced diet is crucial in the prevention and maintenance of T2DM. The impact of a diet high in fat on the pancreas of T2DM patients can be understood by analysis of the changes in pancreatic protein expression, this is possible as proteins are the functional components of cells. This study aimed to investigate the effect of HFD consumption on pancreatic tissues of STZ-induced diabetes rats by using 2-DE analysis coupled with LC-MS/MS.

MATERIALS AND METHODS

Animal Study

Thirty-six male Sprague-Dawley rats were obtained from the Animal Research Centre and Service at Universiti Sains Malaysia (Penang, Malaysia). The rats were housed separately in cages with a controlled environment at 22-24°C and a 12-hr light/dark cycle. The rats had ad libitum access to food and water.

Induction of diabetes

All rats were given one week for adaptation. The rats were then allowed to fast for 12 hr before the induction for diabetes. Diabetes was induced according to Masiello *et al*. (1998). All chemicals were administered to the rats by intraperitoneal (IP) injection. Nicotinamide (NA) in normal saline was administered at first with a dosage of 110 mg/kg. After 15 min, STZ in 0.1 M sodium citrate dihydrate (pH 4.5) was injected with a dosage of 65 mg/kg. At the same time, the control rats were injected with vehicle, saline, and citric buffer. Animals from both diabetic and control groups were monitored for 4 weeks. In the fourth week, 12-hr fasting blood glucose (FBG) levels were obtained by drawing blood from the tail tip of the rats and were measured using a glucometer.

Experimental design

The animals were sorted into four groups, each consisting of six rats. These rats were fed according to the diet plan for 6 weeks. The dietary plan for each rat group is described in Table 1. Control rats were divided into two groups; Group 1 (G1) was fed with ND and Group 3 (G3) was fed with HFD. Besides, induced-diabetic rats were assigned into groups; Group 2 (G2) was fed with ND while Group 4 (G4) was fed with HFD.

Table 1. Dietary plan for each group of rats

STZ induction	Condition	Diets and Treatments
Control	Control	ND
Induced	Diabetic	ND
Control	Control	HFD
Induced	Diabetic	HFD

Diet preparation

A standard rodent chow was used for the ND group and the HFD group was supplemented with

a commercially available fat source (cooking oil, BURUH, Selangor, Malaysia) to achieve a desired percentage of 39% fat by weight. The food pellets were baked at 40°C overnight in an oven.

Harvesting of pancreas organ

Upon completion of the six-week treatment period, euthanasia of the animals was carried out by IP injection of a ketamine-xylazine cocktail (100 mg/kg of ketamine & 10 mg/kg xylazine). The harvested rat pancreases were immediately ground into powder form in liquid nitrogen using a mortar and pestle and then kept at -80°C.

Two-dimensional electrophoresis (2-DE)

Pancreas powder was mixed with thiourea lysis buffer (TLB) [8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 0.4% (w/v) carrier ampholytes, pH 3-10, 50 mM 1,4-dithiothreitol (DTT)] at a powder to buffer ratio of 1:1.5. The mixture was homogenized and centrifuged at 16000 × g for 30 min at 4°C and the supernatant was collected.

The supernatant (or pancreatic protein extract, 0.1 mL) was subjected to protein purification and precipitation using 0.8 mL of 20 mM dithiothreitol (DTT) in cold acetone and 0.1 mL of TCA. The pellet was collected and reconstituted with 150 µL of TLB buffer. It was then quantified using RC/DC protein assay (Bio-RAD, USA) before being analyzed using 2-DE. 2-DE was carried out using IPG strips (linear pH 4-5, 7cm) according to Jiang *et al*. (2011) with slight modification. The focusing parameters were 150 V rapid climb for 30 min, 250 V with a linear climb for 15 min, 1000 V with a rapid climb for 30 min, 4000 V with a linear climb for 1 hr, 4000 V with a rapid climb until 10000 Vhr was reached and hold at 500 V using PROTEAN i12 IEF Cell (Bio-RAD, USA). The strips were then subjected to equilibration processes and finally inserted into vertical mini-slab gel (Mini-PROTEAN® III system, Bio-RAD) for second-phase separation. The separation was carried out at a constant voltage of 120 V until the dye front reached the bottom end of the gel. The gels were then stained using Coomassie blue and destained, The gel images were captured by ChemiDocTM Imaging System (Bio-RAD, USA).

Analysis of 2-DE by PDQuest™ software

PDQuest™ software version 7.3 (Bio-rad, USA) was used for processing and analyzing gel images of 2-DE. The gel images were cropped into the same size (50.9 × 38.6 mm) and arranged into the correct orientation. Gaussian and outliers with 5 × 5 pixels filter size were set to remove electronic artifacts created by the imaging system. Normalization of protein spot intensity was set as total valid spot intensity. Match sets were created to compare the gel images of rat pancreatic protein profiles obtained from different rat groups. In a matchset analysis, common protein spots were identified. Student's t-test was applied by using PDQuest™ software to determine the statistical differences of protein expressions at a 95% significance level. Protein spots that expressed significantly > 2.0-fold were considered as up-regulated, and those that expressed significantly < 2.0-fold were considered as down-regulated.

In-gel digestion

In-gel digestion was performed according to the method of Gam and Aishah (2002) with slight modification. The protein spots of interest were excised from the gel. The gel pieces were hydrated in 30 µL of 100 mM ammonia bicarbonate for 10 min followed by dehydration in 30 µL of Acetonitrile (ACN) for 5 min. The hydration and dehydration steps were repeated three times or until the protein stains were completely removed. Finally, the gel pieces were dried in a vacuum centrifuge (Eppendorf, Germany). The dried gel pieces were added with 30 µL of 100 mM ammonium bicarbonate containing 10 mM DTT and incubated in a water bath at 56°C for an hr. After the supernatant was removed, the gel pieces were incubated in 30 µL of 100 mM ammonium bicarbonate containing 55 mM iodoacetamide for 45 min at room temperature in the dark. The hydration and dehydration steps described previously were repeated twice. The dried gel pieces were incubated for 2 hr on ice in 30 µL of digestion buffer containing 15 ng/mL trypsin. After the buffer was discarded, the gel pieces were incubated at 37°C overnight in 10 µL of digestion buffer without trypsin. The next day, the supernatant was collected into a new Eppendorf tube. A 15 µL of 20 mM of ammonium bicarbonate was added to the gel pieces and was incubated for 10 min. The supernatant was collected. The gel pieces were incubated for 20 min with 5% formic acid in 70% ACN (70:30 of ACN: H_20) at room temperature. The supernatant was recovered. The process was repeated three times. The collected supernatant was blown dry under a continuous flow of nitrogen gas. After it was fully dried, the peptides were kept at -80°C until further analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Samples were reconstituted in 30 µL of 0.1% (v/v) formic acid in deionized water and filtered through a 0.2 µm regenerate cellulose (RC) membrane filter syringe filter (Sartorius, Germany). Easy-nLC II nano liquid chromatography system (Thermo Scientific, USA) coupled with Finnigan LTQ linear ion trap mass spectrometer (Thermo Scientific, USA) was used in this study. The protocol for LC was as follows: pre-column: Easy-Column C18 (20 × 0.10 mm i.d., 5 µm; Thermo Scientific, USA), equilibrated at 3 µL/min for 15 µL; analytical column: Easy-Column C18 (100 × 0.75 mm i.d., 3 µm; Thermo Scientific, USA), equilibrated at 0.3 µL/min for 4 µL; running buffer A: 0.1% (v/v) formic acid in deionized water; running buffer B: 0.1% (v/v) formic acid in acetonitrile. The sample (15 µL) was injected and chromatographically separated at a flow rate of 0.3 µL/min. Elution of samples was done with gradient mode from 5% to 100% of buffer B for 80 min. Eluent was sprayed into a mass spectrometer at 220°C with a source voltage of 2.1 kV. For peptide detection, full scan mass analysis was set from m/z 200-2,000 at resolving power (FWHM) of 60,000 at m/z 400 and acquisition time of 1 sec, with data-dependent MS/MS analyses triggered by 8 most abundant ions from the parent mass list. Rejection of singly or unassigned charged peptides was applied. Collision-induced dissociation (CID) with 35 V was applied for fragmentation.

Data processing for protein identification

PEAKS® Studio Version 7.0 (Bioinformatics Solution, Canada) was used to process the data obtained from LC-MS/MS analysis. The precursor mass and fragment ion mass tolerance were set at 0.5 Da. Variable post-translational modifications (PTM) were set as carbamidomethylation and oxidation (M) with maximum missed cleavage per peptide at 3 were allowed. A significant score (-10 logP) was set at ≥ 20. Swiss-Prot 2019 was used for database matching. The characteristics and function of the proteins were obtained from the Uniprot website (https://uniprot.org).

RESULTS

Comparison between groups

In this study, to understand the impact of HFD on the proteome of rats' pancreas. We have conducted a 2D gel analysis on the proteins extracted from the harvested pancreas. In general, the proteome of all the rat groups was relatively consistent, indicating that types of protein expression were relatively similar, nevertheless, we observed that the intensity of certain protein spots varied from gel to gel, indicating the presence of differently expressed proteins. To identify the identity of these differentially expressed proteins, a detailed comparison was carried out. For comparison purposes, five matched sets of gels were created by using PDQuest™ software namely; A. control rats fed with ND (G1) vs diabetic rats fed with ND (G2); B. control rats fed with HFD (G3) vs diabetic rats fed with HFD (G4); C. control rats fed with ND (G1) vs control rats fed with HFD (G4); D. diabetic rats fed with ND (G2) and diabetic rats fed with HFD (G4).

From PDQuest analysis, 2 out of 4 matched sets detected significant ($p<0.05$) differences in protein expression. These 2 matched sets were between control rats fed with ND (G1) and diabetic rats fed with ND (G2) as well as diabetic rats fed with ND (G2) and diabetic rats fed with HFD (G4). Nevertheless, no significant differences were detected in the comparison analysis of the other 2 matched sets.

Protein spots identification

Figure 1 shows the gel images of control rats fed with ND (G1) and diabetic rats fed with ND (G2). Three protein spots were found to upregulated > 2.0-fold significantly ($p<0.05$) in the G2 group compared to the G1 group. These protein spots were spot 0104, spot 1004, and spot 3101. Quantification analysis of protein spots 0104, 1004, and 3101 were shown in Table 2. where protein spot 0104 was upregulated 2.0-fold, protein spot 1004 was upregulated 9.0-fold, and protein spot 3101 upregulated 2.73-fold, respectively at $p<0.05$.

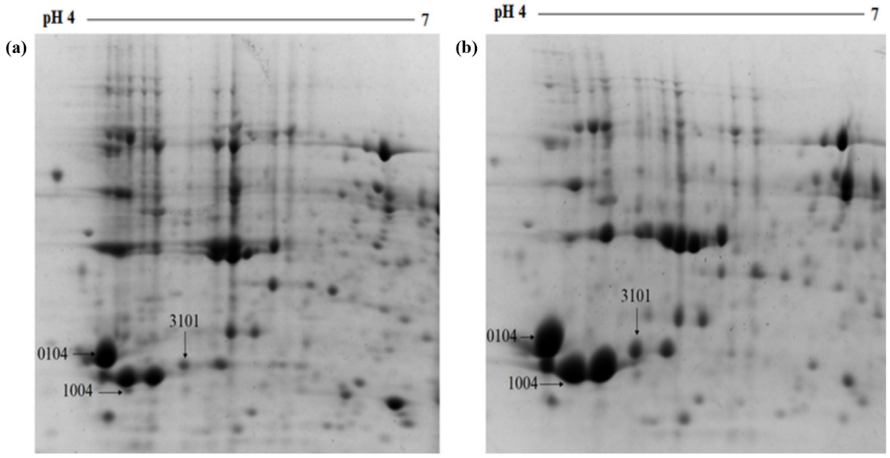

Fig. 1. 2-DE images of rat pancreatic proteins from different diets and treatment groups. Gel image (A): Non-diabetic rats fed with ND (G1); Gel image (B): Diabetic rats fed with ND (G2).

Table 2. Intensity of pancreatic protein spots in G1 and G2

Spot Number	Spot Intensity (ppm)		Fold Change
	G1#	G2#	
0104	52265.1±2269.5	104467.5±9649.9	+ 2.00
1004	1443.4±190.2	12904.8±587.3	+ 9.00
3101	8454.05±981.1	23070.73±4441.22	+ 2.73

#G1: Non-diabetic rats fed with ND; #G2: Diabetic rats fed with ND

Figure 2 shows the gel images of a diabetic rat fed with ND (G2) and a diabetic rat fed with HFD (G4). 2 protein spots were detected upregulated at $p<0.05$ in the G4 group compared to the G2 group, these spots were protein spot 4801 that was 6.51-fold upregulated and protein spot 6702 was 4.52-fold upregulated (Table 3).

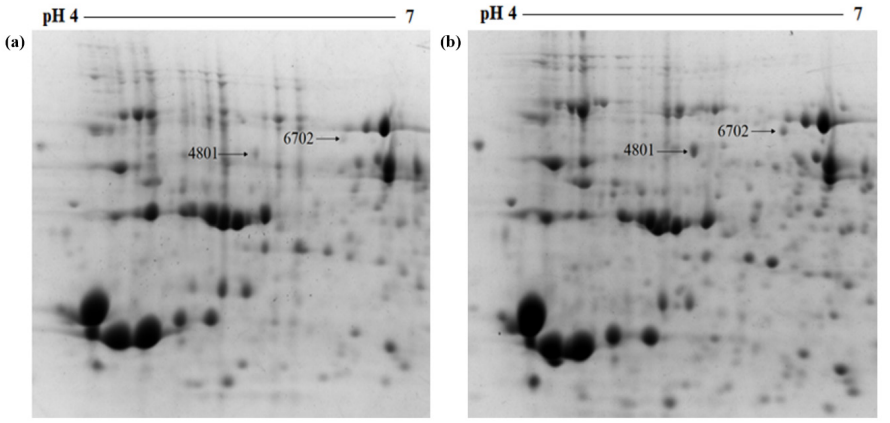

Fig. 2. 2-DE images of rat pancreatic proteins from different diets and treatment groups. Gel image (a): Diabetic rats fed with ND (G2); Gel image (b): Diabetic rats fed with HFD (G5).

Table 3. Intensity of pancreatic protein spots in G2 and G4

Spot Number	Spot Intensity (ppm)		Fold Change
	G2#	G5#	
4801	1291.3±44.5	8409.9±215.4	+ 6.51
6702	774.33±88.6	3163.8±244.1	+ 4.52

#G2: Diabetic rats fed with ND; #G5: Diabetic rats fed with HFD

The upregulated protein spots were excised from the gels, in-gel protein digestion using trypsin enzyme was carried out and the peptides were subjected to LC-MS/MS analysis. Table 4 shows the identity of the proteins. Four protein spots showed a protein hit however one of the spots had no protein hit, the no-hit protein spot belongs to a hypothetical protein, where the sequence was not found in the

protein database. Figure 3 shows an example of the MS spectrum obtained from protein spot 1004. The function of the proteins will be further discussed in the discussion.

Table 4. List of pancreatic proteins identified by LC-MS/MS

Spot Number	Protein Name	Accession	-10logP	MW (kDa)
0104	Anionic trypsin-2	P00763	35.51	25.9
1004	Anionic trypsin-1	P00762	83.08	25.9
3101	No Hit			
4801	Chymotrypsinogen B	P07338	36.13	27.8
6702	Transgelin-3	P37805	24.27	22.5

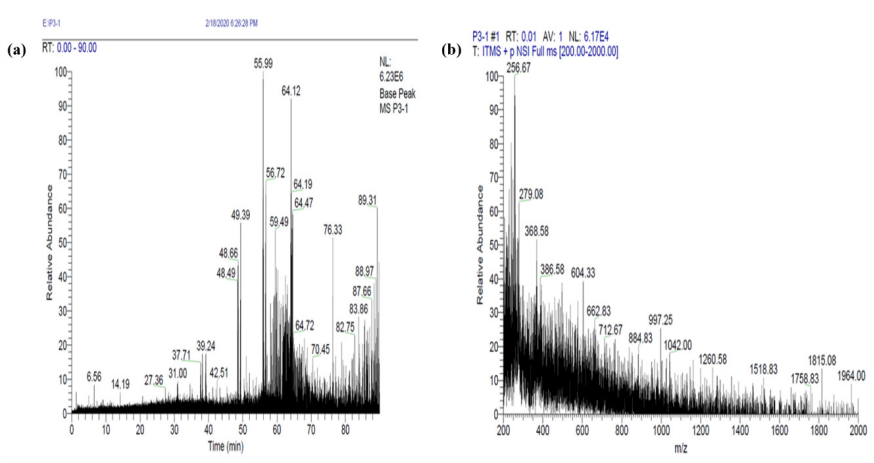

Fig. 3. Base peak (a) and full MS spectrum (b) of protein spot 1004.

DISCUSSION

Dysregulation of blood glucose causes diabetes mellitus, with the pancreas being the key organ that secures hormones to regulate blood glucose, therefore it is directly involved in glucose homeostasis. β-cells of the pancreas secrete insulin that triggers glucose uptake in body cells while α-cells secrete glucagon for stimulation of hepatic glucose production, (Röder *et al*., 2016; Paniccia & Schulick, 2017). Hence, diabetes mellitus is associated with dysregulation of the pancreas.

In this study, each rat group consisted of six animals, and gels were run in duplication on each animal, which led to 12 gels being produced from each animal group. The common protein spots from the 12 gels were taken into producing a master gel for the group. The heterogeneous protein spots were ignored as these protein spots may be formed in individual animals that are not related to the disease condition. Out of the 4 matched sets, only the analysis of 2 matched sets showed significant differential protein expression at a 95% significance level. These 2 matched sets were between control rats and diabetic rats, both were fed with ND as well as between diabetic rats fed with ND and diabetic rats fed with HFD. The other comparison groups showed no significant differences in protein spot intensity at a 95% significance level and therefore were omitted. Protein database search results based on the spectrum acquired from LC-MS/MS analysis have identified the proteins as shown in Table 4, protein spot 0104 was anionic-trypsin 2 and protein spot 1004 was anionic-trypsin 1. The accession numbers for anionic-trypsin 1 and anionic-trypsin 2 are P00762 and P00763, respectively. Additionally, protein spot 4801 was detected as chymotrypsinogen B with the accession number P07338. The Uniprot database indicated that the accession numbers for these 3 proteins were associated with the organism *Rattus norvegicus* (Rat). The origin of these proteins indicated their presence was not due to the cross-contamination of the trypsin enzyme used in in-gel digestion, which was extracted from the bovine pancreas.

Protein anionic trypsin-1 (Figure 1: protein spot 0104), anionic trypsin-2 (Figure 1: protein spot 1004), and protein spot 3101 (no hit) were upregulated in diabetes rats fed with ND compared to control rats fed with ND. Anionic trypsin-1 and anionic trypsin-2 are the precursors of the major form of trypsin enzymes produced by the adult pancreas. Upregulation of these precursors was related to protective response by the pancreas under disease and toxic conditions (Kukor *et al*., 2003; Wan *et al*., 2020). In contrast, the upregulation of anionic trypsin-2 was to induce pancreatic cell autodigestion that leads to pancreatitis *(Wynne et al*.,2019). On the other hand, upregulation of anionic trypsin-1 was also detected in the STZ-induced diabetic rat model. An earlier study on patients with pancreatic cancer showed a

3-fold elevation of circulating anionic trypsin-1 in blood serum (Borgström & Andrén-Sandberg, 1995). In this study. Our study detected significant upregulation of anionic trypsin-1 and 2 in the pancreas of diabetic rats, indicating their potential role in the pancreas's defense against inflammation induced by diabetes.

Two protein spots were detected upregulated significantly (p<0.05) in diabetic rats fed with HFD compared to diabetic rats fed with ND, these proteins were chymotrypsinogen B (Figure 2: protein spot 4801) and transgelin-3 (Figure 2: protein spot 6702). In contrast, no significant changes in protein expression in control rats fed with either HFD or ND were detected, indicating that HFD has a greater impact on diabetes rats' pancreas compared to the control rats.

Chymotrypsinogen B is involved in the digestion of nutrients and regulation of apoptotic processes. In this study, we observed the upregulation of chymotrypsinogen B in diabetes rats fed with HFD, this is in agreement with a previous study that showed upregulation of chymotrypsinogen B was detected in rats fed with a high-fat diet (Snook,1971). Besides, chymotrypsinogen B was also reported upregulated in rats with pancreatitis, where the inflammation of the pancreas was due to miss distribution of digestive enzymes (Sun *et al*., 2011; García-Hernández *et al*., 2012). Therefore, the detection of Chymotrypsinogen B in this study shows that HFD can induce pancreatic inflammation in diabetic rats.

Transgelin-3 is encoded by the TAGLN3 gene and also represents the human NP22 protein. This protein is homologous to transgelin and calponin. Due to the high similarity of amino acid sequences between these homologs, transgelin-3 is assumed to share similar biological characteristics with its homologs, for instance, their interaction with actin that induces actin gelation and regulates actin cytoskeleton. (Wu *et al*., 2021). Besides, transgelin-3 is described as a neuronal protein that is involved in neuronal cytoskeleton organization (Dvorakova *et al*., 2014). Upregulation of trangelin-3 was also detected in the liver of type 1 diabetic subjects (Braga *et al*., 2016). Upregulation of transgelin-3 in diabetes rats is likely induced by HFD.

In this study, we have observed the significant expression of proteins in diabetes rats related to the intake of HFD, nevertheless, due to the limited number of animals used, it can only serve as preliminary data, and a much more detailed study is needed to be conducted before the proteins can be used for the intervention of diabetes.

CONCLUSION

In this study, to investigate the impact of HFD on the pancreas of diabetic rats, we found that the proteome of the pancreas's tissue showed significant differential expression of certain pancreatic proteins between control rats and diabetic rats fed with ND or HFD. These proteins were identified as anionic trypsin-1 and anionic trypsin-2, the upregulation of these proteins indicated the inflammation of the pancreas of diabetic rats upon HFD intake. This is further supported by the upregulation of chymotrypsinogen B, a protein that indicates tissue inflammation, in diabetic rats fed with HFD compared to those fed with ND. On another hand, we also observed the upregulation of transgelin-3 in diabetic rats fed with HFD, where the reason for its upregulated expression is yet to be identified. Hence, these findings may warrant a further study to investigate the impact of HFD on diabetes.

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ETHICAL STATEMENT

This study was approved by the ethical committee of Universiti Sains Malaysia (USM/Animal Ethics Approval /2016/ (717)).

CONFLICT OF INTEREST

The authors declare there is no complicit interest in publishing this piece of work, moreover, the funder does not interfere with the publishing of this work.

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