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Proteomic study of serum proteins in a type 2 diabetes mellitus rat model by Chinese traditional medicine Tianqi Jiangtang Capsule administration

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ABSTRACT

Proteomics technology was for the first time applied to investigate the changes of serum proteins levels in type 2 diabetes mellitus (T2DM) rat model after treated by Chinese traditional medicine Tianqi Jiangtang Capsule (ten normal Wistar rats, ten with T2DM and ten with T2DM administrated by Tianqi Jiangtang Capsule). In addition to two-dimensional polyacrylamide gel electrophoresis (2-DE), serum protein profiling in the three groups was further performed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-TOF/MS). 11 visualized spots were differentially regulated and identified as diabetes-associated proteins. All the samples in three groups were then analyzed by ELISA and estimated the 7 proteins which were found to vary. The distinct effect of T2DM induction on the pattern of rat serum includes the down-regulation of Apolipoprotein E, Apolipoprotein A-I, Ig gamma-2A chain C region, and up-regulation of Transthyretin (TTR), Haptoglobin (Hp), Serum amyloid P-componen (SAP), Prothrombin. The majority of those protein levels were interestingly restored to those of healthy rats after Tianqi Jiangtang Capsule treatment.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the most prevalent chronic diseases in the world, which is characterized by insulin resistance coupled with a failure of pancreatic β cells to compensate by adequate insulin secretion. Because long-term medical attention is required to manage T2DM and limit the development of its devastating complication, researches have been carried out on human subjects with T2DM and a variety of animal models to understand the pathogenesis and develop of new treatments for the cure of diabetes [1,2]. Specific circulating proteins in serum from patients with T2DM have been shown to be altered when compared with healthy individuals [3]. Variation in protein levels can either be viewed as primary changes causing the disease or as secondary effects caused by the disease. It is important to monitor the expression of serum proteins simultaneously during T2DM occurrence.

Recently, blood-related proteomics is emerging and unravelling the plasma and serum proteome. The proteome analysis by two-dimensional polyacrylamide gel electrophoresis (2-DE) is one of the most potent methods of analyzing the complete proteome of organs and tissues in proteomics studies. It allows a fast overview of changes in cell processes by analysis of the entire protein extracts in any medical research projects [4]. Although protein profiling of blood has been successfully used in context of many diseases, researches mainly focused on the variation of proteins between normal subjects and diabetic models by proteomic approaches [5,6]. Only scarce research aimed to study the effect on protein levels by anti-diabetic agents from plants or traditional medicine, such as green tea [7] and Japanese traditional medicine Kampo [8].

In the present study, a Chinese traditional medicine, named Tianqi Jiangtang Capsule, was used as an anti-T2DM drug and orally administered to a T2DM rat model, which was induced by high fatty emulsion and alloxan. Besides biochemical parameters related with T2DM were measured, it is the first time to use proteomics approach to observe the serum proteins alterations in normal rats, rats with T2DM and T2DM rats treated by Tianqi Jiangtang Capsule.

2. Materials and methods

2.1. Herbal materials and preparation method

Tianqi Jiangtang Capsule was purchased from Heilongjiang Baoquan Pharmaceutical Limited Company (Hegang, China). The formula consisted of ten herbal components: Radix Astragali 400 g, Radix Giseng 134 g, Rhizoma Coptidis 200 g, Fructus Ligustri Lucidi 334 g, Radix Trichosanthis 400 g, Caulis Dendrobii 200 g, Cortex Lycii Radicis Bone 267 g, Asiatic Cornelian Cherry Fruit 200 g, Eclip-

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tae Hebra 334 g and Chinese gall 200 g. The former four components were mixed and extracted three times with 60% ethanol for 2, 1 and 1 h, respectively; other components were boiled three times in 2 l of distilled water for 2, 1 and 1 h under reflux system, respectively. The two parts of the aqueous extract were combined and filtered through a cotton cloth to remove solid debris. The filtrated was then centrifuged and filtered to remove undissolved residue. The filtrate was lyophilized into dry powder and packed into 1000 capsules with 0.32 g each pill.

2.2. Animals and experimental design

Wistar rat weighing 180–220 g were obtained from Good Laboratory Practice (GLP) experiment center, Heilongjiang University of Traditional Chinese Medicine and acclimatized for 1 week. The ethical approval for the experiment was followed by the Legislation on the Protection of Animals Used for Experimental Purposes (Directive 86/609/EEC). The animals were kept under standard laboratory conditions with temperature at $23 \pm 2\,^{\circ}C$ and relative humidity at $55 \pm 5\%$.

The induction of experimental diabetes was followed by the method of Ai et al. [9,10]. Briefly, animals were administered with high fat emulsion (10 ml/kg) for 10 days and injected alloxan (120 mg/kg) intraperitoneally at day 11 and day 12, respectively. At day 17, animals with fasting blood glucose levels greater than 16.67 mM were considered diabetic and then included in this study.

Normal and diabetic rats were randomly divided into 3 different groups of 10 animals each, named normal control group, type 2 diabetic mellitus (T2DM) group and treated group, respectively. The normal control group contained normal rats and received distilled water; the T2DM group contained diabetic rats and received distilled water; the therapeutic group contained diabetic rats and received Tianqi Jiangtang Capsule (1.7280 g/kg). The drug solution or vehicle was lavaged once daily in 20 days. Blood samples from rats were collected by orbital puncture at day 0 or day 20, and serum was prepared for proteomics study.

2.3. Sample preparation and two-dimensional electrophoresis

For the proteomic studies, 3 rats in each group were randomly selected. After 1 h of the treatment in each group on day 20, 5 ml blood each rat was collected by orbital puncture. After ultra centrifuged at $40{,}000 \times g$ at $4\,^{\circ}C$ for 10 min, the serum samples were extracted and stored at $-80\,^{\circ}C$. A 2D Clean Up Kit (GE Healthcare Bio-Science, NJ, USA) was used for total protein purification and quantification in various samples. Two-dimensional electrophoresis was performed as described by Gorg et al. [11]. Isoelectric focusing was carried out using IPG phor3 system (Bio-Rad, Hercules, CA, USA). Immobiline 3–10 linear DryStrips (GE Healthcare Bio-Science, USA) were run at 30 V for 8 h, 50 V 4 h, 100 V 1 h, 300 V 1 h, 500 V 1 h, 1000 V 1 h and 8000 V 12 h using rehydration buffer containing 8 M urea, 2% CHAPS, 20 mM DTT and 0.5% (v/v) IPG buffer (Amersha Pharmacia Biotech). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% polyacrylamide gels without a stacking gel in the ETTAN DALT SIX ELECT UNIT 230 (GE Healthcare Bio-Science, NJ, USA). Gels were stained with 0.04% (w/v) Coomassie Blue R-350 in 10% acetic acid and destained with 10% acetic acid. Gel images were scanned using M1600 Thunis Scanner (Beijing, China) and analyzed using Imagemaster 2D Platinum 6.0 software (GE Healthcare Bio-Science, NJ, USA). The protein spots that varied >1.5 fold change and were specific for therapeutic group compared with T2DM group or normal control group were manually labelled and considered for mass spectrometry (MS) analysis. Prediction of spot positions was performed using the pI/Mw program with reference to the Swiss-Prot database (http://ca.expasy.org/tool/pi-tool.html).

2.4. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-TOF/MS) for protein identification

Differential protein spots of interest were excised manually and washed 3 times with in 200 ml aliquots of 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile. In-gel digestion for peptide mass fingerprint (PMF) analysis and mass spectrometric analysis were following protocols of Gamble et al. [12] and Wang et al. [13]. Briefly, each protein spot was dried in a SpeedVac (Savant Instruments, Holbrook, NY) and dehydrated at $37\,^{\circ}C$ for 30 min in 10 ml digestion solution (Roche, Mannheim, Germany), the reaction was stopped by the addition of 5% trifluoroacetic acid (TFA) for 20 min. Trypsinized peptide samples were extracted by 20 ml 5% TFA for 1 h at $37\,^{\circ}C$ and then by 20 ml 2.5% TFA/50% acetonitrile for 1 h at $37\,^{\circ}C$. The combined supernatants were evaporated in SpeedVac again and dissolved in 4 ml 0.5% aqueous TFA for mass spectrometric identification. All mass spectra were obtained on an Ultraflex MALDI-TOF-TOF (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV with α-cyano-4-hydroxy cinnamic acid as the matrix. Spectra were calibrated internally using trypsin autolysis products. All PMFs obtained were used to search the NCBInr database using Mascot Daemon (Matrix Science, London, UK) as a client attached to the Mascot search protocol. The database searches had peptide mass tolerance set at approximately ±0.1 Da and one missed cleavage site.

2.5. Protein analysis by ELISA

Quantification of the proteins, which were identified and found to vary in the three groups by 2-DE and MALDI-TOF-TOF/MS, was done in all the blood samples from the three group by ELISA methods [14]. All assays were based on non-competitive sandwich ELISA method using commercially available kits purchased from R&D, USA.

3. Results and discussion

Before serum proteomic study, T2DM model was successfully built by the method of Ai et al. [10] in present study. The 2-DE and MALDI-TOF-TOF/MS were then used to observe and analyze serum protein profiles from Wistar rats with normal, rats with T2DM and T2DM rats treated by Chinese traditional medicine Tianqi Jiangtang Capsule. 11 protein spots with different concentrations were found in serum and identified as diabetes-related proteins; the level of the proteins which were identified and found to vary were further determined by ELISA.

3.1. Significant differences exist at the proteome level in serum of type 2 diabetes rat model treated by Tianqi Jiangtang Capsule

MALDI-TOF/MS technique with its high reproducibility and sensitivity and good separation capability has been used extensively in blood proteomic studies [15]. Although many circulating proteins in blood are connected to the pathogenesis of the disease in various ways, proteomic researches for diabetes mellitus paid attention mainly on protein profiling of pancreatic islets, β-cells [6]. For the sake of high-abundance serum albumin and tremendous heterogeneity of glycoproteins making detection of low-abundance proteins difficult, only scarce studies using blood from subjects with T2DM were done [16], not to mention the proteins in serum. In present study, MALDI-TOF-TOF/MS was used to profiling serum proteins and differential proteins among normal

Table 1
Identification of differentially expressed protein spots in plasma sample of control group, treated group and type II diabetes rat model group by MALDI-TOF/MS.

Spot no.	Protein name	Gene	Score	Mr (kDa)/pI	Accession no.	No. of matched peptides
1	Apolipoprotein E	Apoe	431	35.73/5.23	P02650	21
2	Apolipoprotein E	Apoe	481	35.73/5.23	P02650	25
3	Apolipoprotein A-I	Apoa1	579	30.04/5.52	P04639	19
4	Apolipoprotein A-I	Apoa1	421	30.04/5.52	P04639	18
5	Haptoglobin	Hp	99	338.54/6.10	P06866	5
6	Transthyretin	Tre	330	15.71/5.77	P02767	10
7	Serum amyloid P-component	Apcs	134	26.16/5.50	P23680	5
8	Serum amyloid P-component	Apcs	192	26.16/5.50	P23680	8
9	Ig γ-2A chain C region	Igg-2a	254	35.16/7.72	P20760	9
10	Ig γ-2A chain C region	Igg-2a	283	35.16/7.72	P20760	10
11	Prothrombin	F2	291	70.37/6.28	P18292	14

Table 2
Quantification of identified serum proteins after proteomics by ELISA assay in normal control group, type II diabetes rat model group and treated group.

Variable	Control group	T2DM group	Treated group
Apolipoprotein E (μg/ml)	0.82 ± 0.01	0.43 ± 0.03[a]	1.53 ± 0.04[b]
Apolipoprotein A-I (μg/ml)	6.81 ± 0.26	4.62 ± 0.24[a]	13.56 ± 0.49[b]
Haptoglobin (μg/ml)	5.88 ± 0.42	13.31 ± 0.36[a]	5.62 ± 0.10[b]
Transthyretin (μg/ml)	214.19 ± 8.03	154.66 ± 1.66[a]	48.64 ± 1.87[b]
Serum amyloid P-component (ng/ml)	24.86 ± 2.15	39.77 ± 2.02	18.97 ± 2.50[b]
Ig gamma-2A chain C region (g/l)	2.45 ± 0.18	1.36 ± 0.13[a]	5.12 ± 0.07[b]
Prothrombin (μg/ml)	10.16 ± 0.06	13.66 ± 0.06	9.74 ± 0.14[b]

[a] $P < 0.01$, T2DM baseline compared with control baseline.
[b] $P < 0.01$, Treated baseline compared with T2DM baseline.

rats, rats with T2DM and T2DM rats administrated by Chinese traditional medicine Tianqi Capsule were analyzed.

The protein profiles of serum in rats of normal control group, T2DM group or treated group were analyzed by 2-DE on 24 cm pH 3–10 IPG strips for the first dimension and 12.5% SDS-PAGE as the second dimension. Proteins were first detected by Coomassie Blue R-350 staining. As shown in Fig. 1, approximately 208, 228, 180 spots were observed from normal control group (Fig. 1A), T2DM group (Fig. 1B) and treated group (Fig. 1C), respectively. Triplicate analytical gels of each group were computationally combined into a representative standard gel. After analyzed with the software of 2D Imagemaster (ImageMaster 2D Platinum 6.0), more than 20 proteins spots showed their abundance change (Fig. 1) and 11 of them (numbered 1–11) were picked and excised for MALDI-TOF-TOF/MS identification (Table 1). According to Mascot search results, spots 1–2, spots 3–4, spots 7–8 and spots 9–10, respectively, corresponded to Apolipoprotein E (ApoE), Apolipoprotein A-I (ApoA1), Serum amyloid P-component (SAP) and Ig gamma-2A chain C region; spots 5, spots 6 and spots 11 were representing Haptoglobin (Hp), Transthyretin (TTR) and Prothrombin. The amounts of all these proteins spots in three groups were further quantified by ELISA and shown in Table 2.

3.2. Quantification analysis by ELISA

In order to determine the levels of 7 proteins as identified by MALDI-TOF-TOF/MS, ELISA assay was used to analyze the levels of these proteins in all the groups of rats. Compared with normal control group, the level of serum proteins ApoE (spots 1–2), ApoA1 (spots 3–4), TTR (spot 6) and Ig γ-2A chain C region (spot 9–10) were dropped significantly in rats of T2DM group (Table 2). However, after 20 days treatment with capsule Tianqi, the amount of these four proteins increased very significantly (Table 2). Several proteins, such as Hp (spot 5) and SAP (spot 7–8) were significantly decreased in treated group, whereas increased in T2DM group (Table 2).

These results were in accord with previous study that in type 2 diabetes mellitus, elevated serum triglyceride (TG) level and lowered high-density lipoprotein (HDL) cholesterol levels often occurred together, which will affect their ability to bind to ApoA1, the level of ApoA1 was decreased [17,18]. The reduced secretion of ApoE might be resulted by reduced level of HDL and ApoA1 [19]. Serum total γ-globulin is elevated in T2DM, which can be a prediction of the development of T2DM in Pima Indians [20] and the

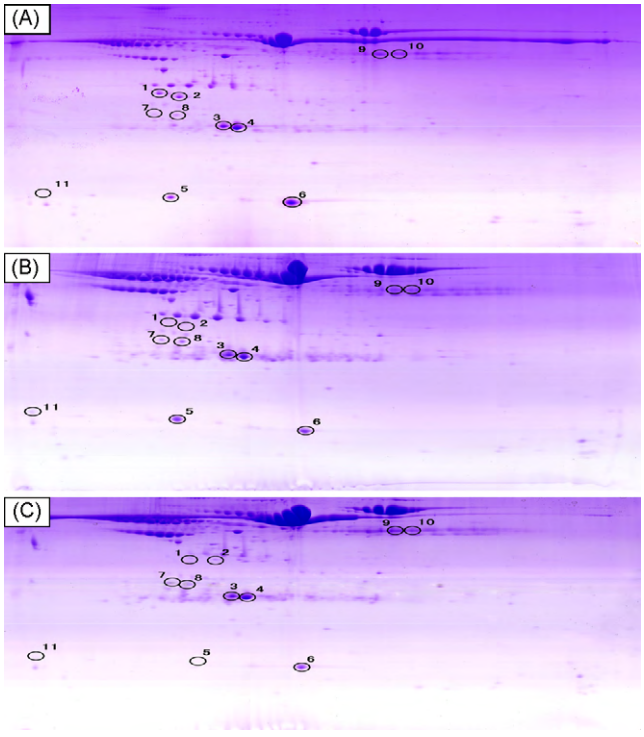

Fig. 1. Comparison of two-dimensional electrophoresis (2-DE) profiles of serum protein in normal control group, type II diabetes rat model group and treated group. Gel was stained with colloidal Coomassie blue. The rectangles highlight expression changes of a major group of storage proteins during germination. Reproducible results were obtained from three independent experiments. A = 2D-images of proteins from normal control group. B = 2D-images of proteins from T2MD group. C = 2D-images of proteins from treated group.

reason of this phenomenon is not clear. It is also reported that TTR levels were decreased in plasma from patients with T2DM, where TTR was an acute-phase protein and can be considered as a robust marker of disease [21]. A causal relation between islet amyloidosis and β-cells failure and insulin insufficiency in later stages of T2DM development might be explained the increased concentration of SAP in T2MD rats [22]. Many reports have established a strong association between Hp phenotypes and complications of diabetes [23].

With 20-day treatment of Tianqi Jiangtang Capsule, the levels ApoA1, ApoE and Ig γ-2A chain C region in serum increased and became 1.5 times higher than normal levels. These results might be due to the anti-hyperlipemia effect of composition of Tianqi Jiangtang Capsule, such as Radix Astragali, Rhizoma Coptidis, Cortex Lycii Radics and Fructus Ligustri Lucidi, all of which can significantly lower the level of blood glucose, TG and improve the metabolism of diabetic animals or humans [24]. However, it is unclear why the levels TTR did not increase after the treatment and further study is needed in this area. In our present study, the Hp level in T2DM rats treated by Tianqi Jiangtang Capsule showed a significant decrease; this might indicate that Tianqi Jiangtang Capsule can decrease the risk of other complications of diabetes development. However, as the functional properties of Hp are type-dependent, we could not know which phenotype of Hp has association with anti-diabetes effect of Tianqi Jiangtang Capsule and further study is needed. Intermittent hyperglycaemia has marked effects on increasing the circulating levels of prothrombin, and acute changes in plasma glucose concentrations may result in a thrombophilic condition as platelet adhesion is also enhanced by hyperglycaemia [3]. This explained the increasing levels of prothrombin in serum of T2DM rats in our study. The levels of prothrombin in rats with T2DM were depressed by administration of Tianqi Jiangtang Capsule.

4. Conclusions

Tianqi Jiangtang Capsule showed an anti-diabetic effect via reducing hyperglycaemia and modifying lipid metabolism. It is the first time to use proteomic method to analyze the differential serum proteins expressed in normal Wistar rats, rats with T2DM and T2DM rats treated by Chinese traditional medicine Tianqi Jiangtang Capsule. Levels of ApoA1, ApoE, Ig gamma-2A chain C region were all up-regulated in serum from T2DM rats after the treatment for 20 days. Levels of TTR, Hp, SAP and prothrombin were depressed in T2DM rats after treated by Tianqi Jiangtang Capsule for 20 days. It is concluded that these differences are probably manifestations of the serum proteins from T2DM rats in therapeutic state. Besides, the effect of Tianqi Jiangtang Capsule on serum TTR and Hp needs further study. As only scarce number of rats was evaluated in this preliminary study, the data require confirmation on a larger number of cases.

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