An inhibition effect on diabetic cardiomyopathy fibrosis of db/db mice through reduction of myocardial protein N-glycosylation by crude 1-DNJ extract from homemade *Bombyx Batryticatus* mori.L

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**ABSTRACT**

The Chinese drug *Bombyx Batryticatus* mori.L which also named as the white stiff silkworm is widely used in clinics, due to the significant antispasmodic and promotional blood circulation effects. In addition, its hypoglycemic effect is also recognized in recent years. From a pathological point of view, the enzymatic glycosylation and non-enzymatic glycation both have important roles in regulating properties of proteins and are associated with Diabetes. With the db/db mouse model, we examined the alterations of N-glycosylation of diabetic myocardium at primary stage and clarify the differences in glycosylation of myocardium before and after with 1-DNJ treatment. Hydrophilic chromatography solid phase extraction enrichment and LC-MS/MS identification was applied to profile the alternations in protein glycosylation. Meanwhile, N-glycan α1, 6-fucosylation alterations were profiled with LCA lectin blot and FITC-labelled lectin affinity histochemistry. Our results showed that AGES, hydroxyproline, CTGF and other serum indicators and fibrosis related cytokines expressional levels were reduced significantly by 1-DNJ in a dose-dependent manner. In order to verify this result, the well-known pathway of TGF-β/smad2/3 was picked out and α1, 6-core fucosylated TGFR- β II was semi-quantified with western blot method. The result sustained the conclusion from LCA lectin affinity histochemistry and lectin blot analysis. The expressional level of
α1, 6-fucosyltransferase mRNA was increased in the myocardium of db/db mice, however, the 1-DNJ administration did not show obvious inhibitory effect on FU8 expression. This unexpected result can be interpreted as 1-DNJ plays the roles by reducing the concentration of substrate rather than inhibiting α1, 6-fucose glycosyltransferase expression. Meanwhile, 1-DNJ crude extract from BBm with some flavonoids accompany can also play the roles of anti-oxidant, and all the chemicals protect the diabetic myocardium from hyperglycemia damage commonly.

Keywords: 1-DNJ, diabetic cardiomyopathy, fibrosis, N-glycosylation, α 1,6-Fucosylation.

1 Introduction

Diabetes mellitus (DM) is a kind of common endocrine and metabolic diseases. It is not only recognized as a long-term chronic plasma hyperglycemia but also accompanied a variety of deadly complications [1-2]. Within all the complications the cardiovascular complication is one of the most harmful on account of the highest morbidity and mortality [3-4]. Type 2 diabetes is associated with a cardiac syndrome called diabetic cardiomyopathy (DCM) which is characterized as left ventricular contractile dysfunction [5], cardiomyocyte hypertrophy [6], interstitial collagen accumulation[7] and fibrosis[8-9]. The pathogenesis of DCM is extremely complicated and its mechanism needs to be further studied. The signaling disturbance of cardiac insulin resistance has been elucidated [10]. Since glucose is involved in the signaling processes of myocardial structural and functional modelling [11], the heart is subjected to face various glucose-mediated challenges included suppression of glucose oxidation, glycogen accumulation and elevation of glycation levels of proteins [12].

Evidence suggests that non-enzymatic glycation of collagen in the myocardial interstitium and microvascular wall to form advanced glycation end products (AGEs) [13] and interacted with some specific receptors. Sustained hyperglycemia can promote the release of some cytokines and growth factors such as connective tissue growth factor (CTGF) and epidermal growth factor-β (TGF-β) [14]. The massive relaxation of CTGF in the course of long-term diabetes will cause a huge expression
of extracellular matrix (ECM) [15-16], which can promote cell hypertrophy and interstitial fibrosis, leading to the change of cardiac functions. Even more, TGF-β can also induce cell hypertrophy and mesangial matrix expansion through a series signaling reactions with receptors [17].

It has been showed that the hexosamine biosynthesis pathway (HBP) contributes to diabetic complications in many tissues of DM patients [18]. Glucose metabolism by the HBP leads to enzymatically regulated O-linked glycosylation to proteins, affecting their biological activities. Hyperglycemia-induced impairments in cardiomyocyte excitation-contraction coupling are associated with sustained elevated O-GlcNAc levels [19, 20]. Inevitably, high levels of glucose entering must be enzymatically driven HBP leading to excess of circulating N-acetylglucosamine (N-GlcNAc) [21]. Compared to O-glycosylation in which oligosaccharides are covalently linked to proteins via serine and threonine residues, the N-glycosidic linkages on Asn are primarily occurs on asparagine residues within the motif Asn-Xxx-Ser/Thr (Xxx is not Proline) [22].

In recent years, asparagine (N)-linked glycosylation has emerged as an essential indicator to evaluate the endocrine and metabolism situation of DM patients, and some N-glycoproteins have been the biomarkers in clinical diagnosis. Earlier, an increase in α1, 3-fucosylation of α1-acid glycoprotein (AGP) was found in the serum and urine of DM patients [23, 24]. Not only that, there was emerging evidence for a role of N-glycosylation in the pathogenesis of voltage-gated Ca²⁺ channels (VGCCs), such as the altered glycosylation of Cav3.2 may contribute to the enhanced T-type current in DRG neurons during diabetes[25]. Serum glycol-proteomic analysis indicated that compared with normal mice, the serum of db/db mice contained more N-glycoprotein with α-1, 6-Fucose glycan structures and the same result was also found in the 2 Diabetes patient serum [26]. Moreover, the well-known pathway of TGF-β/SMAd2/3 which related to cell hypertrophy and mesangial matrix expansion had been clarified that both of the TGF-β receptor and TGβ-Ⅱ receptor are N-glycoproteins, and they were modified with α-1, 6 core fucose. If this modification was blocked, the following steps of TGF-β signaling through the phosphorylation of
regulatory Smad2 and Smad3 (R-Smads) would also be terminated [27]. At present, TGF-β was verified as one of the molecular mediators involved in the progression of fibrosis in DCM [28].

All the studies mentioned above had clarified the most striking changes in the structure and amount of N-glycans targeting at some specific glycoproteins, and some findings provided important implications for the clinical treatment and new drugs’ development. However, the comprehensive N-linked glycosylated proteomic research was not performed yet. Besides, a number of reports highlight that the alterations of asparagine-linked glycosylation expression, but none was correlate with DMC. More serious is that the results were obtained in advanced stages of this disease, but less is known about the direct impact of hyperglycemia on glycan biosynthetic processes and site specific glyco-decoration of cellular proteins during the early onset of the disease. In addition, compared with the more comprehensive and deeply post translational modification research of O-glycosylation of DMC which covers epigenetic modification of gene transcription, signaling and proteins [29], the N-glycosylation research of DMC is relative less, so make a deep detection of differentiation of N-glycosylated proteome among the DMC tissues, normal tissues and the myocardium treated with some drugs is very necessary.

To date, there is no specific therapy available for the development of pre-diabetic cardiomyopathy. In the clinic, it is usually first to control and delay the development of the disease, and then to target at the improvements for every complication of DCM such as oxidative damage [30], arrhythmias [31], heart failures [32], and fibrosis [33]. In addition, the early symptoms of DCM were not obvious and the existed treatments methods have some limitations, and can’t be effective prevent its development timely. So there is an urgent need to find new drugs and ways that can effectively prevent and treat diabetic cardiomyopathy, aiming to further improve and enrich the treatment strategy of diabetic heart disease.

1-Deoxynojirimycin (1-DNJ) is a kind of piperidine alkaloids and its chemical name is (2R,3R,4R,5S)-2-Hydroxymethyl-piperidine-3,4,5-triol. This chemical is an alpha-glucosidase inhibitor with antiviral action. It has been reported that the N-linked
complex oligosaccharides formation could be inhibited by 1-DNJ [34].

In this study, we used the widely recognized and approved type 2 DM medical models of 5 weeks old db/db mice by gavage with the 1-DNJ extract with the purity of 33.3% for 6 weeks, depending on the N-glycosylation proteomics research methods, we verified that 1-DNJ can be used to reduce the blood glucose, inhibit the lipid metabolism and relieve cardiomyopathy and fibrosis caused by diabetes. Not only that, it is also verified that this chemical with the inhibition effect of complex N-glycan forming and deprivation the chance of α1,6-fucose to be linked on the glycan, although the inhibition was mild and there was no dose-dependent relationship. It is also verified that the TGF-β/SMAd2/3 pathway signaling was disturbed owing to the core fucose glycosylation of TGF-β/βI receptors was reduced. Unexpectedly, 1-DNJ has no obvious effect on the expression of α1, 6-fucosyltransferase (FUT8). The aim of this study was to reveal the 1-DNJ molecular protection mechanism of diabetic cardiomyopathy in mice and to find new drug targets for clinical therapeutics and drug development. Our works provided a new understanding of the 1-DNJ protection effect on DCM from hyperglycemia damage and fibrosis formation. All about this study provided new avenues for clinical treatment.

2 Materials and methods

2.1 Reagents and Drugs

_Bombyx Batryticatus_ mori. L. (Abbreviated as BBm) was prepared by our team. The insects of _Bombyx mori_.L were purchased from Sericulture Institute of Sichuan Academy of Agricultural Sciences (Sichuan, China), and the mulberry leaves were purchased from mulberry planting base in Huzhou City, Zhejiang Province. The fungus of _Beauveria bassiana_ was purchased from China General Microbiological Culture Collection Center (CGMCC). After the expansion culture of _Beauveria bassiana_, the generated spores were washed with physiological saline and sprayed on the surface of silkworms. The preparation was under the condition of 95% relative moisture and 26-28°C. The silkworms were infected the fungi and then became stiff until the bodies were wrapped with the white mycelium. The stiff silkworms were
picked out and dried in an electric blast drying oven at 30°C.

Trypsin, PNGaseF (PNGF) was from New England Biolabs (Ipswich, MA). Chemical reagents of methanol, acetonitrile, iodacetamide (IAA), 1, 4-dithiothreitol (DTT), trifluoroacetate (TFA), formic acid (FA) were obtained from Sigma-aldrich (Shanghai, China). Ammonium bicarbonate (ABC) and urea were from Bio Basic Inc. (Ontario, Canada). The standard substances of 1-DNJ, kaempferol, quercetin and rutin were purchased from Aladdine Reagents (Shanghai, China). Strong cation exchange resins and other chemicals as well as the reagents were from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Hydrophilic chromatography beads (5 μm, 120 Å) were from Agela Technologies (Tianjin, China). LCA-FITC reagent was from VETOR LABS (California, USA). Antibodies were purchased form Abcam (Massachusetts, USA).

2.2 1-DNJ extract preparation and purity detection

The drugs were smashed into powder and then were refluxed with distilled water (1:10, w/v) for 1 h. The filtrates were collected and the residues were refluxed in water (1:10, w/v) for another 0.5 h. Two batches of filtrates were combined. Afterwards, the extract was precipitated by adding same volume of ethanol, and the supernatant was obtained by centrifugation (5000rpm, 20min). The ethanol was reclaimed by rotary evaporator and the residual liquid was adsorbed by 732-H⁺ cation exchange resins. The resins were washed with deionized water for three times and the DNJ was eluted with 10 times column volume of 0.5 M ammonia spirit. The elution was lyophilized and the 1-DNJ was reconstituted with methanol. With the use of Evaporative Light Scattering Detector (ELSD) and LC-NH₂ column (4×250mm, 5μm), the detection was conducted with a system of acetonitrile and water (80 : 20) as mobile phase and the flow rate was 0.8 mL/min. The column temperature was 40°C and the vaporizer temperature was 100°C. The nitrogen flow rate was set as 2.3-2.5 SLPM. Other chemicals were determined with HPLC system with UV detector at 360 nm, the analysis column was ODS-C18 column (4.6×250mm, 5μm), mobile phase was consisted of 0.1% acetic acid (A) and acetonitrile (B) with the gradient elution mode as below: 0.01-20.00min, B: 30%-85%; 20.01-30.00min, B: 85%-85%; 30.01-35.00,
2.3 Animals and administration

5 weeks old C57BLKS/J and C57BLKS/db/db mice were bought from Laboratory Animal Center of Peking University (Beijing, China). Animals were maintained in a SPF lab with controlled temperature (23±2°C) and humidity (30-60%). Air ventilation frequency was 15 times per hour, and the air replacement was controlled as exhaust volume of 2000-2500 cubic meters per hour, meanwhile, the air flow rate was less than 0.25 meter per second. The noise in the lab was less than 30 decibels. The animals were cared in accordance with animal experiments welfare ethics review requirements of Hebei University and the number of the ethical code approval was 2018001. Every group of mice was consisted with 4 males and 4 females, during the feeding, animals ate and drank freely. C57-J mice of control group (Control) were treated with sterile water instead of drug orally, and db/db mice of low dose therapy group (DMTL) were treated with 1-DNJ 10.0 mg/kg orally. The db/db mice of high dose therapy group (DMTH) were treated with 1-DNJ in dose of 5.0 mg/kg. The model group (DM) was consisted with db/db mice treated with 0.2 mL sterile water. This administration was continued for 6 weeks.

2.4. Clinical biochemistry indicators determination and histopathology analysis

At the end of the experiment, all mice were anesthetized with pentobarbital sodium (120 mg/kg), and the bloods were collected from abdominal aortic. The serum was obtained by the centrifugation (3000 rpm, 15 min) and the key biochemical indicators included fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG) glycated hemoglobin (HbA1) and AGEs were assessed with ELISA kits (Beyotime, Beijing).

The cardiac was removed from the chest immediately accompanied with washes of precooled PBS. One half of the heart tissue was stored in liquid nitrogen and the other half was preserved in formalin solution. After fixation, the tissues were made into histopathological sections and these sections were stained with HE or FITC labelled LCA lectins (1:2000) respectively. The cardiac tissues saved in liquid nitrogen no more than 24 hours were taken out and smashed into powder which can be facilitate to
the subsequent extractions of proteins and total RNA.

2.5 Proteins extract and digestion

About 100mg frozen powder was used to extract the proteins with 500μL lysis buffer (10mM Tris-HCl with 4% SDS and 1% Sodium orthovanadate, pH=8.0). With the aid of sonicator, the proteins were extracted completely, and the concentration was determined with Bradford method [35]. A half of the extract was used for ELISA kits analysis, the rest was used for mass spectrometry based shot-gun proteomic analysis. About 500mg proteins were denatured with heating at 95°C for 10 min, and then was digested in to peptides with FASP method[36].

2.6 Glycosylated peptides enrichment and N-glycan resection

The glycosylated peptides were enriched with solid phase extraction with hydrophilic chromatography and the N-glycan was removed from the peptide with endoglycosidase PNGF. All the procedures were carried out just like the publication of our team as before [37]. After lyophilization, all the peptides were reconstituted with 0.5% FA and the concentration was detected with Nano-drop 2000 (Thermo Scientific, USA).

2.7 proteomic analysis with LC - MS/MS

About 700 ng peptides were analyzed by nano-LC–MS/MS. The samples were separated with Easy-1000 Nano-liquid phase systems, mobile phase A was 0.5% formic acid and the mobile phase B was the mixture of ACN and 0.5% FA at the volume portion of 98 to 2. The flow rate was 300 nl/min; B phase gradient for 60 minutes rose from 5% to 50 %; The Q-Exactive mass spectrometer (Thermo Scientific, USA), positive ion mode scanning; Scan range is 300-1400 m/z; A resolution of 7000; Secondary mass spectrometry (DDA) for the data dependence model, HCD fracture energy for 27; Select primary atlas 75 ion signal is strongest in secondary mass spectrum scanning; Time is set to the 18 s dynamic ruled out.

2.8 Database retrieval conditions and statistical analysis toolkits

Using Proteomic Discovery 1.3 series software to exchange the extension named as raw files to mgf files and using pFind 3.1.0 to search matched proteins through peptides sequence and modifications information in database of
Ref_mouse_20171201. Total protein database search condition was as bellows: the mass spectrum level accurate mass number deviation was 20 PPM or less, secondary search quality deviation was 15 mmu; For trypsin digestion type; Fixed modification was cysteine urea methylation and the variable modifiers were as bellows: methionine oxidation, protein N-term acetylation, asparagine deamination, the maximum allowed missing cleavage site number was 2. The analysis toolkits included pFind software (http://pfind.ict.ac.cn/), IBM-SPSS19.0, MeV-4.9.0, DAVID Functional Annotation, etc.

2.9 Lectin blot analysis

The samples with concentration detection were denatured at 95°C for 10min. Then about 20ul aliquots were separated with one-dimensional SDS-PAGE. The proteins were transferred on PVDF membrane under the condition of 120 volts for 60min in ice bath. The PVDF membranes were blocked with 5% BSA. During every wash step, the membrane was rinsed gently for 3 times. After that, the membrane was incubated with biotinylated LCA lectins at 4°C overnight. After three times washes, the membrane was developed with avidin -labeled DBA developing kit. Finally, the bands were quantified with Image-J analysis software.

2.10 Western-blot analysis

As the lectin blot procedures, the PVDF membranes were blocked with 5% BSA and then were incubated with primary antibodies overnight at 4°C. After 3 times cleaning, the membrane was incubated with horseradish peroxidase conjugated secondary antibody for an hour at room temperature for 1 h. After that, the PVDF membrane was cleaned for 3 times. The protein bands from four groups of samples were detected using an ECL kit (CWBIO, Beijing) and the proteins expressed were quantified using Image-J analysis software.

2.11 Real time PCR analysis of FUT-8

FUT-8 mRNA expressional quantification was performed with Real-time fluorescence quantitative PCR using the SYBR Primer Script RT-PCR Kit (Takara, Otsu, Shiga, Japan) according to the manufacturer’s protocol. The primers were synthesized by BGI Genomics (Shenzhen, China) as follows: FUT8, the forward
primer (from 5’ to 3’) was with the sequence as GCTACCGATGACCCTGCTTTG and the reverse primer sequence was CCGATTGTGTAATCCAGCTGAC. GAPDH, the forward primer sequence (from 5’ to 3’) was GCACCGTCAAGGCTGAGAAC and the reverse primer sequence was TGGTGAAGACGCCAGTGGA. The levels of gene expression were calculated with DDCt method after normalization to GAPDH and all samples were analyzed in triplicate.

3. Results

3.1 1-DNJ enrichment and purity testing

From Figure-1, we can see that the Chinese drug BBm produced by our team had a good appearance. The stiff silkworm bodies were wrapped with a lot of white mycelium (figure B). From 5.0 kg BBm, we obtained 231.55g solid material from 75% ethanol extract. Totally, there was 19.72g solid substance was eluted from 732 type strong cation exchanged resins column. The 1-DNJ was determined with HPLC and the content was 33.26% (figure C and D). Besides, some flavonoids such as rutin, quercetin and kaempferol were detected and the content was 2.8%, 6.5% and 3.5% respectively (figure E and F).

3.2 Effects of 1-DNJ on clinical biochemistry indicators of db/db mice

From Figure-2, we found it was interesting and surprised that each indicator’s expressional differences among these four mice groups were with the same tendency. In other words, TC, TG, FPG, HbA1 and AGEs in DM groups were expressed with the highest levels. After treated with 1-DNJ crude extracts, the levels of these indicators of DMTH group and DMTL group were both decreased significantly (P<0.05) compared with the model group, and the positive effects were related to the dose of 1-DNJ. This result showed that 1-DNJ can improve the metabolic status of db/db mice by inhibiting glucose absorption of glucose.

3.3 Myocardial histopathology examination

From the histologic sections, we can see that the left ventricular myocardium of DMTH group were not absolutely as clear and smooth as the control group, however, there were not any obvious hypertrophies or hyperplasia can be found in the cells. The myocardial tissue of DMTL group mice presented with some swellings and
hypertrophies, meanwhile, the edges of some cells were unclear and the nucleus also appeared shrinkages. DM group mice myocardium in the pathological sections exhibited obvious hypertrophy and hyperplasia. What was worse was that the ECM increase and thickening began to emerge. Besides, the DM group mice myocardium presented with some cell necrosis and interstitial fibrosis. This result was verified with GTGF and hydroxyproline quantitative expression. From Figure-3, we can see that compared with control group, the CTGF and hydroxyproline both upregulated significantly (P<0.01) in DM group. 1-DNJ administration exhibited obvious down regulating effect of CTGF (p<0.01) and hydroxyproline (p<0.01) which were both considered as the biomarkers related to hypertrophy, hyperplasia and fibrosis.

3.5 Site specific N- glycosylated proteome identification and motif analysis

In order to compare the N-glycosylated proteomic differences and improve the accuracy of identification, we extracted the intensity of N-glycosylated peptides based a Perl program combined with the official Xcalibur. The peptide with intensity as “null” was removed. After the correction the results became as follows, for the control group, 633 non-redundant N-glycosylated peptides with 664 sites were identified from 726 spectra which were corresponding to 392 protein groups. Similarly, 733 non-redundant N-glycosylated peptides with 762 sites from 851 spectra were identified as corresponding to 475 protein groups. For the DMTH group, 799 non-redundant N-glycosylated peptides with 832 sites were identified from 970 spectra, and they were corresponding to 475 protein groups. Notably, there were 839 non-redundant N-glycosylated peptides with 872 sites in 1630 spectra corresponding to 437 protein groups were identified of the DM group.

Venny 2.1.0 based overlap analysis revealed that only 127 identified N-linked proteins were co-expressed from these four groups. Subcellular location analysis exhibited that most of the N-glycosylated proteins were located in extracellular matrix, plasma membrane or membrane bonded organelle. At the meanwhile, there were 96 proteins with the molecular function annotated as “protein bindings”, such as Na⁺/K⁺ transporting polypeptide, CD48 antigen, Fc IgG low affinity receptor, cell adhesion molecular, activated leukocyte cell adhesion molecular, and so on. From Figure 4 we
could see the unique protein distribution of four groups of mice myocardial tissues. There were 41 unique proteins related to cell adhesion and metabolic process as well as differentiation in control group and there were 51 unique proteins related to cell growth, cell adhesion and metabolic process in DMTH group. There were 98 unique proteins related to glycoprotein metabolic process, carbohydrate derivative metabolic process and extracellular matrix organization of DMTL group and there were 163 unique proteins related to cell adhesion, angiogenesis, regulation of cell motility and regulation of cell migration in DM group.

We also made the Gene oncology analysis, the results indicated that the biological process was described with many aspects, according to the Benjamini & Hochberg corrected \( p \)-value, we chose the top 10 reliable classified aspects (Benjamini coefficient between \(1.3 \times 10^{-59}\) and \(1.6 \times 10^{-21}\)), such as biological adhesion, cell adhesion, locomotion, regulation of locomotion, regulation of cellular component and so on. The cellular component (Benjamini coefficient between \(1.5 \times 10^{-115}\) and \(1.3 \times 10^{-63}\)) were corresponding to the extracellular region part, extracellular vesicle, membrane bonded vesicle, cell surface intrinsic component membrane and so on. Similarly, the molecular function of these proteins (Benjamini coefficient between \(1.9 \times 10^{-27}\) and \(2.1 \times 10^{-12}\)) could be summarized as cell adhesion molecule binding, glycosaminoglycan binding, integrin binding, heparin binding, carbohydrate binding and so on.

Gene symbol cluster analysis in Figure-5 showed the relationship between DM group and the DMTL group was closer than any other two groups. In anticipation, the control group with the most distant relationship with others owing to the different genetic backgrounds. The network relationship analysis indicated that the identified N-glycosylated proteins subcellular locations were extracted as membrane part and extracellular region. This result hinted that the hyperglycemia status may promote the proteins of plasma membrane, interstitial cells and extracellular matrix N-glycosylated.

The N-glycosylation sites analysis was performed with iceLogo program (http://www.twosamplelogo.org/). When setting the parameters, we took asparagine as
the center, and then six amino acids were taken forward and backward respectively. The motif of amino acid sequence was of N-X-T of the N-glycosylation sites is greater than the presence of N-X-S. More importantly, the glycosylated site of control group was presented as N-X-T/S while the N-glycosylated sites of other three groups site with the amino acid sequences as N-X-T/S/C. This difference may be due to the different glycemia level and genetic background.

Consistent with the four group expressional trends of serum biochemical markers, some N-glycosylated proteomic expressions were down regulated by the intervention of 1-DNJ for 6 weeks. Compared with the model group, the profiles of DMTH group were deduced much more significant than the DMTL group. There were some glycosylated proteins presented in figure-6. It is noteworthy that these glycoproteins molecular functions were correlated to adhesion, hyperplasia and fibrosis, such as integrin β [33, 34], laminin [35], CD36, CD38 and Collagen VI alpha (GOL VI-α). These potential biomarkers expressional differences prompted us that the hyperglycemia and glucose handling obstacles were the internal factors for the triggers and promotions of myocardium structural and functional changes.

3.5 Lectin affinity fluorescence histochemical analysis

The N-glycan α1, 6-fucosylated profiling with FITC-labeled LCH lectin affinity histochemical analysis was presented in Figure 7. All the image of the sections showed that the extracellular and interstitial matrix was stained obviously. The glycoproteins with α-1, 6 fucose structures were mainly distributed in the myocardium cytoplasmic membrane and intracellular stoma as well as serous membranes in connective tissue. From the fluorescence intensity we can see that the proteomic expression with α1, 6-fucosylated glycan in DM group myocardium was upregulated significantly and the coronary artery walls were thickened obvious compared with the control group. Moreover, some wrinkled cardiomyocytes can be seen form the section. Similarly, the DMTL group also presented with strong fluorescence intensity of extracellular matrix, and the difference between control group and DMTL group was also very significant. The fluorescence of DMTH group was relative weak, and the cell morphology was relative normal compared with the control group.
3.6 Lectin-blot analysis and α1, 6-fucosyltransferase mRNA expressional quantification

Lectin blot image in Figure 7 indicated that α1,6-fucosylatioal level in DM, DMTH and DMTL group were all higher than the control group, however, the intergroup differences among these three groups were not so significant, especially in the low molecular weight region and high molecular weight region. During 40-55KD, the stained binds number of DMTH and DM group were both bigger than DMTL group and the grayscale values calculated by image J software indicated that, in this region, the expressed α1,6-fucosylated proteins were quantified as DMTH>DM>DMTL. Meanwhile, the results of mRNA quantified by Real-time fluorescence quantitative PCR showed that α1, 6-fucosyltransferase namely FUT-8 in db/db mice myocardium were more than C-57/J mice. Although the expressions of the two administered groups were slightly lower than that of the model group, the intergroup differences were not significant. Interestingly, the FUT-8 mRNA quantification result was consistent with the lectin blot profition.

3.7 TGF-β/SMAd2/3 pathway proteins profiled with western-blot analysis

The results of western blot analysis were shown in Figure-8. From the images and calculations, we can see the trends of proteins abundances of TGF-β/SMAd2/3 pathway expressed in these four groups were similar. Compared with the control group, the expressional levels of ALK-5 (namely TGFβ-Ⅱ), TGFRβ-Ⅱ, smad2/3 or P-smad2/3 in db/db mice myocardium were promoted significantly. 1-DNJ crude extract administration can inhibit the expressions of these proteins mentioned above, and compared with the DM group, the effects of high-dose treatment were much more significant. This result indicated that the 1-DNJ crude extracts can be used to inhibit myocardium hypertrophy, extracellular matrix expansion and myocardial fibrosis of DCM patients in clinical.
Figure-1 BBm preparation and 1-DNJ extract purity detection with HPLC

A: The subcultured standard strains of Beauveria bassiana. B: Infected silkworms became stiff and white. C: Liquid chromatogram of standard substance of 1-DNJ. D: Liquid chromatogram of 1-DNJ extracted form Bombyx Batryticatus. E: Several flavonoids were identified and assayed in 1-DNJ crude extracts by HPLC.
Figure-2 Box plot analysis the effects of 1-DNJ on some key clinical biochemical indicators of db/db mice.

A: Effect of 1-DNJ on serum total cholesterol of mice. B: Effect of 1-DNJ on fast plasma glucose of mice. C: Effect of 1-DNJ on serum triglyceride of mice. D: Effect of 1-DNJ on advanced glycation end products of mice. E: Effect of 1-DNJ on glycated hemoglobin of mice. All the above data comparing was used ANOVA analysis method (\( \bar{x} \pm S \)). VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05).
Figure 3: Left ventricular muscle histopathology and key biomarkers expression contradi distinction in myocardium of 4 groups of mice. A: Histopathological sections were stained with HE (10×40). B: Hydroxyproline expression measurement with ELISA kits (X ± S. VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05). C: CTCG expression measurement with ELISA kits (X ± S. VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05).
Figure-4 Basic bioinformatics analysis of N-glycosylation profiling of mice myocardial tissue based on hydrophilic chromatography enrichment and LC-MS/MS technology. A: Represented one spectrum of N-linked peptides total ion chromatogram (TIC) in this analysis. ①～⑤ represented oxonium ion fragmentation of N-Acetyl glucosamine (GlcNAc). B: Mass spectrometry based N-glycosylation matched results of four group sample obtained from database search (FDR<0.01). C: Overlap relationship of identified N-glycoproteins from these four groups. D: Cluster analysis of four groups N-glycosylation on peptide level.
Figure-5 Subcellular location, gene symbol cluster and N-glycosylation motif analysis.

A: Identified proteins with N-glycosylation subcellular locations. B: Cluster analysis of four groups proteins based the experssional amount and Gene symbol. C: Motives of the identified N-glycoproteins based Icelogo analysis.
Figure-6 The presentation of representative N-glycoproteins expressional differences that can be significantly down-regulated by 1-DNJ.

A-D: CD38, CD36, COL-α1, COL-α3, LAM-α2, LAM-γ1, ITG-β1, ITG-β2 expressional quantification based on the peptide MS2 intensity analysis corresponding to A1, A2, B1, B2, C1, C2, D1, D2. (X ± S. VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05).
Figure-7 Lectin affinity fluorescence histochemical analysis of cardiomyocytes proteomic N-glycosylational characterization.  

A: FITC labeled LCA lectin affinity fluorescence histopathology sections image.  

B: LCA lectin blot analysis.  

C: FUT-8 mRNA expressional measurement with Elisa kits (X ± S. VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05).
Figure 8: TGF-β/SMAd2/3 pathway protein expressional semi-quantitative detection with western-blot of four groups of db/db mice myocardium.

A represented the images of western-blot analysis which were developed with ECL kits and films. B to E represented the semi-quantitative values comparison of four groups with ANOVA analysis of three independent measurements; The values were obtained with VS GAPDH. (X±S. VS Control: ** represented P<0.01; * represented P<0.05; VS DM: ## represented P<0.01; # represented P<0.05).

4 Discussion

DCM refers to myocardial disease that occurs in DM and can’t be explained by hypertension, coronary heart disease, valvular heart disease, and heart disease. Compared with non-diabetic heart disease, the diabetic coronary atherosclerosis usually finally resulted in hypertrophy and transmural myocardial infarctions [38, 39]. From the therapeutic point of view, once the coronary atherosclerosis happened, the prognosis would be bad and the mortality would be enhanced. Although most of DCM patients were very stable in the long-term early stage, some patients died soon in the...
later stage. So far, there are seldom effective therapies for the diabetic cardiomyopathy. At present, the clinical treatment is to control the blood glucose level firstly, then the treatments for complications such as myocardial damage, cardiac arrhythmias, heart failure and antithrombotic will be conducted in the process.

Compared with other chemical drugs, the nature compound of 1-DNJ is easily to be obtained. Many plants in nature can synthesize 1-DNJ and the content of 1-DNJ in mulberry leaves is relatively high. More importantly, mulberry, as economic plants of traditional agricultural representatives, the leaves are extremely accessible. Not only that, many mulberry eating insects were rich in DNJ, such as silkworms. The enrichment effect of silkworms is so significant that with the same weight, the content of DNJ in silkworms is 3-4 times higher than mulberry leaves. Unlike silkworm, BBm was the product full with fungal fermentation. The active ingredients in BBm are more easily to be absorbed in clinic. Moreover, some fungal metabolites become the new active ingredients which could play more extensive clinical roles. The mechanism of hypoglycemic effect of 1-DNJ was that this chemical possessed $\alpha$-glucosidase activity inhibition. The carbohydrates in food were hydrolyzed into sucrose or maltose and when these disaccharides are going to be hydrolyzed into glucose by $\alpha$-glucosidase the chemical reaction was hindered by 1-DNJ. The mechanism is that 1-DNJ has a greater affinity to glucosidase than disaccharides. Since the disaccharides can’t be hydrolyzed into glycose which can be absorbed by intestinal epithelial cells the disaccharides were fed directly into the large intensity [40,41]. So, 1-DNJ can be used to treat diabetes and diabetic complications, obesity and related disorders.

The reason we chose 5-week-old db/db mice as study subjects was that we are more concerned with the changes in biochemical markers in early stage and the post-translational modification status differences of early myocardial proteins. From fifth to tenth week, the mice body weights and blood glucose values were detected every week, in contrast, other important serum biochemical indicators determinations were conducted before the end of experiments. All the test results showed that the 1-DNJ crude extract administration did have significant therapeutic effects especially
for the high dose group.

Histopathological analysis suggest that in the primary stage of diabetes, db/db mouse myocardium changes in physiological structure were not obvious, this phenomenon was consistent with the clinical practice, in another words, the significant changes of myocardium structure will not take place until the patient's cardiovascular function declined to a certain extent. This conclusion was verified by hyperplasia and fibrosis related cytokines of CTGF [42] and hydroxyproline [43] expressional quantification with ELISA analysis.

The left ventricular myocardium glycosylated proteomics profile showed that there was a difference about the amount of N-glycoprotein collected and identified in different groups of samples. First, the number of identified protein groups of DM group was not the highest among these four groups, although at the peptide and glycosylation sites level the number of DM group was biggest. Second, 1-DNJ did have the inhibit effect on myocardial N-glycosylation in db/db mice, and the effect was positively correlated with dose administration. Third, the overlap of proteomic profiles in the four groups was very small, and this suggested that our experimental research methods were still with some bias.

In this study, we applied the hydrophilic chromatography base solid phase extraction approach combined with LC–MS/MS analysis with collision induced dissociation (CID) and high-energy collision dissociation (HCD) for both the mapping of N-glycosites and characterization of site-specific N-glycosylation. Totally 1241 N-glycosylation sites from 816 non-redundant protein groups were identified. Most of these glycosylated proteins were with subcellular location at membrane or extracellular matrix, which provided a solution for site-specific N-glycosylation characterization of myocardium of mice. IceLogo analysis showed that based on the difference in gene background, the myocardium protein N-glycosylation motif of C57-J mice showed as N-X-S/T while the N-glycosylation motif of db/db mice in the tenth week showed as N-X-S/T/C.

*Lens culinaris agglutinin* (LCA) lectin was with the specific recognition to fucose α 1-6 acetylglucosamine (GlcNAc) structure, especially to the core fucose α1-6 GlcNAc.
structure, the affinity was more than three times than the former [44].

Alpha-(1,6)-fucosyltransferase is an enzyme that is encoded by the FUT8 gene. To examine the alteration of myocardium N-glycans in type 2 diabetes, the lectin affinity fluorescence histopathology and lectin blot analysis were conducted in our experiments. In order to profile the myocardium α1,6-fucose glycosylation of these four groups, we used real-time fluorescence quantitative PCR to profile the FUT-8 mRNA expression. The level of α1, 6-fucosyltransferase mRNA was increased in the myocardium of the db/db mice. Although the expressions of the 1-DNJ administered groups were slightly lower than that of the model group, the intergroup differences were not significant. In order to verify the effect of 1-DNJ on inhibition of α1, 6-fucose glycosylation, the core fucosylated TGFR-β II expression in the well-known pathway of TGF-β/SMAd2/3 was picked out and presented with western-blot method, and the results proved the accuracy and authenticity of LCA lectin affinity histopathology and lectin blot analysis. Interestingly, the FUT-8 mRNA quantification result was consistent with the lectin blot presentation. Based on the above results we can conclude that 1-DNJ administration with little effect on FUT-8 expression. However, the FITC labelled LCA lectin affinity histopathology showed that the fluorescence intensity of DMTH group was weaker compared with DM and DMTL group. The first explanation was the anti-oxidation and inhibiting liver P450 enzyme activity effects [45] of flavonoids such as quercetin and kaempferol in the high-dose group resulted low expression of proteoglycans or glycolipidssome, and the lectin non-specific adsorption of these polymers was reduced. The second explanation was the indirect synergistic effect that the substrate concentrations of glycosyl ligands in myocardium were inhibited by high doses of 1-DNJ administration. In addition, N-linked glycan synthesis starts at the endoplasmic reticulum and ends in Golgi apparatus [46]. The biological process can be summarized as the followed: At the beginning, the dolichol-bound oligosaccharide precursor Glc3Man9GlcNAc2 was transferred to Asn residues on nascent polypeptides by the oligosaccharyltransferase (ost) and then the oligosaccharide undergoes trimming of the glucose and of some of the mannose residues, first in the ER and then in the Golgi, followed by the addition
of branching N-acetylglucosamine and additional sugars, such as galactose, fucose and sialic acid, by Golgi glycosyltransferases to form hybrid and complex N-glycans. It has been reported that the N-linked complex oligosaccharides formation could be inhibited by 1-DNJ [47]. Due to the α-glucosidase inhibitor effect, the formation of Glc3Man9GlcNAc2-PP-dolichol in intestinal epithelial cells in culture was greatly reduced by 1-DNJ. Moreover, this reagent capable of inducing misfolding of nascent glycoproteins and inhibitor the calnexin rescue cycle, and finally resulted in glycoproteins degradation. So 1-DNJ possess the ability of N-glycosylation disruptive effect that to a certain extent. Not only that, the flavonoids in the crude extract can also be played the roles of anti-oxidant and anti-inflammatory, and all the chemicals protect the diabetic myocardium from hyperglycemia damage commonly.

5 Conclusion

At the primary stage, there were no significant pathological changes in cardiac myocytes and extracellular matrix of db/db mice, but the expressions of cytokines related to cell hypertrophy and hyperplasia were up-regulated. 1-DNJ crude extract of BBm delays glucose production by inhibiting glucosidase activity to interrupt the process that the glucose-mediated attack on the replacement collagen deposited in the diabetic heart providing structural substrate for extracellular AGE formation in the hyperglycemic disease state. Besides, 1-DNJ can effectively reduce the N-linked glycosylation level of myocardium proteins and improve cardiomyocyte extracellular matrix thickening. The α-1, 6 fucose glycosylation levels of db/db mice myocardium were also reduced by 1-DNJ, and this chemical may play the roles by reducing the concentration of substrate rather than inhibiting α1, 6-fucose glycosyltransferase expression.

Conflicts of Interest

There are no conflicts of interest.

Acknowledgments
The authors express their appreciation to all participants of this study. This work was supported by funds from “Hebei Province Natural Science Fund Project”, grant number: H2014201042. This work was also supported by funds from Sub-topics of “Public welfare scientific research project of the State Administration of Traditional Chinese Medicine of People's Republic of China” (201507004), grant number: ZYBZHI-C-JL-24-01.

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