Can Silkworm be Used as a Diabetic Foot Animal Model?

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Keywords: Low-Cost Animal Models, Diabetic Foot Ulcers, Silkworm, Proteomics, Signaling Pathways

Abstract. It has been reported that the hyperlipidemic silkworms are useful for evaluating the hypoglycemic activities of candidate drugs against type II diabetes. At present, the accepted simulation method is the combination of high-fat diet and streptozotocin, which is the surgical removal of foot skin induced by common foot ulcer models in mice. Is it possible to use silkworm to establish diabetic foot animal model in combination with the above methods? We found that it can cause skin necrosis of the feet by pierce silkworm feet with surgical needles. This necrosis can be aggravated by further feeding with high sugar diet. After G.D (feed contains 7.5% glucose) treatment, 26.88% of the patients in the high sugar control group and 10% in the N.D (Feed does not contain glucose) group. In order to further understand the mechanism of skin necrosis of foot induced by glucose healing, Lable-free quantitative proteomics analysis is used to detect differential protein between G.D groups and N.D groups. Glucose significantly increased the risk of diabetic foot in silkworm, which was 2.5 times higher than that in blank control group. Through proteomic analysis, the mechanism may be through the effects of glucose induced high expression of carbonic anhydrase 7 (CA7), which blocking the binding of TGF-beta to receptors, slowed down the healing of silkworm foot skin. This will provide a low-cost animal model for screening diabetic foot drugs or therapies, and will be conducive to overcoming diabetic foot.

1. Introduction

Diabetic foot is a chronic complications of serious diabetes mellitus which is characteristic of high cost of treatment, and it is also the first cause of amputation in many countries. About 60,000 diabetic patients are amputated each year because of foot ulcer [1]. It costs more than $25 billion a year to treat chronic refractory wounds in the United States [2]. Therefore, the search for new targets and strategies for the treatment of diabetic foot is not only a global challenge, but also a major event related to the protection of limited medical resources and social development [3]. At present, scholars have built several skin wound model of breed animals [4], such as domestic dogs, miniature pigs, rabbits, etc., is the most commonly used mouse model [5-6]. At present, the most widely used is the surgical removal of abdominal skin in mice as an ideal trauma model.

Is there an animal with a lower price, faster time, similar to the human body, no ethics and other problems of the diabetic foot model? The answer is yes, silkworm can be used as a diabetic foot model. Compared with the above animals, Bombyx mori has a particularly obvious advantage, the first is cheap, the cost of modeling is greatly reduced, the price of a single silkworm is cheap and each silkworm has a wide range of options, compared with the above animals, it also has an absolute advantage in quantity. Second, the silkworm is very suitable for making silkworm diabetic foot model, because it is about 8 days from molting to callon at the fourth instar, and the whole silkworm skin area increases. It's 27 times bigger. Third, silkworm has a similar human tissue and

organ fat body, equivalent to the function of the liver. Fourth, there is no ethical problem, Bombyx mori belongs to invertebrates [7-9].

In this paper, proteome Lable-free method [10] was used to analyze the protein difference between blank group and diabetic foot group, the possible signal pathway was found out by database search, and then the targeted proteome was detected by PRM technique. To find out which signal pathways are caused by the diabetic foot model of Bombyx mori, and whether these pathways are consistent with the etiology of diabetic foot in human beings. If there is a similar prescription, it may provide a low-cost animal screening model for the treatment of diabetes mellitus.

2. Materials and Methods

2.1 Sample Collection Treatment

The silkworm is divided into two groups, 120 in each group. The blank group is given ordinary mulberry leaves N.D, the diabetic model was established by spraying mulberry leaves with glucose solution in the glucose group G.D. After spraying mulberry leaves with glucose solution for 12 hours, the tissue glucose increased and the model was established. On the first day of the 5th instar silkworm foot puncture, the leg wound was formed in both groups, resulting in diabetic foot infection [11-12]. Tissue glucose was measured every 12 hours. After the experiment, the number of diabetic foot and rotting foot in the two groups was counted.

2.2 Protein Extraction and Peptide Enzymolysis

The protein was extracted from the skin of Bombyx mori foot by SDT (4% (w/v SDS, 100mMTris/HClpH7.6,0.1MDTT) lytic method, and then the protein was quantified by BCA method.

2.3 Lc-Ms/Ms Data Acquisition Using Q-Exactive Mass Spectrometer

Each sample was separated by HPLC liquid phase system Easy nLC. The sample was analyzed by analytical column (Thermo scientific EASY column,10cm, ID 75 μm, 3 μ m, C 18 - A 2) was separated, and the flow rate was 300 nL/ min. The samples were separated by chromatography and analyzed by Q-Exactive mass spectrometry [13].

2.4 Protein Clustering Analysis and Protein Interaction Network Analysis

Find the interaction between target proteins based on the information in the STRING (http://string-db.org/) database and use C YtoScape software (version number: 3.2.1) generates and analyzes the interaction network.

2.5 Parallel Reaction Monitoring (Prm) Analysis

The samples separated by HPLC were analyzed by PRM mass spectrometry with Q-Exactive HF mass spectrometer (Thermo Science).

3. Results

3.1 Establishment of Diabetic Foot Model of Silkworm

Fig. 1B shows that there is a great difference between the blank group and the high glucose group. the blood glucose value of the high glucose group is more than 10.0mmol/L, we think that the establishment of the diabetes model is successful. The protein molecular weight distribution map of each group was preliminarily analyzed by electrophoresis. As shown in Fig. 1C, the protein molecular weight of diabetic group was 45KD, 65KD and 116KD, which was significantly higher than that of blank group. This is related to Japanese scholars' report that JNK protein is highly expressed in type 2 diabetes model established by feeding silkworm, and the molecular weight of JNK protein is in 46KD to 54KD [7,8]. The JNK protein can be clearly seen by figure 1C

electrophoresis. The concentration was much higher than that of the blank group. It was further found that the protein was numbered Q0N2R8. The molecular weight of 20KD was significantly lower than that of the blank group.

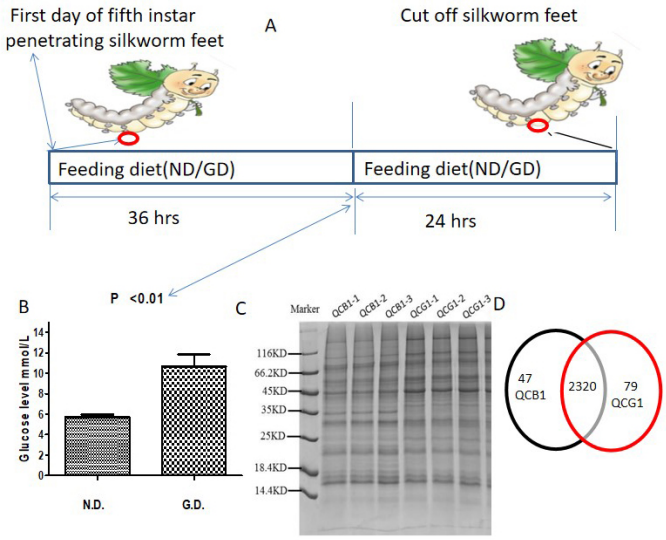

Figure 1 General Workflow of Diabetic Foot Ulcer Model

(Note: A: Establishment of animal model B: Changes in tissue fluid glucose of silkworm in blank group and glucose group. C: SDS-PAGE electrophoresis pattern of each sample (loading amount 300μl). QCB1 represents the first blank group, QCG1 represents the glucose group, and the following 1, 2 and 3 represent three parallel samples fo r detection. D: Summary of Protein Identification Results)

The skin of Bombyx mori feet was taken and the protein differentiation was analyzed by Lable-free method. A total of 2446 proteins were identified in this project. Compared with the blank group, 66 proteins with big difference were obtained in the high glucose group, including 28 proteins with more than 2.0 times up regulation and 38 proteins with a down regulation of less than 0.5 times.

3.2 Verification of Diabetic Foot Model of Silkworm

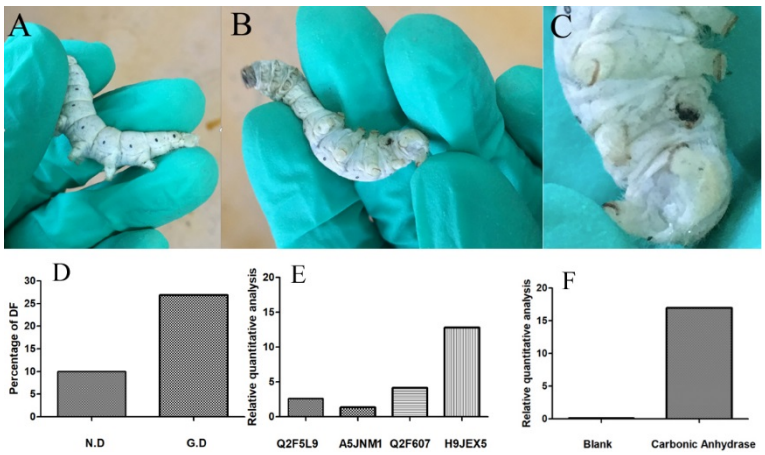

Figure 2 Confirmation and Analysis of Diabetic Foot in Silkworm

(Note: A: The skin healing of silkworm foot can be observed after puncture. B: The necrosis caused by the skin puncture of diabetic foot. C: shows the enlarged picture of necrosis. D: The percentage of diabetic foot in normal group and gluoces group. E: Differential protein by Parallel Reaction Monitoring. F: Major differences in enzymes)

Fig. 2A is a normal diabetic foot trauma model. The skin healing of silkworm foot can be observed that healing condition is good by puncture. Fig. 2B shows the necrosis caused by diabetic foot skin puncture, and fig. 2C shows the magnification of necrosis. Fig. 2D shows that the

proportion of foot necrosis in the group with diabetes mellitus was significantly higher than that in the blank group, and the proportion of the necrosis was significantly lower than the high glucose group, which was close to that in the blank group. Fig. 2E is a parallel reaction monitoring and verification of the target protein after Lable-free analysis [14,15], combined with diabetic foot signal pathway investigation, and it was found that the contents of Q2F5L9, A5JNM1, Q2F607, H9JEX5 in high glucose histone were significantly higher than those in blank group. Fig. 2F by classifying the above differential proteins, it was found that carbonic anhydrase was the most significant difference between the high glucose group and the blank group.

3.3 Functional Analysis of Differential Proteins in Silkworm Diabetic Foot Model

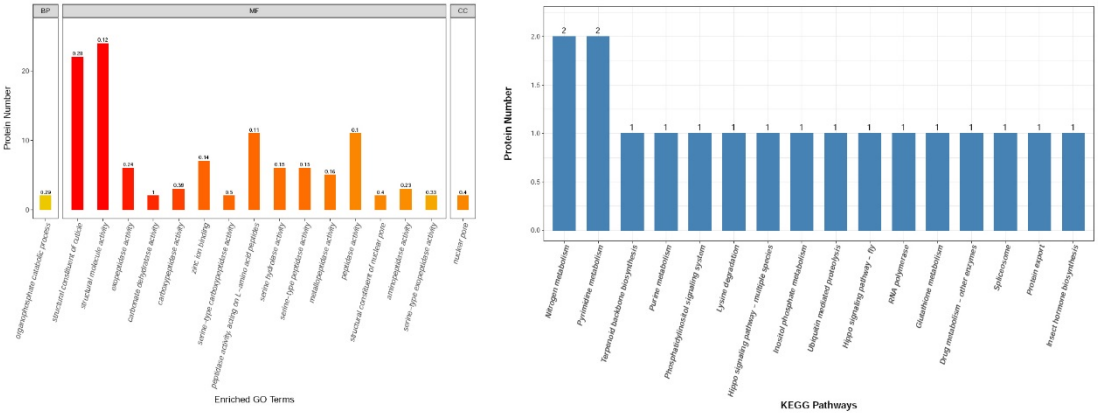

Figure 3 Enriched Go Terms and Kegg Pathway Analysis

As shown in Fig. 3, the results of analysis showed that these differential proteins were mainly involved in metabolic process, immune system process, cell process, etc. Among which metabolic process, cell process, stimulation reaction process and localization process were more obvious. The results of analysis showed that the molecular functions of organophosphate catabolic process and other important biological processes, structural molecule activity, exopeptidase activity,carbonate dehydratase activity and carboxypeptidase activity in QCG1 and QCB1 changed significantly[14]. The cell composition was analyzed, and it was found that the cell, extracellular region, cell membrane, cell composition and membrane composition had significant changes. The rate is more than 5%.

Pathway analysis is the most direct and necessary way to understand the biological process of cells, the occurrence mechanism of traits or diseases, the mechanism of drug action and so on. The abscissa in Fig.3 represents the functional classification of enriched GO, which is divided into biological processes (Biological Process, BP), molecular functional (Molecular Function, MF) and cell components (Cellular Component, CC) three categories; ordinate represents the number of differential proteins under each functional category. The KEGG pathway enrichment of differentially expressed proteins in QCG1_vs_QCB1 of the comparison group was analyzed by Fisher accurate test. The results showed that there were significant changes in Nitrogen metabolism and other important pathways [15]. The main protein that affects this channel is Fig. 1E, which namely involves protein carbonic anhydrase.

3.4 The Schematic Graph Reflects Possible Signaling Mechanisms in the Normal and Diabetic Wounds.

It was found that the big difference between high glucose group and blank group was carbonic anhydrase 7. The concentration of high glucose group was 12.28 times higher than that of blank group (Fig. 2E, PRM quantitative determination). The total carbonic anhydrase (including Q2F607 and H9JEX5) was 16.44 times higher than that of blank group (Fig. 2F). Scholars have reported that carbonic anhydrase inhibitors have been used to treat gastric ulcers for many years and have worked well [16-17]. Ulcer is the limited defect and ulceration of skin or mucous membrane surface tissue. The stomach ulceration is mainly due to the defect of the gastric mucosa and the ulceration, and the

diabetic foot is due to skin defect and festering, these two have very similar causes. It was found that glucose induced the increase of carbonic anhydrase surface in the skin of Bombyx mori foot, while the content of carbonic anhydrase in the skin of silkworm foot in the blank group was relatively low, which indicated that the inhibitor of carbonic anhydrase might be used to treat diabetic foot of Bombyx mori.

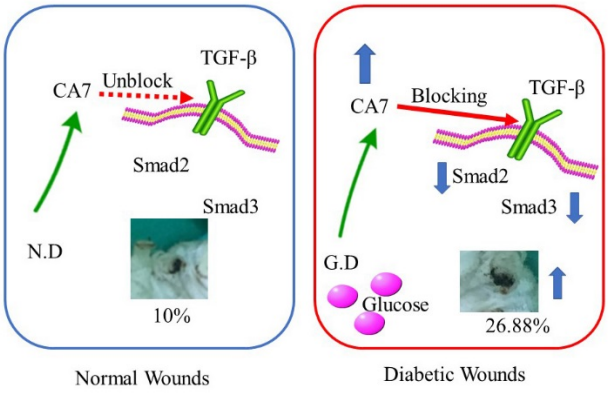

Figure 4 The Schematic Graph Reflects Possible Signaling Mechanisms in the Normal and Diabetic Wounds.

4. Discussion

The repair and healing of diabetic foot ulcers is a complex and orderly biochemical process mediated by many cytokines and involving a variety of cells [18], which mainly includes inflammatory reaction phase, proliferation phase and remodeling stage. This study Combining the above two methods, the diabetic foot model was established by Bombyx. It was found that the probability of diabetic foot necrosis induced by high glucose was more than 2.5 times higher than that in the blank group. In order to study the mechanism of foot necrosis induced by high glucose, the proteome method was used to analyze the protein difference between the high glucose group and the blank group, and a total of 126 differential proteins were obtained. The hierarchical clustering algorithm, GO function and KEGG pathway analysis of these differential proteins were carried out. It was found that the potential signal pathways of high glucose induced Bombyx foot ulcer were as follows.

High glucose can induce the metabolism of nitrogen in the tissue of the foot of the silkworm, thus delaying the healing of the skin. The metabolic pathway of nitrogen is an important influence symbol of the Diabetic foot [15]. NO is released under the stimulation of various media in the inflammatory reaction, and the NO is also an important potential adjustment factor after the burn, and the NO content after the burn is significantly reduced. In this paper, the expression of the related protein of nitrogen metabolism in the skin of the foot of the silkworm was confirmed, and the expression of this protein was consistent with the nitrogen metabolism of the wound tissues before and after the burn. The quantity of the protein Q2F607 and H9JEX5 of the silkworm foot which is fed by using a surgical intervention and high sugar feeding was significantly higher than that of the blank group. Both protein Q2F607 and H9JEX5 belong to carbonic anhydrase, which can provide an acidic environment, that is, when their expression is up-regulated, the content of NO in the skin of silkworm feet is significantly decreased [24]. When the mouse model was used, the expression of H9JEX5, belonging to protein Carbonic Anhydrase7 was also up-regulated on the seventh day after breakage, which was consistent with the results of silkworm animal model.

It is found that transforming growth factor- β (TGF- β) is a multifunctional cytokine in wound repair [19]. It is considered to play a key role in wound repair. During the repair of diabetic wounds, it was found that the signal expression of TGF- β 1 in chronic refractory wounds was irregular, the function was abnormal and the number was decreased, and the expression of VEGF β 1 was significantly decreased, especially in chronic refractory wounds. It is TGF- β RI, which loses the ability to respond to TGF- β 1 and delays wound healing [19].

If the up-regulation of carbonic anhydrase expression happens to block the binding of TGF- β to its receptors R1 and R2, it affects the TGF-β1/Smad3 signal transduction pathway [20]. It was confirmed that TGF- β 1/Smad3 signal transduction pathway was involved in wound healing and showed transient and rapid activation after injury. Smad3 played a partial negative regulatory effect on the promoting function of TGF- β 1, and Smad3 inhibited the self-positive feedback of TGF- β 1. This coincides with the experimental fact that glucose leads to an increase in the incidence of foot ulcers in silkworm.

High glucose can induce the metabolism of pyrimidine and other enzymes in the skin of Bombyx. The content of protein H9JKV3 in the foot of Bombyx which is carried out surgical and fed with high sugar diet was significantly higher than that in the blank group. Pyrimidines may also have therapeutic potential in wound healing, as ATP and UTP have been shown to have many characteristics of wound healing factors. Pyrimidines are involved in the proliferation of keratinocytes, and keratinocytes cooperate with growth factors (such as pdgf, TGF alpha and egf) that stimulate wound healing [21]. Protein H9JKV3 is involved in the high expression of deoxyuridine 5' triphosphate nucleotide hydrolase, but the relationship between the enzyme and skin healing of diabetic foot has not been reported. This group will continue this research in the future research.

In summary, this study provides a low-cost animal model for testing drugs or therapies for diabetic foot. Glucose significantly increased the risk of diabetic foot in silkworm, which was 2.5 times higher than that in blank control group. Through proteomic analysis, the mechanism may be through the effects of nitrogen and pyrimidine metabolism signaling pathways. However, this study lacks a comprehensive analysis of up-regulated, down-regulated proteins, but only analyzes the reported databases currently and finds that it may affect the signal pathway. In the future, our group will conduct further in-depth researches. Improving the animal model as soon as possible to contribute to the eradication of diabetic foot.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81774327, 81660145). We thank Drs. Zan Zhicheng and Shanghai Applied Protein Technology for their detection and discussions on the work.

References

[1] M.S. Pinzur, M.P. Slovenkai, E. Trepman, N.N. Shields, Guidelines for diabetic foot care: recommendations endorsed by the Diabetes Committee of the American Orthopaedic Foot and Ankle Society. Foot Ankle Int. 26 (2005) 113-119.

[2] A.J. Boulton, L. Vileikyte, G. Ragnarsontennvall, J.A.P. Apelqvist. The global burden of diabetic foot disease. Lancet, 366 (2005)1719-1724.

[3] M.P. Hanolkar, S.C. Bain, J.W. Stephens, The diabetic foot. Q J Med. 101 (2008) 685-695.

[4] D.L. Steed, C. Attinger, T. Colaizzi, M. Crossland, M. Franz, L. Harkless, A. Johnson, H. Moosa, M. Robson, T.Serena, P. Sheehan, A. Veves, L. Wiersma-Bryant, Guidelines for the treatment of diabetic ulcers. Wound Repair & Regeneration, 6 (2010) 680-692.

[5] Y.F. Jiang, X.B. Fu, Progress in the study of chronic refratory wounds. Infect Inflamm Rep. 1 (2011) 59-61.

[6] Y.F. Jiang, Z.R. Xu, X.B. Fu, Healing diabetic foot ulcers step by step. Int J Lower Extrem Wound. 11 (2012) 307-310.

[7] M. Yasuhiko, S. Kazuhisa, Evaluation of anti-diabetic drugs by using silkworm, Bombyx mori. Drug Discoveries & Therapeutics, 1 (2016) 19-23.

[8] Y. Matsumoto, M. Ishii, Y. Hayashi, et al. Diabetic silkworms for evaluation of therapeutically

effective drugs against type II diabetes. Scientific Reports, 5(2015) 10722.

[9] B.P. Liu, Y. Yue, Y. Yany, Y. Jin, Oral Administration of Silkworm-Produced GAD65 and Insulin Bi-Autoantigens against Type 1 Diabetes. PLOS ONE, 1(2016) e0147260.

[10] J.M. Cox, Mann. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 26 (2008) 1367-1372.

[11] J.M. Kim, J.Y. Chung, S.Y. Lee, E.W. Choi, M.K. Kim, C.Y. Hwang, H.Y. Youn. Hypoglycemic effects of vanadium on alloxan monohydrate induced diabetic dogs. Journal of Veterinary Science. 7(2006) 391-395.

[12] Y.F. Xie, Y. Li, L.B. Dai, H.Z. Ye, P.H. Liang, C.K. Li, Effect of acupuncture on NO content of wound tissue in early burn. Chinese Acupuncture and Moxibustion. 23 (2003) 239-240.

[13] J. Cox, M.Y. Hein, C.A. Luber, I. Paron, N. Nagaraj, M. Mann, Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics. 13 (2014) 2513-2526.

[14] S.I. Ibrahim, A.D. Amodu, A.S. Ene-Ojo, U.A. Ismaila, M. Fakhruddeen, Effect of Hyperglycemia on Erythrocyte Carbonic Anhydrase and Lactic Acid in Type II Diabetic Subjects. Journal of Diabetes Mellitu.6 (2016) 158-165.

[15] L. Lindblom, J. Cassuto, L. Yregard, U. Mattsson, P. Tarnow, R. Sinclair. Role of nitric oxide in the control of burn perfusion. Burns: journal of the International Society for Burn Injuries. 26 (2000) 19-23.

[16] G.M. Buzás, Cl.T. Supuran, The history and rationale of using carbonic anhydrase inhibitors in the treatment of peptic ulcers. In memoriam Ioan Puşcaş (1932-2015). Journal of Enzyme Inhibition and Medicinal Chemistry. 31 (2015) 527-533.

[17] J.L. Zhang, Q.C. Zheng, H.X. Zhang, Theoretical improvement of the specific inhibitor of human carbonic anhydrase VII. Computational Biology and Chemistry. 35 (2011) 50-56.

[18] A.J.M. Boulton, The diabetic foot: grand overview, epidemiology and pathogenesis. Diabetes Metab Res Rev. 24 (2008) S3-S6.

[19] D. Wang, G. Zhang, X. Chen, T. Wei, C.X. Liu, C. Chen, Y.H. Gong, Q.L. Wei, Sitagliptin ameliorates diabetic nephropathy by blocking TGF- β1/Smad signaling pathway. International Journal of Molecular Medicine. 41 (2018) 2784-2792.

[20] X.J. Jia, Z. Tang, W.W. Shuai, Z.R. Zhang, J.Y. Li, L. Chen, J. Cao, W. Yin, Endogenous peptide LYENRL prevents the activation of hypertrophic scarderived fibroblasts by inhibiting the TGF-β1/Smad pathway. Life Sciences. 231 (2019)116674.

[21] E. Gendaszewskadarmach, M. Kucharska, Nucleotide receptors as targets in the pharmacological enhancement of dermal wound healing. Purinergic Signalling. 7 (2011) 193-206.