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Effect of *Lycium barbarum* Polysaccharide on the Improvement of Antioxidant Ability and DNA Damage in NIDDM Rats

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The effects of polysaccharide extracted from *Lycium barbarum* (LBP) on blood glucose, oxidative stress and DNA damage in rats with non-insulin dependent diabetes mellitus (NIDDM) were studied. The results show that LBP treatment (10 mg/kg·d) for 4 weeks led to decreased levels of blood glucose, malondialdehyde (MDA) and nitric oxide (NO) in serum of fasting rats; and to increased serum level of superoxidedismutase (SOD). Furthermore, LBP could reduce cellular DNA damage in peripheral lymphocytes of NIDDM rats. The DNA damage was determined by using the single cell gel (comet) assay with alkaline electrophoresis and was quantified by measuring tail length and tail moment. These results suggest that LBP can control blood glucose and modulate the metabolism of glucose, leading to significant improvement of oxidative stress markers (SOD, MDA) in rats with NIDDM. And that, LBP decreases DNA damage possibly *via* a decrease in oxidative stress levels. In conclusion, LBP as a dietary supplement may prevent the development of complications or even tendency to carcinogenesis in NIDDM rats.

Key words——*Lycium barbarum* polysaccharide; oxidative stress; free radicals; non-insulin dependent diabetes mellitus; nitric oxide; DNA damage

INTRODUCTION

Diabetes mellitus is a major health problem. The World Health Organization has estimated that by the year 2010 diabetes will affect 221 million people worldwide,[1] and most cases (90—95%) as NIDDM, a disease that secretion of insulin is relative shortage. The treatment or preventing complications of NIDDM has spent vast amounts of resources in all countries. Dietary supplements have been used extensively both as pharmacological supplements, food ingredients, in processed foods to aid weight control, and the regulation of glucose control for diabetic patients.[2] Recently, the increasing interest has been attracted for development and utilization of antidiabetic plants because they have less side effects and are more economic, especially in developing countries.[3] *Lycium barbarum* polysaccharide (LBP) extracted from the traditional Chinese herb *Lycium barbarum*, is found to have bioactivities such as anticancer, antioxidant and hypoglycemic activities.[4] LBP could clean out the free radicals and restrain the DNA damage of testicle cells caused by the oxidative stress.[5]

Metabolism abnormality and hyperglycemia result in the increase of oxidative stress and increased oxidative stress is a widely accepted factor in the development and progression of diabetes and its complications.[6] Chronic hyperglycemia is the primer of a series of cascade reactions causing the overproduction of free radicals.[7] Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to the damage of cellular organelles, increased lipid peroxidation and development of insulin resistance.[8] Malondialdehyde (MDA) is a kind of lipid peroxidation, could reflect the degree of oxidation in the body.[9] As free radicals impair not only lipids and proteins, but also cause oxidative damage to DNA, we attempted to examine the level of DNA strand breaks as well as DNA repair processes using comet assay modifications in NIDDM rats. Nitric oxide (NO) is generated from L-arginine in inflamed tissue by inducible nitric oxide synthase (iNOS). Under the stimulus of cytokines, invading macrophages and the β-cell themselves may produce the large amounts of NO, leading to β-cell dysfunction and death.[10] NO leads to nuclear DNA damage including base deamination, nitrosation and oxidation.[11] DNA strand breakage produced by NO has been shown in mammalian cells.[12] Oxidative

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DNA damage has been studied for several years in diabetic population with important findings of increased DNA injury expressed as strand breaks DNAsb.[13,14].

Streptozotocin (STZ) has drawn attention as a potential source of oxidative stress. STZ can generate NO.[15] In our research, the diabetes model rats were made by feeding high fat diet and subjecting to intraperitoneal injecting STZ. Despite a growing amount of data which show oxidative stress plays a major role in the etiology of NIDDM and its complications, the relationship among NO, the parameters of oxidative stress (SOD, MDA) and DNA damage has not been assessed so far in this disease. Epidemiological data suggest a possible relationship between diabetes mellitus and cancer risk, but the mechanism underlying this association is not clear.[16,17] Since oxidative stress play a major role in complication of diabetes, any antidiabetic agents should be able to decrease both oxidative DNA damage and cancer incidence. Furthermore, there is no one previously published study known to us that used the comet assay to investigate the effects of herb on increasing DNA damage repair in diabetic rats. Therefore, the primary objective of the present study was to explore the therapeutic effects of LBP on the improvement of NIDDM rats through increasing antioxidation and alleviating DNA damage in peripheral lymphocyte and to provide scientific evidence for development of LBP as a potential natural oral antidiabetic agent or functional food.

MATERIALS AND METHODS

Plant Material The fruits of *Lycium barbarum* were collected in Ningxia Hui Autonomous Region which is the well-known production area of *Lycium barbarum* in China and were authenticated at the Agricultural College of Northwest A & F University. A specimen was deposited in the herbarium of the Botany Department.

Preparation of *Lycium barbarum* Polysaccharide According to our previous method,[18] the dried fruits were ground to powder, and the powder were refluxed two times (each time for 1 h) to remove lipids with petroleum ether (60—90℃), then refluxed again with 80% ethanol two times (each time for 2 h) to remove monosaccharide and oligosaccharide. The residue was extracted three times in hot water (90℃) and then combined filtrate to concentrate through decompressing using a rotary evaporator (ShangHai, China), after then, added little dose of activated carbon to decolor. After filtering, the filtrate was added 95% ethanol and deposited for one night. The precipitation was washed using 95% ethanol, 100% ethanol, acetone and aether respectively for time after time. In the end, the precipitation was collected and dried in vacuum, giving polysaccharide. The yield of the extract was 7.23% (w/w).

Experimental Animals Thirty-five male wistar rats, weighing 230—250 g, were provided by the Animal Department of Beijing Institute of Traditional Medical and Pharmaceutical Sciences. All investigations were carried out in accordance with the ''Guiding Principles in the Care and Use of Animals'' and the University of Yanshan Protocol for Animal Studies. By methods in reference,[19] thirty-five male rats were used, in which ten were chosen out randomly as control, the rest were fed on high fat diet.[20] After exposure to the high fat diets for 3 weeks, rats were intraperitoneally injected with 0.5% STZ solution at the dose of 50 mg/kg weights. Seventy-two hours after injection, the blood sample was collected from tail vein in the fasted rats, and the level of serum glucose was determined. The serum glucose level of over 16 mmol/l was defined as diabetic model rats. Then these rats were divided into two groups, model control group and LBP group. The LBP group rats were treated by oral infusion with LBP (10 mg/kg·d, the dose chosen according to our previous study[18]) dissolved in normal saline and model control group rats received normal saline (10 mg/kg·d) for 4 weeks. All rats were individually housed in stainless cages and kept in an isolated room at a controlled temperature (18—25℃) and ambient humidity (50—80%). Lights were maintained on a reversed 12 h light/dark cycle.

Drugs and Reagents STZ from Sigma Co; 0.5% STZ solution was prepared with 0.1mol/l citric acid and sodium citric acid buffer (pH 4.4) before used. Reagents for SOD, MDA and NO were obtained from Nanjing Jiancheng Biology Engineering Research Institute. Ficoll Paque Lymphocytes separation medium (Amersham Biosciences, AB). Phosphate buffer saline (PBS buffer, Oxide, Basingstoke, Kent, UK). Tris (tris (hydroxymethyl) aminomethane hydrochloride), disodium ethylenediamine tetraacetate (EDTA), dimethyl sulfoxide (DMSO), Triton X-100, and ethidium bromide (EtBr) were

purchased from Sigma (St. Louis, MO). Low melting point agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL (Grand Island, NY).

Method of Measurement Blood was collected from the cut tip of the tail of all rats to test blood glucose. The serum glucose was determined by a one touch II micro blood glucose instrument (American Life Scan Company). At the end of the experiment, blood was collected from the eyes of the rats to test other biochemical indexes. The test methods were done according to the reagent protocols prepared by the manufacturing firms.

Lymphocytes Preparation Rat peripheral lymphocytes were isolated from total blood using a Ficoll Paque Lymphocytes separation medium. The lymphocytes were washed with PBS and suspended in buffer at approximately 2×10^5 cells/ml. Cell viability determined using the Trypan blue assay was $\geq98\%$.

The Alkaline Comet Assay The alkaline comet assay procedure in this study was a modification of the method described by Singh et al.[21] Essentially, 100 μl of 0.5% normal melting point agarose was pipetted onto frosted microscope slides and allowed to solidify under a coverslip, which was then carefully removed. Approximately 10000 cells were suspended in 75 μl low melting point agarose gel; the cell suspension was rapidly pipetted onto the first agarose layer, and gently spread by placing a coverslip on top. This was allowed to solidify on an ice tray for 5 min. After removal of the coverslip, the slide was immersed in freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, with 1% Triton X–100, and 10% DMSO) and incubated for 2 h at 4℃. The slides were removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank. The tank was filled with fresh, cold electrophoresis solution (1 mM EDTA and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage before electrophoresis. Electrophoresis was conducted at 4℃ for 20 min using 25 V and a current of 300 mA. Following electrophoresis, the slides were washed in Tris buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali. Finally, the slides were stained with 75 μl ethidium bromide (EB, 20 μg/ml).

Image Analysis of Slides Slides were stored in a light-proof box containing tissues moistened with PBS and viewed within 12 h of staining. Observations were made using a fluorescence microscope (Olympus BX60F-3, Olympus Optical, Japan) equipped with an excitation filter 515—560 nm and a barrier filter 590 nm. The microscope was connected to a computer through a charge coupled device (CCD) camera to transport images to a software (CASP, Poland) for the comet assay analysis.[22] The image analysis software provides a full range of densitometric and geometric parameters describing the complete comet, as well as the head and tail portions. All experiments were performed three times and for each experimental point fifty 'comets' were randomly captured, avoiding the edges and eventually damaged parts of the gel. Tail moment was used to evaluate DNA damage. The tail moment, expressed in arbitrary units, has been calculated by multiplying the percent of DNA (fluorescence) in the tail by the length of the tail.[22,23]

Statistical Analysis Results are presented as means ± S.D. Data were analyzed using the statistical function of SigmaPlot 4.01 and SigmaStat 2.03 (SPSS Inc., Chicago, IL, USA). The median tail moment values were used in a one-way analysis of variance test (ANOVA). If a significant F-value of $p\leq0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted. Differences between two groups were statistically evaluated by the Paired t-test.

RESULTS

The Effects of LBP on Blood Glucose in Rats with NIDDM After making model, the levels of blood glucose in rats of model control and LBP groups accord with diabetes mellitus standard. During LBP treatment for 4 weeks, the level of blood glucose was measured for once every week. The results are showed in Table 1. The blood glucose levels in the LBP group rats showed no significant differences at the end of the first week of drug administration ($p>0.05$), but the blood glucose levels of rats in LBP group decreased 19.3% ($p<0.05$), 30.4% ($p<0.01$) and 34.9% ($p<0.01$), respectively compared with that of NIDDM model group by the end of the second, third and fourth week of drug administration. The above results indicate LBP could effectively decrease hyperglycemia in NIDDM rats.

The Effects of LBP on the Levels of SOD, MDA and NO in NIDDM Rats After LBP administration for 4 weeks, the effects of LBP on the levels of

Table 1. Effects of LBP on Blood Glucose (mmol/lc) in Rats with NIDDM ($\bar{x}\pm$S.D.)

Group	*n*	1 week	2 weeks	3 weeks	4 weeks
Normal control	10	3.591±0.472**	4.283±0.515**	4.321±0.526**	4.594±0.378**
Model control	10	24.537±2.312	26.934±1.779	29.627±2.108	30.876±1.980
LBP	10	23.643±3.251	21.731±2.258*	20.625±4.057**	20.107±3.083**

* $p<0.05$ compared with the non-insulin dependent diabetes mellitus model control group. ** $p<0.01$ compared with the non-insulin dependent diabetes mellitus model control group.

Table 2. Effects of LBP on the Levels of SOD, MDA and NO in NIDDM Rats ($\bar{x}\pm$S.D.)

Group	*n*	SOD (NU/ml)	MDA (nmol/lc)	NO (umol/lc)
Normal control	10	149.64±10.28**	5.92±1.73**	5.44±0.76**
Model control	10	115.37±7.63	9.84±1.61	19.75±3.88
LBP	10	142.56±5.47**	5.01±1.29**	12.84±4.31*

* $p<0.05$ compared with the non-insulin dependent diabetes mellitus model control group. ** $p<0.01$ compared with the non-insulin dependent diabetes mellitus model control group.

Table 3. The Effect of DNA Damages Repair in Lymphocytes of NIDDM Rats Detected by Comet Assay after LBP Treatment for 4 Weeks ($\bar{x}\pm$S.D.)

Group	*n*	Percent of lymphocytes with tails	Tail length (μm)	Tail moment
Normal control	10	9.15±1.06**	10.41±1.32**	1.64±0.72**
Model control	10	87.89±3.21	70.94±2.38	29.96±1.25
LBP	10	48.23±2.18**	27.39±1.06**	4.95±0.56**

** $p<0.01$ compared with the non-insulin dependent diabetes mellitus model control group.

SOD, MDA and NO in NIDDM rats are given in Table 2. The results show that SOD in LBP group significantly increased 23.57% ($p<0.01$), compared with that of NIDDM model group, and MDA significantly decreased 49.1% ($p<0.01$) compared with that of NIDDM model group. The decrease of NO in LBP group 34.99% ($p<0.05$) compared with that of NIDDM model group. The results suggest that LBP has preferably antioxidation.

The Effects of LBP on the Improvement of DNA Damage in NIDDM Rats Table 3 showed results of comet assays, in control group, most cells had no detectable comet tail. But in NIDDM rats, lymphocytes were damaged apparently. Alkaline SCGE revealed that most of single strand DNA in NIDDM rats as fragment migrated into the comet tail. After LBP treatment for 4 weeks, alkaline SCGE revealed a small amount of fragmented single strand DNA. These fragments are displayed as a comet tail, while the majority of DNA remains non-fragmented and localized within the cell nucleus, appearing as a comet head. The tail length and tail moment decreased, and the number of lymphocytes with tails decreased after LBP treatment for 4 weeks.

DISCUSSION

Lycium barbarum, a well-known traditional Chinese medicinal herb, our previous study has confirmed LBP can improve insulin resistance in NIDDM rats.[18] Diabetes mellitus is a metabolic disease characterized by hyperglycemia. There is evidence that hyperglycemia may disrupt natural antioxidant defense mechanisms.[24] The occurrence of NIDDM and its complications can be associated with an increased level of oxidative stress, which results from hyperglycemic through glycoxidation and sorbitol system activation.[25] We show here a reduced blood glucose gain in NIDDM rats treated by LBP for 4 weeks.

Under conditions of oxidative stress damage to cellular biomolecules including proteins, lipids and DNA can occur. The defense of the organism against oxidative stress is ensured by a lot of antioxidant substances and enzymes (flavonoids,[26] vitamin E, SOD,

glutathione peroxidase). Several investigations have focused on the antioxidant status and oxidative stress in NIDDM.[27,28] Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. The levels of these defense mechanisms are altered in diabetes and, therefore, the ineffective scavenging of free radicals plays a crucial role in determining the extent of tissue injury. The combination of protective effect exerted on antioxidants and free radicals scavenging are of utmost importance in preventing or showing down the complications of diabetes. In this study, after making model, the level of SOD (a scavenger of free radicals) was lower in NIDDM rats than that in control group rats. It was out of accord with the level of SOD in NIDDM was higher than that of normal.[29] The cause was probably higher blood glucose could combine with SOD. Altogether, in the present study, the results of oxidative stress parameters showed significantly lower MDA and increased SOD compared with model group rats after treatment with LBP for 4 weeks. This suggests that LBP has effective antioxidative properties and could well scavenge excess free radical and decrease the level of lipid peroxidation.

Endothelial dysfunction is considered an intrinsic element in the pathogenesis of diabetic angiopathies. Some studies suggest that diabetes-induced endothelial dysfunction is associated with upregulation of reactive oxygen species (ROS) within diabetic arteries. In prolonged duration of disease, a contributing factor may be the eventual reduction in SOD gene expression in diabetic rat aorta.[30] The free radical NO is derived from endothelium[31] and it is considered as vasodilator that participates in the general homeostasis of the vasculature. NO is a unstable radical and converted to nitrite/nitrate anion (NO_2^-/NO_3^-) in a very short time which is more stable product. However, excess NO can exert cytotoxic and cytostatic effects.[32] Biswass et al. have reported that in the resting state the levels of NO were higher in diabetic as compared to normal polymorphonuclear leukocytes (PMNL), and diabetic serum factor provoked a significant generation of NO in normal PMNL.[33] The development of diabetic blood vessel complications is closely related to the increased generation of superoxide anion ($O_2^{\cdot-}$) and NO.[34] NO and ROS can react to form peroxynitrite anion ($ONOO^-$), which in turn rapidly decomposes to hydroxyl anion (OH^-) and nitrogen dioxide (NO_2), the former being a highly reactive and toxic compound.[35,36] So far, there is not a considerable research on both antioxidative systems and NO pathways in same group of diabetic rats. Our experimental results show for the first time that LBP treatment (10 mg/kg·d) for 4 weeks led to decreased levels of MDA and NO in serum of fasting rats, and to increased serum level of SOD.

Diabetes is accompanied by increased oxidative DNA damage but the pattern of increased cancer risk seems unusual.[37] There is no explicit evidence that a correlation between NIDDM and pancreatic cancer and that oxidative DNA damage is a biomarker of subsequent cancer development. But, it follows that agents that decrease the amount of oxidative DNA damage should decrease the risk of cancer development.[38] The Single Cell Gel Electrophoresis (SCGE) assay or 'Comet Assay' has been shown to be a very sensitive method for the evaluation of DNA damage (primarily single strand breaks and alkali labile damage) in individual cells.[22,39] Studies with the comet assay have shown increased levels of oxidative DNA breakage in peripheral blood lymphocytes of diabetic patients with poor glycemia control, but not in patients with normal glycemia.[16,40] Hyperglycemia can enhance cytokine-induced NO production and induce generation of ROS, resulting in oxidative stress.[41] The vascular and other complications of diabetes mellitus are frequently suggested to involve oxidative damage resulting from the hyperglycemia and /or hyperlipidemia. MDA, a kind of peroxidation products is known to interact with DNA.[42] DNA damage can be produced by the attack of several different ROS, including OH^- and $ONOO^-$.[43] Besides oxidative DNA damage, other types of DNA damage such as DNA-protein cross-links, and adducts to proteins due to advanced glycation end products (AGEs)[44,45] can contribute to diabetes-associated cancers. Diabetic patients may have a risk of carcinogenesis by oxidative DNA damage due to endogenous aldehydes, especially glyoxylic acid (GA), in relation to hyperglycemia. To acquire an overall prospect, we designed the present study, providing information about LBP improving NIDDM and the mechanism of the relations between the oxidative stress and DNA damage in NIDDM rats. DNA damage in lymphocyte is thus a useful marker of oxidative stress, so our study indicates that LBP decreases DNA damage possibly via a decrease in oxidative stress levels.

In conclusion, our experimental results indicate LBP as a dietary supplement possesses antioxidative properties and can ameliorate DNA damage in NIDDM rats. And hypoglycemia is a major LBP target in the steps from enhancement of blood glucose level to DNA damages. These suggest LBP can as a potential natural oral antidiabetic agent use in clinic for treating and postponing the appearance of complication and carcinogenesis in NIDDM patients.

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