—Regular Articles—

Hypoglycemic Effects and Mechanisms of Action of *Cortex Lycii Radicis* on Alloxan-Induced Diabetic Mice

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(Received March 16, 2007; Accepted July 2, 2007)

Cortex Lycii Radicis (CLR) has been used as a traditional Oriental medicine as an antipyretic and to treat pneumonia, night-sweats, cough, hematemesis, inflammation, and diabetes mellitus for centuries. This study aimed to determine the effects of CLR on alloxan-induced diabetic mice and its mechanisms. Based on thin-layer chromatography (TLC) assay, the main compounds of CLR include an organic acid, flavone, alkaloid, polysaccharide, anthraquinone, and saponin. The mice were divided into four groups: normal control (NC), diabetes control (DC), diabetes+high-dose CLR (200 mg kg^{-1}), and diabetes+low-dose CLR (100 mg kg^{-1}). The diabetic mice were administered CLR daily for 28 days. The CLR treatment resulted in significant decreases in fasting blood glucose, total cholesterol, and triglycerides. CLR also showed a tendency to improve body weight gain in diabetic mice. Furthermore, the serum insulin level of each group was assayed, and the DC group had a lower serum insulin level than the NC group. Insulin levels were dose dependently raised in the CLR-treated groups compared with the DC group. According to single-cell gel electrophoresis and LD_{50} analysis, CLR was nontoxic to the animals. The results indicate that CLR alleviates the blood glucose and lipid increases associated with diabetes and improves the abnormal glucose metabolism and increases insulin secretion by restoring impaired pancrease βcells in alloxan-induced diabetic mice. The results suggest that CLR has hypoglycemic potential and could be useful in diabetes therapy.

Key words——diabetes; *Cortex Lycii Radicis*; hypoglycemia; hypolipidemia; insulin

INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disease and affects 5% of the world population.[1)] It is becoming the third human ''killer'' following cancer and cardiovascular and cerebrovascular disease. DM leads to metabolic abnormalities and is characterized by hyperglycemia associated with hypolipidemia resulting from defects in insulin secretion, insulin action, or both. Despite numerous preventive strategies and medical therapies, 300 million people worldwide are expected to develop DM by 2025.[2)] Plant products are frequently considered to be less toxic and have fewer side effects than synthetic ones.[3)] A growing number of people are turning to alternative therapies including herbal medicines. Traditional Chinese medicine has been used in clinical practice for several centuries. However, the compounds and precise antidiabetic mechanisms of most herbs remain to be determined.

Cortex Lycii Radicis (CLR), the dried root bark of *Lycium barbarum Linn.*, which belongs to the Solanaceae family, is also called Chinese Wolfberry Root-bark or Bark of Boxthornroot. CLR has been extensively used in traditional Chinese herbal medicine for centuries due to its variety of biological activities, as an antipyretic and to treat pneumonia, night-sweats, cough, hematemesis, inflammation, and DM,[4–8)] but the mechanism of action and its compounds have not been clarified.

In this study, we analyzed the compounds of CLR extracts and investigated its hypoglycemic effects. Activities of the extract were determined by comparing the changes in blood glucose, body weight, serum triglyceride (TG) and total cholesterol (TC) levels in alloxan-induced diabetic mice. Furthermore, the serum insulin levels in four groups of mice were assayed to determine the antidiabetic mechanism of CLR. The toxicity of CLR was assayed in single-cell gel electrophoresis and LD_{50} experiments.

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MATERIALS AND METHODS

Reagents and Herb A Glucose Analyzer (GT-1640) and strips were purchased from Arkray Inc. (Kyoto, Japan). Alloxan was obtained from Sigma Co. (Loutis, USA). GF254-TLC plates were bought from Qingdao Haiyang Chemical Co (Qingdao, China). Other chemicals were of analytic grade. Dried CLR was purchased from a local drugstore and authenticated by specialists.

Preparation of CLR Extracts and Characterization The dried CLR was ground to a fine powder. The ground samples (1000 g) were immersed in a 10-fold volume of dH_2O, boiled at 80℃ for 1 h, and then the water extract was collected. The process was repeated once, and the extracts were combined and concentrated with a rotary evaporator and vacuum-dried[9] to yield 9.1% (w/w) of the extract. The extract was examined using thin-layer chromatography (TLC) analysis to identify the main compounds. The CLR solution was dotted on the TLC plates, and n-butanol: acetic acid: H_2O (20 : 5 : 7) was used as the solvent system,[10] and then the indicators were sprayed on the plates. The plates were heated at 105℃ for 10 min in an oven. Nine indicator systems were used to identify the compounds of CLR.[11]

Preparation of Experimental Animals Male ICR mice weighing 18–22 g were provided by the Animal Department of the Beijing Institute of Traditional Medical and Pharmaceutical Sciences. The mice were housed in stainless steel cages at a controlled temperature (22±2℃) and 60–65% relative humidity with a normal 12-h light and dark cycle. Eight mice were randomly selected as normal controls, and the rest were fed a high-fat diet. After exposure to the high-fat diet for 3 weeks, the mice were fasted overnight with free access to water and injected intraperitoneally with alloxan 200 mg/kg body weight (bw) dissolved in sterile normal saline solution.[12–15] Seventy-two hours thereafter, the fasting blood glucose level of the mice was determined using the glucose oxidase method[16] with a Glucose Analyzer. A blood glucose level greater than 11.1 mmol/l was defined as DM. Thirty-two mice (8 normal mice, 24 alloxan-induced diabetic mice) were chosen and divided into four groups: the normal control group (NC), diabetes control group (DC), diabetes+low-dose CLR group (DM+CLR LD), and diabetes+high-dose CLR group (DM+CLR HD).

Acute Effects of CLR on Blood Glucose Levels On day 1, the acute blood glucose test was performed to assess the hypoglycemic effects of CLR. After overnight fasting with free access to water, the mice were orally administered CLR suspension dissolved in dH_2O via gavage at the dose of 100 mg/kg bw in the DM+CLR LD group and 200 mg/kg bw in the DM+CLR HD group. The same volume of dH_2O alone was administered in the NC and DC groups. Tail blood samples were taken at 0, 0.5, 1, 1.5, 2.0, and 4 h after the administration of CLR or H_2O. Blood glucose levels at the various time points were measured using the glucose oxidase method.

Long-term Effects of CLR on Blood Glucose Levels The DM+CLR LD group was given CLR extract 100 mg/kg bw dissolved in dH_2O and the DM+CLR HD group was given a dose of 200 mg/kg bw daily by gavage for 28 days. The control mice (NC and DC groups) were given the same volume of dH_2O alone. On days 0, 7, 14, 21, and 28, blood samples were collected the from the tail vein following overnight fasting and blood glucose levels were measured. At the same time, the bw of each mouse was recorded.

Assay of Serum TG, TC, and Insulin Levels On day 29, the mice were fasted overnight, blood samples were collected in a sterile tube by sinoocular puncture under ether anesthesia and left to stand at room temperature for 2 h, then centrifuged at 1500×*g* for 15 min at 4℃. The supernatant was immediately separated from the pellet to prepare serum samples to determine the levels of TG and TC using an automated chemistry analyzer (Olympus, Japan), following the manufacturer's instructions (Center of Medical Science and Technology, Capital Medical University, Beijing, China). Serum insulin levels were then determined with an insulin-ELISA kit (Insulin ELISA kit, Adlitteram Diagnostic Laboratories Co., USA) according to the manufacturer's instructions.

LD_{50} Experiment Twenty-four normal mice were divided into four groups of 6 animals each (3 females and 3 males) weighing about 18–22 g. The mice were administered CLR extract orally at doses of 800, 1200, 1600, and 2000 mg/kg bw. Then the mice were observed for gross behavioral, neurologic, autonomic, and toxic effects at short time intervals for 24 h. Food consumption and feces and urine output were also examined at 2 h, and then at 6-h intervals for 24 h.

Single-cell Gel Electrophoresis Experiment Sin-

gle-cell gel electrophoresis (SCGE) was perfirmed according to the method of Singh *et al*. with some modification.[17] Blood was collected from 10 mice, and lymphocytes were separated from whole blood using a Ficoll-Paque lymphocyte separation medium and then suspended in PBS.[18] Cells were incubated in RPMI 1640 (10% fetal bovine serum) medium and exposed to the test compounds, which included dH_2O control (same volume), CLR (50, 100 μg/ml, final concentration), or H_2O_2 (5 μmol/l), cultured at 37℃ in a 5% CO_2, 95% air incubator for 1 h. Then the cells were centrifuged at 4℃ and suspended in a small volume of PBS. The cells were mixed with 0.5% low melting-temperature agarose at 37℃, and then placed on precleaned microscope slides covered with a thin layer of 0.5% normal melting-point agarose. The slides were covered with a third layer of low melting-point agarose. The slides were immersed in a lysing solution for 1 h to lyse the cells and permit DNA unfolding. Electrophoresis was conducted at 25 V for 20 min. After electrophoresis, the slides were washed gently to remove alkali and detergents with Tris buffer, rinsed with distilled water, and then stained with ethidium bromide (20 μg/ml). The slides were evaluated under a fluorescence microscope (Nikon). Four different cultures were analyzed, and the tail lengths of 100 cells per culture were evaluated and categorized. The assay was repeated three times to avoid selection bias.

Statistical Analysis Statistical analyses were performed using the SPSS statistical software package. Data are expressed as mean±S.E. The effects of CLR on acute and long-term blood glucose levels were determined using two-way ANOVA repeated measures, followed by the Tukey test. Differences in body weight and blood lipid and serum insulin levels among diabetic control and CLR low/high-dose groups were analyzed using one-way ANOVA, followed by the Scheffe test, and the difference between the NC and DC groups was compared using Student's *t*-test. Results were considered significantly different at the level of $p<0.05$.

RESULTS

Components of CLR in the TLC Assay Results of TLC are shown in Table 1. There were six main compounds in CLR, including an alkaloid, saponin, polysaccharide, anthraquinone, flavone, and organic acid, but no hydroxybenzene, terpene, steroid, or lignin were detected.

Effects of CLR on Acute Blood Glucose Test Results There was no significant difference observed at 0 min among the DC and CLR-treated groups (Fig. 1). However, the administration of CLR decreased the blood glucose levels in the DC mice. The dose-dependent hypoglycemic effects of CLR became significant at 1 h following oral administration, reached the peak at 1.5 h ($p<0.01$), and was still significant at 4 h. There was no significant change in blood glucose levels in the DC group during the acute glucose test ($p>0.05$). The rates of decrease in blood glucose in the LD and CLR HD groups were 27.4% and 30.7% compared with the 0 h blood glucose levels, respectively. The results indicate that CLR has acute hypoglycemic effects.

Effects of CLR on Long-term Blood Glucose Test Results During CLR treatment for 4 weeks, blood glucose levels were measured once weekly. The results are summarized in Table 2. Before the induction of diabetes, there was no significant difference in blood

Table 1. Compounds in CLR Extract in TLC Analysis

Indicator	Examined components	Ratio of flow (Rf)	Color
Iodine/potassic iodide	Alkaloide	0.2525	Brown
Ferric trichloride/water	Hydroxybenzene	—	—
Acetic anhydride/sulfuric acid	Terpene and steroid	—	—
Phosphomolybdic acid/ethanol	Saponin	0.2447	Dark blue
Phenol/sulfuric acid	Polysaccharide	0.2783	Brown
10% KOH	Anthraquinone	0.2473	Orange
10% NaOH	Flavone	0.2418	Yellow
Sulfuric acid/ethanol	Lignin	—	—
Bromophenol blue/ethanol	Organic acid	0.2198	Dark yellow

—, means without any spot on the plates.

Table 2. Effects of CLR on Blood Glucose in the Long-Term Blood Glucose Test

Group	Blood glucose levels (mmol/l)				
	Day 0	Day 7	Day 14	Day 21	Day 28
NC	5.28±0.14	5.18±0.13	5.14±0.22	5.15±0.19	5.24±0.14
DC	13.58±0.44	13.03±0.39	13.31±0.41	12.69±0.35	12.94±0.26
DM+CLR LD	13.21±0.37	12.08±0.32	10.21±0.26*	8.58±0.25*	7.33±0.25*
DM+CLR HD	13.46±0.38	12.10±0.34	9.83±0.30*	8.13±0.23*	6.61±0.15*

Each value represents mean±S.E. of eight mice per group. * Statistically significant difference vs. DC group ($p<0.01$).

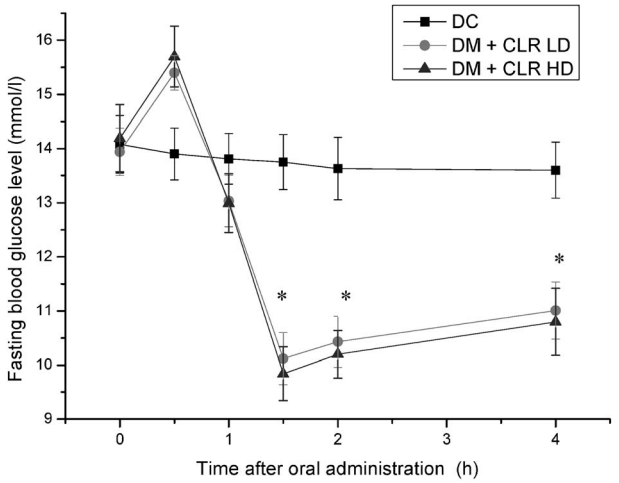


Fig. 1. Effects of CLR on Blood Glucose in the Acute Blood Glucose Test

Each value represents mean±S.E. of 8 mice in each group. *Statistically significant difference vs. DC group ($p<0.05$).

glucose levels among the groups ($p>0.05$). Blood glucose levels in the CLR-treated groups showed no significant differences at the end of the first week of drug administration ($p>0.05$), but those in the two CLR-treated groups were all lower than that in the DC group ($p<0.01$) after 14 days of treatment. On day 28, blood glucose levels in the CLR LD and HD groups decreased by 36.72% and 41.89%, respectively. The NC and DC mice did not show any significant variation in blood glucose level throughout the experimental period ($p>0.05$). These results indicate that CLR decreases hyperglycemia in diabetic mice.

Effects of CLR on Body Weight Changes in body weights in control and experimental groups are listed in Table 3. There was no significant difference in the initial body weights among the four groups ($p>0.05$). After feeding a high-fat diet for 3 weeks, the difference between the NC and DC groups was significant ($p<0.05$). CLR administration improved the weight gain compared with the DC mice. By the end of the experiment, the body weight of the NC group

Table 3. Effects of CLR on Body Weight of Mice

Group	Body weight (g)		
	Before model	After model	After CLR
NC	22.55±0.68	27.73±0.76*	35.56±0.50
DC	22.18±0.64	31.36±1.04	33.16±0.70
DM+CLR LD	22.03±0.74	31.7±1.02	37.96±0.76**
DM+CLR HD	22.68±0.67	31.28±0.86	38.06±0.53**

Each value represents mean±S.E. of eight mice per group. * Statistically significant difference vs. DC group ($p<0.05$). ** Statistically significant difference vs. DC group ($p<0.01$).

was significantly increased. In contrast, the mice in the DC group had slightly increased body weight during the experimental period ($p>0.05$). Following CLR treatment for 4 weeks, the body weight of mice in both CLR-treated groups was significantly increased compared with that in the DC group ($p<0.01$).

Effects of CLR on Serum TC and TG Levels Serum TG and TC levels were determined on day 29, and the results are summarized in Table 4. The serum TG and TC levels were significantly higher in the DC group than in the NC group ($p<0.01$). TG and TC levels were decreased with CLR administration; TG levels in DM+CLR LD and HD groups were lower than that in the DC group ($p<0.01$), while TC levels in the DM+CLR LD and HD groups were decreased compared with that in the DC group ($p<0.05$, 0.01, respectively). The hypolipidemic effects of CLR were not dose dependent, although the levels of TG and TC in the DM+CLR HD group were lower than those in the DM+CLR LD group ($p>0.05$).

Effects of CLR on Serum Insulin Levels The serum insulin levels in the four groups were determined on day 29, and the results are summarized in Fig. 2. The serum insulin level in the NC group was higher than that in the DC group, which indicates that alloxan damages pancreas islet cells. With 28 days of CLR

administration, serum insulin levels in the CLR-treated groups were significantly higher than that in the DC group ($p<0.05$), which suggests that CLR improved insulin secretion in diabetic mice. In the CLR HD group, the insulin level was higher than that of the CLR LD group, indicating that CLR improves the function of islet cells and stimulates insulin secretion.

Table 4. Effects of CLR on TC and TG levels

Group	TG (mmol/l)	TC (mmol/l)
NC	1.659±0.031**	2.786±0.062**
DC	2.050±0.059	3.321±0.065
DM+CLR LD	1.859±0.035**	3.086±0.067*
DM+CLR HD	1.781±0.054**	3.035±0.064**

Each value represents mean±S.E. of eight mice per group. * Statistically significant difference vs. DC group ($p<0.05$). ** Statistically significant difference vs. DC group ($p<0.01$).

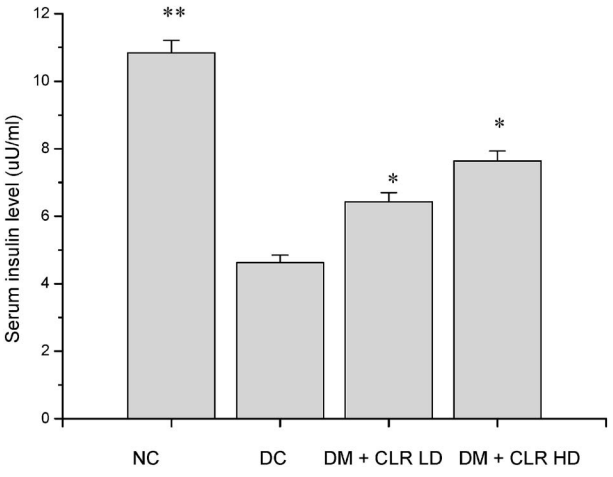


Fig. 2. Effects of CLR on Serum Insulin Level

Each value represents mean±S.E from 8 mice. * Statistically significant difference vs. DC group ($p<0.05$).

LD_{50} Experiment The behavior of the treated mice appeared normal during the experiment. No toxic effects were found at up to 10-fold the effective dose of the water extract, and no death occurred in any group. Only the consumption of food was increased at 8- and 10- fold higher doses but returned to normal after 4 h.

SCGE Cultured lymphocytes from the four groups were assayed with SCGE. The results are shown in Fig. 3 and Table 5. The percentage of DNA in the tail [(2.5×cells0+12.5×cells1+30×cells2+60×cells3+90×cells4)/Σcells] was calculated to express the amount of DNA damage.[18] The results showed that CLR-treated lymphocytes were not damaged, and the images were similar in dH_2O-treated cultures, but H_2O_2-treated cells were heavily damaged.

DISCUSSION

We used the TLC assay to identify the main compounds of CLR, which included an alkaloid, saponin, polysaccharide, anthraquinone, flavone, and organic acid, but no hydroxybenzene, terpene, steroid, or lignin compounds. According to other reports, polysaccharide and flavone are usual antidiabetic compounds in herbs.[11,12,19,20] Further work on the isolation and purification of each compound from CLR will be done to identify the hypoglycemic compounds.

Alloxan is cytotoxic to the pancreatic β cells and thus is an effective diabetes-induction agent. It has been widely used to induce DM in experimental animal models, allowing the investigation of hypoglycemic agents in the treatment of diabetes.[21,22] Alloxan injection consistently produces symptoms of DM including hyperglycemia, decreased insulin lev-

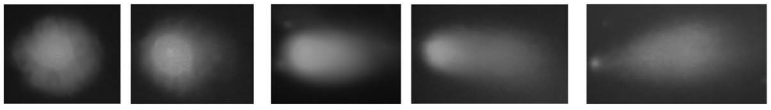
Scores	Cell 0	Cell 1	Cell 2	Cell 3	Cell 4
Percentage DNA in the tail	<5	5-20	20-40	40-80	>80
Average	2.5	12.5	30	60	90
Images					

Fig. 3. Single-Cell Gel Electrophoresis Images of Damaged Lymphocytes

Visual classification of DNA damage, according to the relative proportion of DNA in the tail (cells 0–4), obtained in SCGE. Cell 0 represents undamaged cells, and cell 4 represents the most heavily damaged cells.

Table 5. DNA (%) in the Tail of Lymphocytes in the SCGE Assay

Score	Cell 0	Cell 1	Cell 2	Cell 3	Cell 4	DNA in the tail (%)
dH_2O culture	286	14	0	0	0	3.84
CLR (50 μg/ml)	285	15	0	0	0	3.93
CLR (100 μg/ml)	288	12	0	0	0	2.9
H_2O_2 (5 μmol/l)	0	6	147	135	12	45.55

Cell 0 represents the number of undamaged cells, and cell 4 represents the number of the most heavily damaged cells.

els, polyuria, and weight loss.[22]

In the DC group, the blood glucose level remained high during the experimental period. After diabetic mice were treated for 1.5 h with CLR extract, the blood glucose level decreased significantly, and the hypoglycemic effect was maintained for 4 h. The results indicate that CLR had acute hypoglycemic activity. Furthermore, when the diabetic mice were treated for 28 days with low and high doses of CLR extract, blood glucose levels decreased, and the effect was dose dependent, suggesting that CLR has long-term hypoglycemic activity.

The body weight is usually decreased in diabetes.[23–25] In our study, a significant decrease in body weight was observed on day 28 after alloxan-induced diabetes compared with that in the NC group. The oral administration of CLR for 28 days improved the body weight loss.

DM is a metabolic disorder that usually affects carbohydrate, fat, and protein metabolism, followed by multiorgan injury in the later period, and hyperlipidemia is associated with hyperglycemia.[26,27] In the DC group, the levels of TC and TG were increased significantly compared with those in normal mice. In the CLR-treated groups, TC and TG levels were significantly decreased. The results indicate that CLR extract not only has significant hypoglycemic activity, but also has hypolipidemic effects in alloxan-induced diabetic mice fed a high-fat diet.

Alloxan damages pancreatic β cells, resulting in a decrease in endogenous insulin secretion, which then decreases the utilization of glucose by the tissues.[28] We have found that CLR decreased the level of blood glucose and increased the concentration of serum insulin in alloxan-induced diabetic mice. The possible mechanism of action of CLR extract could be correlated with promoting insulin secretion by closure of K+-ATP channels, membrane depolarization, and stimulation of Ca^{2+} influx, an initial key step in insulin secretion.[29] A further study should be designed to address this hypothesis. Under normal conditions, insulin increases the receptor-mediated removal of low-density lipoprotein cholesterol and the decrease in insulin activity causes hypercholesterolemia in DM. Hypercholesterolemia and hypertriglyceridemia were reported to occur in diabetic rats.[30] Accumulation of TGs is one risk factor for coronary heart disease (CHD). The significant increase in the level of TGs in the plasma of DC mice might have been due to the lack of insulin. Since under normal conditions, insulin activates the enzyme of lipoprotein lipase and hydrolysis of TGs,[31] CLR may reduce the TG level by increasing the plasma insulin level in alloxan-induced diabetic mice and thus prevent the progression of CHD.

The SCGE assay is a rapid, simple, visual, and sensitive technique for measuring DNA breakage in individual mammalian cells.[17,20] Cells embedded in agarose on microscope slides are subjected to lysis, unwinding of DNA, and electrophoresis at high pH. After staining with a fluorescent dye, cells with DNA damage display increased migration of genetic material from the cell nucleus. The damage is quantified by measuring the displacement between the genetic material of the nucleus (comet head) and the resulting tail. The torsional moment of the tail has been suggested to be an appropriate index of induced DNA damage when considering both the migration of the genetic material as well as the relative amount of DNA in the tail. We performed SCGE of CLR-treated cells. Both the SCGE and LD_{50} results showed that CLR had no toxic effects on the animals.

It can be concluded that the extract of CLR has hypoglycemic potential by stimulating insulin secretion. Thus CLR could be a candidate therapeutic pharmaceutical for the treatment of DM. Further investigations should be carried out on the purification and identification of the antidiabetic components of CLR.

Acknowledgements This work was supported by the 2004 Qinhuangdao Scientific Research Grant (Project No. D08).

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