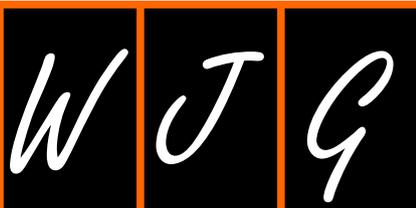


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Basic Study

Inhibition of N-methyl-N-nitrosourea-induced gastric tumorigenesis by Liuwei Dihuang Pill in db/db mice

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Abstract**AIM**

To investigate the inhibitory effect of Liuwei Dihuang Pill (LDP) on gastric tumorigenesis induced by N-methyl-N-nitrosourea (MNU) in diabetic mice.

METHODS

Four-week-old mice were divided into four groups: A, 12 db/m mice treated with MNU and saline, as the non-diabetic control; B, 12 db/db mice treated with MNU and saline, as the diabetic control; C, 12 db/db mice treated with MNU and metformin, as the positive control; and D, 12 db/db mice treated with MNU and LDP. MNU was administered for 20 wk to induce gastric carcinogenesis. LDP was administered for 10 wk for improvement of insulin resistance. Body weight and food intake were measured every week. Blood samples were collected for assays of fasting blood glucose, insulin, insulin-like growth factor (IGF)-1, adiponectin and leptin. Stomach tissues were collected for histopathological analysis, immunohistochemical staining of Ki67, quantitative reverse transcription-polymerase chain reaction and western blotting.

RESULTS

The incidence of MNU-induced gastric dysplasia was significantly elevated in diabetic (db/db) mice relative to the control (db/m) mice. The incidence of gastric

dysplasia was significantly reduced by LDP with suppression of cell proliferation, as demonstrated by a decrease in Ki67 staining. Hyperglycemia, hyperinsulinemia and serum IGF-1 were inhibited by LDP. Expression of IGF-1 and insulin receptor mRNAs was decreased, phosphorylation of IGF-1 receptor and AKT protein was reduced in the stomach tissues by LDP. In addition, adiponectin was increased and leptin was decreased in the serum by LDP.

CONCLUSION

LDP decreased risk of gastric dysplasia in type 2 diabetic mice by down-regulation of IGF and insulin activity and correction of adipokines disorders.

Key words: Diabetes; Gastric cancer; Liuwei Dihuang Pill; Insulin; Insulin-like growth factor

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Core tip: Type 2 diabetes is reported to increase risk of gastric carcinogenesis, partly by hyperinsulinemia, hyperglycemia, excessive activation of insulin-like growth factor (IGF)-1, and disorders of adipokines. In this study, we demonstrated that Liuwei Dihuang Pill decreased the risk of gastric tumorigenesis in type 2 diabetic mice by alleviating insulin resistance and decreasing IGF-1 and insulin activity, followed by down-regulation of the IGF-1/AKT signaling pathway. The improvement in adiponectin and leptin may also contribute to the effects.

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INTRODUCTION

Gastric cancer is one of the most common malignancies, particularly among populations of East Asia. The prevention and treatment of gastric cancer remain a challenge due to the absence of effective strategies. Multiple risk factors, such as *Helicobacter pylori* infection, high salt intake, smoking and obesity, contribute to the initiation of gastric cancer^[1,2]. Recent studies in humans have demonstrated that type 2 diabetes may be a new risk factor for gastric cancer. The incidence of gastric cancer is affected by duration of diabetes and some anti-diabetic drugs. Given the high prevalence of type 2 diabetes and gastric cancer worldwide, control of gastric cancer in diabetic patients has become a new challenge.

The etiology of gastric cancer remains unknown in type 2 diabetic patients. However, hyperinsulinemia and

insulin-like growth factor (IGF) may play a central role in the promotion of tumorigenesis. A large Japanese case-control study has demonstrated that hyperinsulinemia and serum C peptide are positively correlated with increased risk of gastric cancer^[3]. A high level of insulin increases the bioavailability of serum IGF-1. Both insulin and IGF-1 activate the mitogenic signaling pathways, including the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase pathways^[4]. In addition, imbalance of adipokines in diabetes and obesity, such as the high level of leptin and low level of adiponectin, is associated with the risk of cancer^[5].

Strategies targeting those factors may decrease the risk of cancer. Metformin is an effective drug in the control of insulin resistance in type 2 diabetes. Population studies have revealed that long-term use of metformin can decrease the risk of gastric cancer^[6]. The antitumor effect of metformin may be mediated by activation of the AMP-activated protein kinase pathway or inhibition of the IGF-1 receptor signaling pathway^[7,8]. In the present study, metformin was selected as a positive control in the control of gastric tumorigenesis.

The choice of effective and safe drugs remains limited for gastric cancer. Low-grade gastric dysplasia is a precancerous lesion in the early phase of gastric cancer. Inhibition of the dysplasia is able to block advancement of the precancerous lesion into gastric cancer^[9]. We propose that this strategy may be used in the control of gastric cancer in diabetes and obesity. Traditional Chinese medicine (TCM) represents a rich resource in the treatment of diabetes and cancer^[10,11], and Liuwei Dihuang Pill (LDP) is one of the examples.

LDP is made from a TCM formula that is often prescribed for treatment of diabetes. Studies in rodents have suggested that LDP reduces serum insulin as well as leptin *via* weight loss and fat reduction^[12,13]. LDP also has beneficial effects against certain types of cancers. Several studies have demonstrated that LDP inhibits spontaneous tumorigenesis and suppresses liver tumors^[14,15]. In clinical practice, long-term application of LDP prevents epithelial dysplasia of the esophagus^[16]. These results suggest that LDP is an effective therapy for both type 2 diabetes and cancer. Despite this knowledge, there is no report on LDP activity in the risk control of gastric cancer in patients with type 2 diabetes.

In the present study, we addressed the issue in diabetic db/db mice, which have a high susceptibility to the carcinogen N-methyl-N-nitrosourea (MNU) for gastric cancer^[17-19]. The mice were treated with MNU in drinking water to induce gastric dysplasia. LDP was tested for its ability to inhibit gastric tumorigenesis in the model.

MATERIALS AND METHODS

Animals

Thirty-six male db/db mice and 12 control (db/m) mice

Table 1 Compositions of Liuwei Dihuang Pill

Chinese name	Latin name	Parts used	Weight, g
Shu di huang	<i>Rehmannia glutinosa</i> Libosch	Root	160
Shan zhu yu	<i>Cornus officinalis</i> Sieb	Fruit	80
Shan yao	<i>Dioscorea opposita</i> Thunb	Rootstock	80
Ze xie	<i>Alisma orientale</i> (<i>G. Samuelsson</i>) Juz	Rootstock	60
Fu ling	<i>Poria cocos</i> (Schw.) Wolf	Sclerotium	60
Mu dan pi	<i>Paeonia suffruticosa</i> Andrews	Bark	60

Plant name as verified at <http://www.theplantlist.org>.

at age 4 wk were obtained from the Nanjing University Animal Laboratories, China (Certification No. SCXK 2015-0001). The animal protocol was designed to minimize pain or discomfort to the animals. The mice were maintained in specific pathogen-free conditions with a 12-h light-dark cycle, room temperature of 25 °C ± 1 °C, and relative humidity of 60% ± 5% in the animal facility of Shanghai Jiao Tong University. The mice had free access to regular chow diet and water. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

LDP water extract and dosage

LDP was purchased from Tongrentang Group (Beijing, China). LDP is made of six raw medicinal materials (Table 1) and manufactured in accordance with the quality control standards in the Chinese Pharmacopoeia. The bioactive components of LDP were certified according to the high-performance liquid chromatography-fingerprints, which included loganin, paeoniflorin, paeonol, gallic acid, and morroniside^[20,21]. The LDP was ground, extracted ultrasonically with distilled water for 30 min, and filtered through a screen with 90 µm aperture. LDP is taken orally at 0.15 g/kg per d of herbal materials in humans, and this dosage is equal to 1.8 g/kg per d in mice. LDP was administered through the drinking water at a final concentration of 0.09 g/mL (about 9 g/kg per d) for 10 wk. Metformin (Sino-American Shanghai Squibb Co., Shanghai, China) was dissolved in distilled water at a concentration of 5 mg/mL, as described in a previous report^[22].

Gastric tumor model

MNU (Sigma, St. Louis, MO, United States) was administered to the mice through drinking water 3 times weekly at a concentration of 120 ppm in light-shielded bottles. The administration was performed every other week for 20 wk as described previously^[23].

Study design and samples collection

Following 1-wk acclimation, the mice were randomly divided into four groups. Group A, 12 control (db/m) mice were treated with MNU for 20 wk followed by saline treatment for 10 wk to serve as the non-diabetic control. Group B, 12 diabetic (db/db) mice were treated

with MNU for 20 wk followed by saline treatment for 10 wk to serve as the diabetic control. Group C, 12 diabetic mice were treated with MNU for 20 wk followed by metformin treatment for 10 wk, as the positive control. Group D, 12 diabetic mice were treated with MNU for 20 wk followed by LDP treatment for 10 wk. At the end of treatment (30 wk), all mice were fasted for 12 h and blood samples were collected from the left ventricle for fasting blood glucose (FBG) measurement.

All animals were euthanized with 1% pentobarbital sodium. Serum was collected and stored at -80 °C for biochemical analysis after centrifugation of the blood at 1000 g for 10 min. The stomach was removed and divided into two parts along the greater curvature. One part was quickly frozen in liquid nitrogen and then stored at -80 °C for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis. The other part was fixed in neutral-buffered formalin (40 g/L) for 24 h for histological assessment.

Histological examination of gastric lesions

Fixed stomach tissue was cut longitudinally into three strips. After dehydration and rinsing in xylol, the strips were embedded in paraffin, sectioned at 4-µm thickness, and stained with hematoxylin and eosin. Gastric lesions were classified as chronic gastritis, gastric dysplasia and gastric carcinoma. These lesions were determined independently by two pathologists. The incidence of gastric lesions in each group was presented as percentage of mice with gastric lesions. If two gastric lesions were found in the same stomach, each lesion was counted.

Immunohistochemistry

Paraffin-embedded stomach sections were deparaffinized, rehydrated, and then immersed in 3% hydrogen peroxide to quench endogenous peroxidase. The sections were blocked with 10% goat serum for 20 min followed by incubation with a rabbit anti-Ki67 antibody (1:100; Proteintech, Rosemont, IL, United States) at 4 °C for 12 h. After washing, the tissue sections were incubated with a biotin-conjugated secondary antibody for 60 min and were visualized using diaminobenzidine. Ki67-positive cells were stained brown in the nucleus and were counted in ≥ 10 fields in each slide to obtain the average value.

Biochemical analyses

FBG was measured using a OneTouch glucometer (ACCU-CHEK Performa; Roche, Shanghai, China). Serum IGF-1 and fasting insulin (FINS) were determined with enzyme-linked immunosorbent assay (ELISA) kits (Shibayagi Co. Ltd., Shibukawa, Japan). Serum adiponectin and leptin concentrations were determined with ELISA kits from Crystal Chem (Downers Grove, IL, United States). The homeostatic model assessment of insulin resistance (HOMA-IR) was used to determine insulin resistance by the formula:

Table 2 Sequences of primers for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IGF-1	CTGGACCAGAGACCCCTTIGC	GGACGGGGACTTCTGAGTCTT
IGF-1R	CATGTGCTGGCAGTATAACCC	TCGGGAGGCTTGTTCCTCT
IGF-2	ACAACCTCGATTGAACCACATTC	GAGAGCTCAAACCATGCAAACCT
IGF-2R	TGAATGGTGATCCTTGCCCTC	CCGGTAGCTGTTGGTCTGTC
Insulin receptor	TCAAGACCAGACCCGAAGATT	TCTCGAAGATAACCAGGG
GAPDH	CCAATGTGTCCTCGTGGATCT	GTTGAAGTCGACAGGAGACAACC

HOMA-IR = (FBG × FINS)/22.5. The liver function was examined with serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The renal function was evaluated by measuring the levels of blood urea nitrogen (BUN) and creatinine (Cr) using commercial kits (Nanjing Jiancheng Bioengineering, China).

RNA extraction and qRT-PCR

Total RNA was extracted from the stomach tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and subjected to treatment with DNase I (Takara, Tokyo, Japan) to avoid genomic DNA contamination. RNA (1 µg) was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Takara). Real-time PCR was conducted with SYBR Green PCR Master Mix (Takara) using ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, United States). Primers sequences are shown in Table 2. Gene expression was normalized to the expression of GAPDH gene using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The stomach tissues were ground in liquid nitrogen, homogenized on ice using lysis buffer containing protease inhibitors, and centrifuged at 12000 rpm for 15 min to obtain the supernatant. The concentration of total protein was determined with a BCA protein assay kit (Beyotime Biotechnology Company, Beijing, China). Protein from each mouse was separated at 50 µg/sample using 8% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States). The membrane was blocked in tris-buffered saline with 0.1% Tween-20 and 5% fat-free milk, incubated with anti-phospho-IGF-1R (Tyr1135/1136; Cell Signaling Technology, Danvers, MA, United States), anti-IGF-1R (Proteintech), anti-phospho-AKT (Ser473; Cell Signaling Technology), anti-AKT (Proteintech), and β-actin (Cell Signaling Technology) at 4 °C overnight. After being washed in tris-buffered saline with 0.1% Tween-20 3 times, the membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit antibodies at room temperature for 1 h, and then visualized using an enhanced chemiluminescence kit (Millipore). The protein abundance was determined using the intensity of protein bands using Image-J 1.46r software (National Institutes of Health, Bethesda, MD, United States), and

normalized to β-actin (loading control).

Statistical analysis

Data were expressed as mean ± SE and analyzed using SPSS version 19.0 software (IBM Corp., Armonk, NY, United States). Differences among the groups were evaluated with ANOVA, Bonferroni multiple comparisons test, and Fisher's exact test. Results were considered to be statistically significant at $P < 0.05$ and highly significant at $P < 0.01$. The statistical methods were reviewed by Xu-Hong Hou from the Clinical Epidemiology Center of Shanghai Diabetes Research Institution.

RESULTS

General observations

The diabetic mice had higher body weights than non-diabetic mice. There was no significant reduction in body weight in the diabetic mice treated with LDP compared to the untreated mice (Table 3). In the positive control, the body weight was significantly reduced by metformin. The diabetic mice had higher level of serum ALT relative to the non-diabetic mice. Moreover, both LDP and metformin significantly reduced serum ALT (Table 3). There was no significant difference in serum AST, BUN and Cr among the four groups, suggesting that LDP did not cause toxicity in the liver and kidney. LDP-treated mice had normal appearance without hair depilation, subcutaneous hematoma, and skin ulcers. There was no difference in mortality between LDP-treated and untreated groups (Table 4). All these data suggest that the mice had good tolerance to LDP without toxicity.

Reduction of gastric dysplasia by LDP

It is reported that diabetes increases and metformin decreases the incidence of gastric cancer^[24]. The incidence of gastric dysplasia was significantly increased in the diabetic mice compared to the non-diabetic mice (67% vs 17%; Table 4, Figure 1A and 1B). The incidence was markedly reduced by LDP (10% vs 67%; Table 4 and Figure 1E) or metformin (10% vs 67%; Table 4 and Figure 1D). Metformin was used as the positive control for suppression of gastric tumorigenesis in the diabetic mice. Two diabetic mice had gastric tumors in the untreated group (Figure 1C), while tumors were not observed in the treated groups.

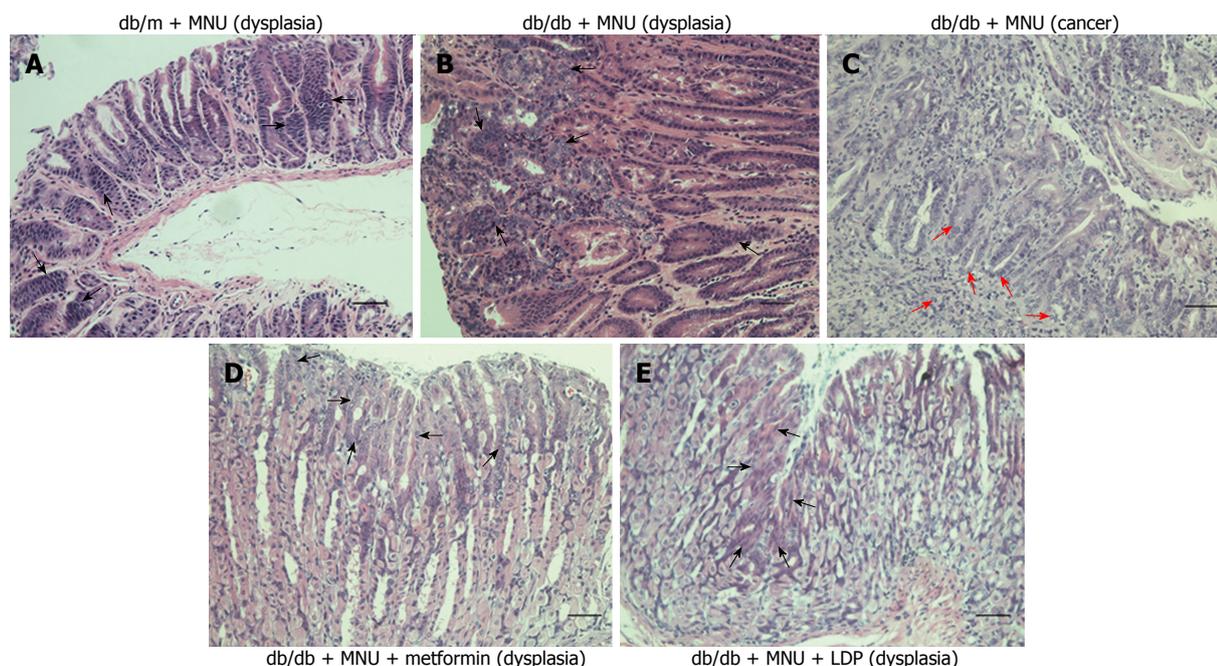


Figure 1 Hematoxylin and eosin staining of stomach tissue. A: Gastric dysplasia in non-diabetic control group (db/m); B: Gastric dysplasia in diabetic control group (db/db); C: Gastric cancer in diabetic control group (db/db); D: Gastric dysplasia in metformin-treated group (db/db); E: Gastric dysplasia in LDP-treated group (db/db). The black arrows indicate cell dysplasia with irregular and hyperchromatic cell nuclei. The red arrows show invasive gastric cancer cells in the submucosa. Bar represents 50 μ m. LDP: Liuwei Dihuang Pill; MNU: N-methyl-N-nitrosourea.

Table 3 Effects of Liuwei Dihuang Pill on body weights and serum biochemical parameters

Group	Body weights at week 20, g	Body weights at week 30, g	ALT, U/L	AST, U/L	BUN, mmol/L	Cr, μ mol/L
db/m + MNU + saline	24.80 \pm 1.08	25.56 \pm 1.78	8.98 \pm 1.78 ^e	8.91 \pm 1.45	4.80 \pm 0.26	13.94 \pm 4.17
db/db + MNU + saline	59.22 \pm 5.61	61.18 \pm 2.10	53.31 \pm 8.31	10.23 \pm 0.90	4.65 \pm 0.52	18.44 \pm 6.28
db/db + MNU + metformin	59.44 \pm 3.38	48.90 \pm 4.99 ^b	23.40 \pm 2.37 ^b	10.79 \pm 1.52	4.64 \pm 0.51	9.45 \pm 3.07
db/db + MNU + LDP	62.79 \pm 5.03	57.96 \pm 3.34	38.08 \pm 4.81 ^a	11.30 \pm 1.14	4.85 \pm 0.45	9.00 \pm 1.50

Data are presented as mean \pm SE. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 vs db/db + MNU + saline group by one-way ANOVA. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDP: Liuwei Dihuang Pill; MNU: N-methyl-N-nitrosourea.

Table 4 Incidence of gastric dysplasia and carcinoma in the experimental mice

Group	Survival	Mortality	Histopathology findings (no. of mice)		
			Chronic gastritis	Gastric dysplasia	Gastric carcinoma
db/m + MNU + saline	12	0	5 (42)	2 (17) ^a	0
db/db + MNU + saline	9	3 (25)	6 (67)	6 (67)	2 (22)
db/db + MNU + metformin	10	2 (17)	6 (60)	1 (10) ^a	0
db/db + MNU + LDP	10	2 (17)	5 (50)	1 (10) ^a	0

Data are presented as *n* (%). ^a*P* < 0.05 vs incidence of gastric dysplasia in db/db + MNU + saline group. Fisher's exact test was used for comparison among groups. LDP: Liuwei Dihuang Pill; MNU: N-methyl-N-nitrosourea.

However, the difference was not significant among the four groups, which may have been related to the small number of mice in each group. The incidence of chronic gastritis did not differ among the four groups. These data suggest that the incidence of gastric dysplasia was markedly decreased by LDP in the diabetic mice.

Decreased expression of Ki67 by LDP

Ki67 is a well-known antigen located in the nucleus and a marker of cell proliferation. We examined the

marker to measure dysplasia. The percentage of Ki67-positive cells was significantly increased by the carcinogen MNU. The induction was attenuated by LDP or metformin (Figure 2). These results suggest that diabetes promoted gastric mucosal proliferation in the MNU tumor model. The proliferative activity was inhibited by LDP and metformin.

Decrease in fasting insulin and IGF-1 by LDP

Hyperinsulinemia, hyperglycemia and IGF-1 elevation

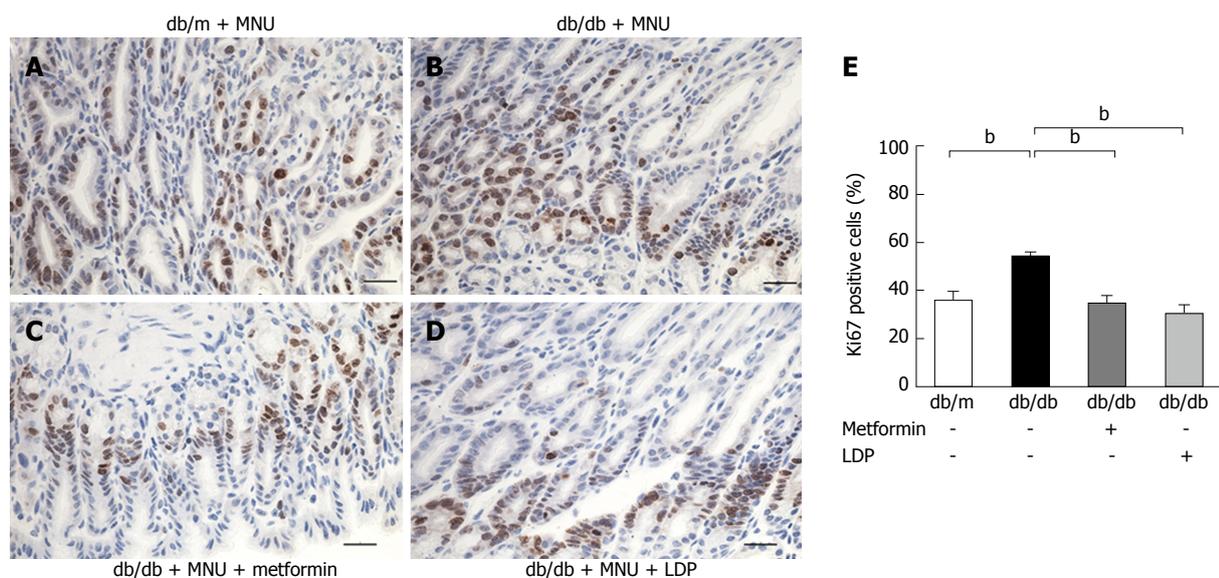


Figure 2 Cell proliferation assay with immunohistological staining of Ki67 in gastric dysplasia. A: Ki67 expression in non-diabetic control group ($n = 2$); B: Ki67 expression in diabetic control group ($n = 6$); C: Ki67 expression in metformin-treated group ($n = 1$); D: Ki67 expression in LDP-treated group ($n = 1$); E: Ki67-positive cells in each group. Results are presented as mean \pm SE. ^a $P < 0.01$ vs control. Bar represents 25 μ m. LDP: Liuwei Dihuang Pill; MNU: N-methyl-N-nitrosourea.

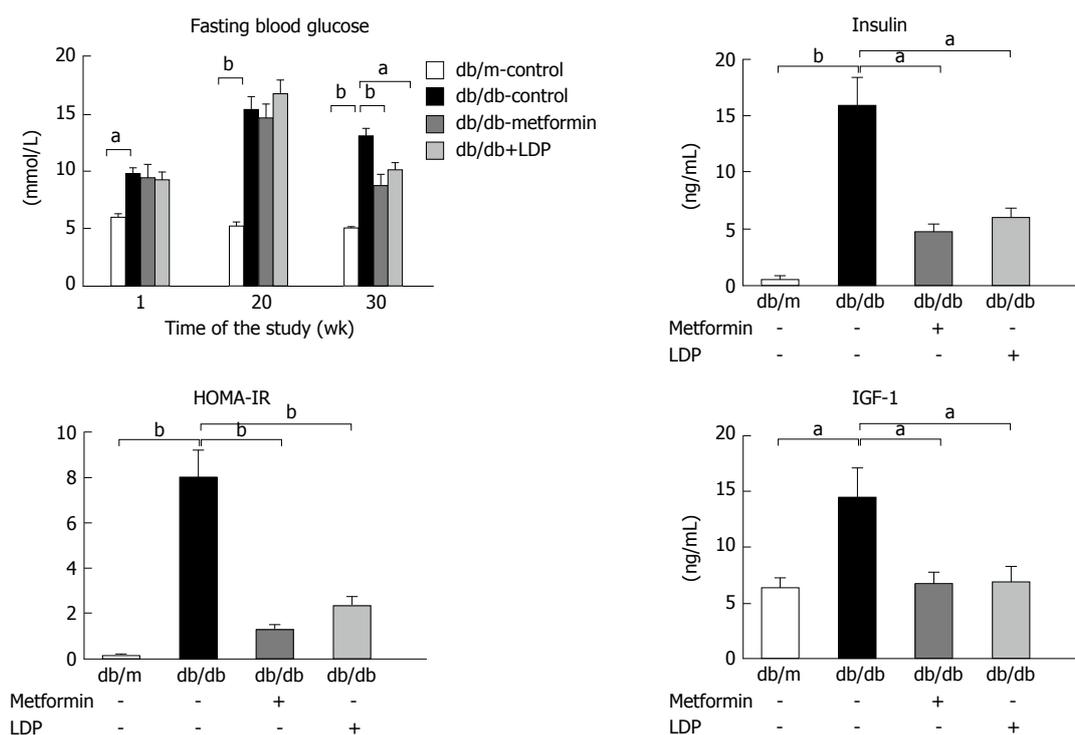


Figure 3 Effects of Liuwei Dihuang Pill on fasting blood glucose, insulin, insulin-like growth factor and insulin resistance. The fasting blood glucose at baseline (weeks 1 and 20) and week 30 was measured. The levels of insulin and IGF-1 were measured with ELISA kits. HOMA-IR was calculated for insulin resistance. Data represent the mean \pm SE ($n = 9$). ^a $P < 0.05$, ^b $P < 0.01$ vs control. ELISA: Enzyme-linked immunosorbent assay; HOMA-IR: Homeostatic model assessment of insulin resistance; IGF-1: Insulin-like growth factor-1.

are important pathological characteristics of type 2 diabetes. These factors have potent activity in the promotion of tumor growth and their activity was significantly elevated in diabetic mice. The elevation was significantly inhibited by LDP or metformin (Figure 3). Insulin resistance was significantly improved by LDP or metformin, as suggested by a reduction in

HOMA-IR (Figure 3), indicating that the LDP activity is related to improvement of insulin sensitivity.

Expression of IGF and insulin receptor

IGF-1 and insulin exert mitogenic effects on cancer cells through activation of their receptors. In this study, their receptor mRNAs were examined by qRT-PCR. The

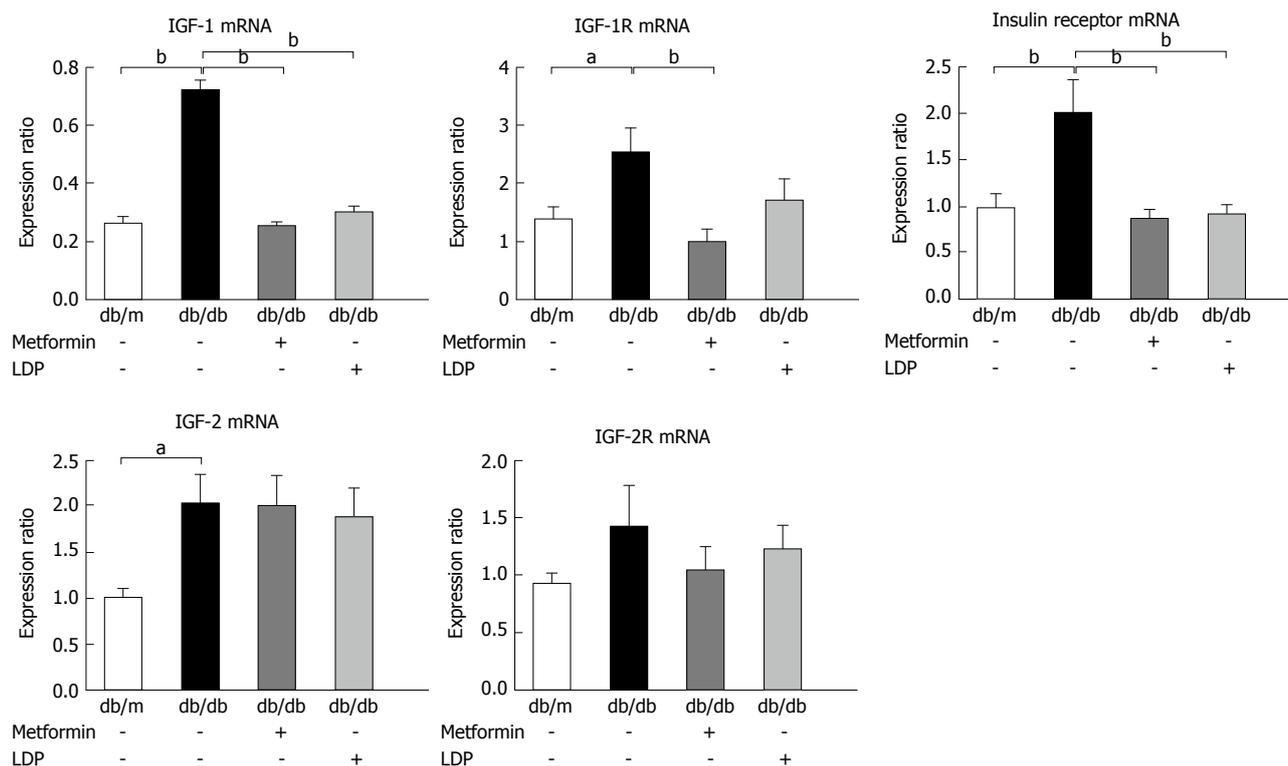


Figure 4 Effects of Liuwei Dihuang Pill on gene expression in gastric tissues. mRNA expression of IGF-1, IGF-1R, IGF-2, IGF-2R and insulin receptor was determined in stomach tissues by qRT-PCR. mRNA expression was normalized to that of GAPDH. Data represent the mean \pm SE. ^a $P < 0.05$, ^b $P < 0.01$ vs control. IGF-1/2: Insulin-like growth factor-1 or 2; IGF-1/2R: Insulin-like growth factor-1 or 2 receptor; LDP: Liuwei Dihuang Pill; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

diabetic mice exhibited an increase in the expression of IGF-1, IGF-1 receptor (IGF-1R), insulin receptor, and IGF-2 mRNAs (Figure 4). LDP inhibited the increases in IGF-1 and insulin receptor, but not in IGF-1R, IGF-2 and IGF-2R (Figure 4). A similar activity was observed for metformin in the regulation of gene expression (Figure 4).

Decreased activation of IGF-1R and AKT proteins in stomach

Insulin and IGF-1 activate their receptor signaling pathways during promotion of carcinogenesis. As a key signaling molecule, AKT plays a central role in cell growth, cell survival, cancer progression and metastasis. Phosphorylation of AKT and IGF-1R was examined along with total AKT and IGF-1R proteins. Phosphorylation was significantly higher in the diabetic mice than in the non-diabetic mice. The signals were significantly down-regulated by LDP or metformin (Figure 5). These data suggest that LDP inhibits activation of the IGF-1/IGF-1R/AKT signaling pathways in the gastric dysplasia tissue of diabetic mice.

Decreased serum leptin and increased serum adiponectin

Adipokine change in the context of type 2 diabetes may be associated with gastric carcinogenesis. Serum adiponectin and leptin were examined in this study to investigate adipokine activity. Adiponectin was

decreased and leptin was increased in diabetic mice compared with non-diabetic mice (Figure 6). Metformin significantly reduced the level of leptin, but did not markedly increase serum adiponectin. LDP significantly increased adiponectin and decreased leptin in the serum of diabetic mice (Figure 6). These data imply that LDP may significantly improve imbalance of adipokines in the diabetic mice.

DISCUSSION

In this study, we established a model of gastric dysplasia in diabetic mice by administration of MNU. Gastric dysplasia was enhanced by type 2 diabetes, which was associated with hyperinsulinemia and IGF-1 elevation. The carcinogenic change was significantly inhibited by LDP in the diabetic mice, as indicated by the reduction in Ki67 signaling. The inhibition was associated with a reduction in serum insulin and IGF-1, suggesting improvement of insulin sensitivity by LDP. Insulin sensitivity was improved by LDP as indicated by the HOMA-IR and the pattern of adipokine change in LDP-treated diabetic mice. These data suggest that LDP may reduce the risk of gastric cancer by improvement of insulin sensitivity in diabetic mice.

Our results suggest that LDP may improve insulin sensitivity in the absence of weight loss. Hyperinsulinemia is the most prominent feature of type 2 diabetes, as a result of insulin resistance. The high

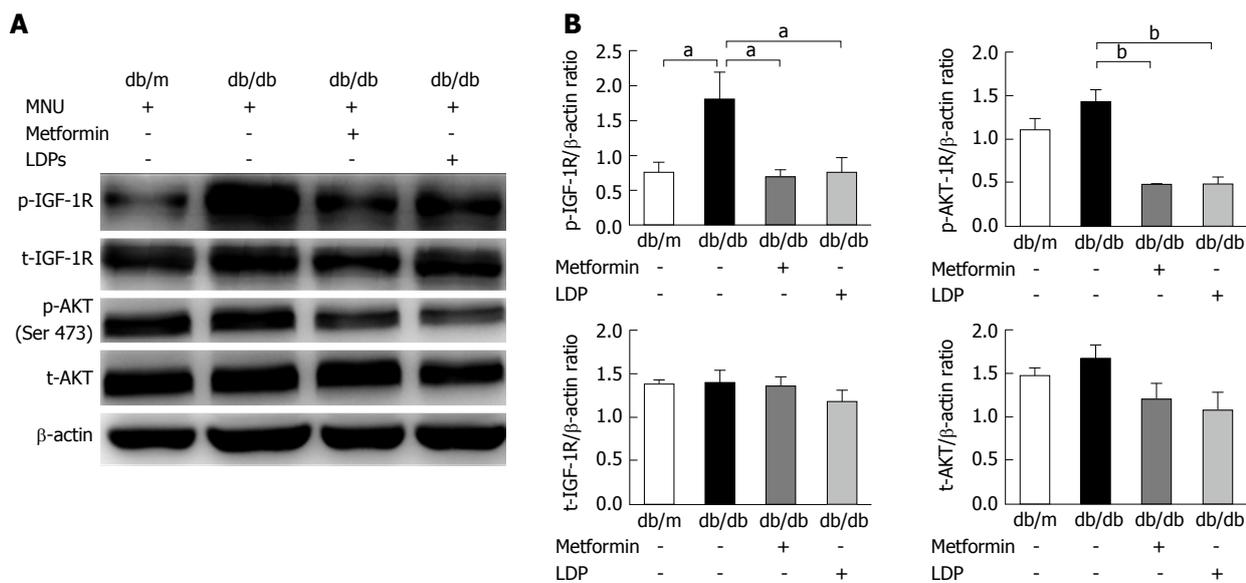


Figure 5 Effects of Liuwei Dihuang Pill on insulin and insulin-like growth factor signaling pathways. A: A representative blot is presented. IGF-1R, AKT and their phosphorylated status were determined by western blotting. β -actin served as a loading control; B: Intensity of protein bands was determined with Image-J 1.46r software. The expression ratios of target proteins relative to β -actin were calculated. Data represent the mean \pm SE. ^a $P < 0.05$, ^b $P < 0.01$ vs control. IGF-1R: Insulin-like growth factor-1 receptor.

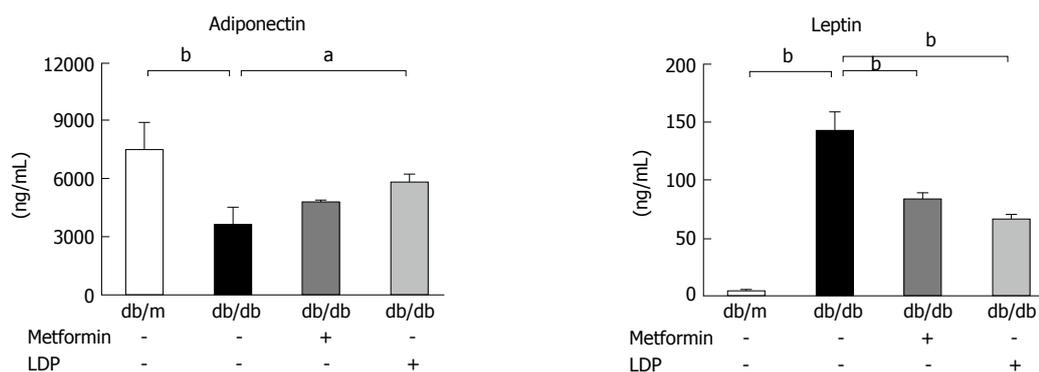


Figure 6 Effects of Liuwei Dihuang Pill on serum adiponectin and leptin. Serum levels of adiponectin and leptin were examined with ELISA kits. Values are the mean \pm SE. ^a $P < 0.05$, ^b $P < 0.01$ vs control. ELISA: Enzyme-linked immunosorbent assay; IGF-1: Insulin-like growth factor; LDP: Liuwei Dihuang Pill.

level of insulin is a risk of gastric cancer for promotion of cell proliferation^[3]. This possibility is supported by a study in mice, in which hyperinsulinemia promoted gastric tumor growth in diet-induced obesity^[25]. LDP increased insulin sensitivity and reduced hyperinsulinemia through weight loss in one study^[12]. However, weight loss was not observed in our study. The discrepancy might be a result of differences in LDP dosage and the animal models. Our data suggest that LDP improves insulin sensitivity without weight loss, although weight loss may contribute to the effect under certain conditions.

A reduction in IGF-1 may have contributed to the cancer preventive effect of LDP in diabetic mice. IGF-1 is a potent growth factor in tumor cells in addition to insulin. IGF-1 promotes cell proliferation and inhibits apoptosis through activation of IGF-1R. Both IGF-1 and IGF-1R are overexpressed in human gastric cancer in most conditions^[26]. Inhibition of the signaling

pathway is associated with suppression of gastric cancer growth and cancer metastasis^[27]. IGF-1 level was elevated in the serum, expression of IGF-1 and its receptor was increased in the lesion tissue of db/db mice. Those alterations were attenuated by LDP in the gastric cancer model. The conclusion was supported by the decrease in phosphorylation of IGF-1R and AKT. IGF-2 and IGF-2R are members of the IGF family. IGF-2 can promote gastric cancer proliferation like IGF-1, while IGF-2R has a tumor-suppressive effect through clearance of IGF-2^[28,29]. Our results suggest that expression of IGF-2 and IGF-2R was not changed by LDP, although IGF-2 expression was elevated in the diabetic mice. These results suggest that inhibition of IGF-1 activity contributes to the LDP effect in the suppression of gastric dysplasia.

AKT is activated by both insulin and IGF-1 in their signaling pathways. AKT promotes tumor formation by stimulation of cell proliferation and inhibition of

apoptosis. These activities are related to activation of cyclin D1 and inhibition of caspase-9, respectively. In gastric cancer, abnormal activation of AKT is widely observed and AKT has become a key target of antitumor agents^[30]. Inhibition of phospho-AKT may represent another antineoplastic mechanism of LDP.

In addition to the insulin/IGF signaling, alteration of adipokines may contribute to the chemopreventive effects of LDP in our model. Adiponectin and leptin are two important adipokines that link type 2 diabetes to cancer. Adiponectin has an anti-inflammatory activity in addition to regulation of cell metabolism, proliferation and apoptosis^[31]. Contrary to adiponectin, leptin has proinflammatory activity^[32], which may increase the cancer risk. Human studies have revealed that a low level of adiponectin and high level of leptin are associated with an increased risk of gastric cancer^[33,34]. Correction of the adipokines' imbalance may contribute to the inhibition of gastric carcinogenesis by LDP. In this study, the effects of LDP on the adipokines are consistent with those reported by Perry *et al.*^[12] in obese rats^[35]. These findings suggest that restoration of the balance of adiponectin and leptin may contribute to LDP activity.

In our study, LDP did not exhibit toxicity, as determined by body weight, mortality, appearance and physical activity of the mice. In addition, there was no significant increase in other parameters including AST, BUN and Cr in LDP-treated mice, suggesting that our dosage of LDP did not generate any adverse effect on liver and renal function. These results suggest that LDP is safe for treatment of type 2 diabetes and control of gastric tumorigenesis.

In summary, LDP exhibited excellent activity in the risk control of gastric tumorigenesis in diabetic mice. The activity was observed without any toxicity. Inhibition of gastric tumorigenesis was associated with reduction in hyperinsulinemia, serum IGF-1, and local IGF-1 signaling in the gastric tissue. Improvement of adiponectin and leptin imbalance may also contribute to the tumor control effect of LDP. The current study shed light on the potential of LDP in the management of both gastric dysplasia and type 2 diabetes.

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COMMENTS

Background

Accumulating studies suggest that type 2 diabetes increases the risk of gastric cancer, and some antidiabetic drugs, such as metformin, reduce the incidence of gastric cancer in patients with type 2 diabetes. Traditional Chinese medicine Liuwei Dihuang Pill (LDP) has a history of thousands of years in treating diabetes, and modern pharmacological research shows that LDP also prevents gastrointestinal tumors. However, it remains unknown whether and how LDP inhibits incidence and progression of diabetes-related gastric cancer *in vivo*.

Research frontiers

The increased incidence of gastric cancer in type 2 diabetes may be associated with insulin resistance, hyperinsulinemia, excessive activity of insulin-like growth factor (IGF)-1, chronic inflammation, and abnormal alteration of adipokines. Therefore, targeting these abnormal metabolic alterations may be a promising strategy for reducing risk of gastric cancer in type 2 diabetes. Recent studies suggest that LDP reduces insulin resistance and hyperinsulinemia, and improves imbalance of adipokines. Investigation of the effects of LDP on the insulin/IGF-1 axis and adipokines will facilitate our understanding of the underlying mechanism of LDP in preventing gastric tumorigenesis in type 2 diabetes.

Innovations and breakthroughs

In the present study, LDP inhibited the early phase of gastric carcinogenesis in diabetic and obese mice, partly by alleviating insulin resistance, reducing insulin/IGF-1 activity and restoring adipokine abnormality. To the best of our knowledge, this is the first study to show the chemopreventive effect of LDP on diabetes-related gastric tumorigenesis and the underlying mechanism.

Applications

LDP may be a potential candidate for preventing gastric tumorigenesis in type 2 diabetic individuals and has value in clinical applications.

Terminology

IGF-1 is a peptide hormone with a similar structure to insulin. Both insulin and IGF-1 exert their mitogenic effects by binding to their receptors. The activated insulin receptor and IGF-1 receptor mediate two pivotal signaling transduction pathways, phosphoinositide 3-kinase/protein kinase B and mitogen-activated protein kinase. Both pathways are involved in cancer cell growth, proliferation, apoptosis and angiogenesis.

Peer-review

This paper describes mechanisms of LDP for prevention of development of gastric dysplasia. This paper has several points to be revised before accepted for publication.

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