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Combination of Luteolin and Solifenacin Improves Urinary Dysfunction Induced by Diabetic Cystopathy in Rats

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Background: The purpose of the present study was to assess the effect of luteolin and solifenacin on diabetic cystopathy (DCP) and to investigate the mechanism of action. A novel link between the overexpression of c-Kit in the bladder and voiding dysfunction was identified in rats with DCP.

Material/Methods: A rat model of DCP was successfully established by intraperitoneal injection of streptozotocin and a diet high in glucose and lipids, and animals were treated with luteolin and solifenacin. The effect of luteolin and solifenacin on urinary dysfunction in DCP rats was investigated by assessing bladder pressure and performing a volume test. The protein levels of c-Kit, stem cell factor (SCF), p110, and phosphorylated p110 in the bladder were detected by Western blot analysis and immunohistochemical staining.

Results: In DCP rats, the protein levels of c-Kit, SCF and phosphorylated p110 in the bladder were significantly increased. However, oral treatment of DCP rats with luteolin combined with solifenacin resulted in effective improvement of overactive bladder and reduced the protein expression of c-Kit, SCF, and phosphorylated p110. Moreover, the effect of luteolin combined with solifenacin on maximum voiding pressure and residual urine volume was improved compared to that of luteolin alone.

Conclusions: Luteolin improved overactive bladder in DCP rats, which may be due to SCF/c-kit inhibition, as well as the down-regulation of the phosphoinositide-3 kinase signaling pathway. Moreover, solifenacin enhanced the potential pharmacological effect of luteolin in the treatment of DCP.

MeSH Keywords: Diabetes Complications • Luteolin • Phosphatidylinositol 3-Kinases • Proto-Oncogene Proteins c-kit

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Background

Diabetic cystopathy (DCP) is a common urological complication, traditionally described as a triad of poor bladder emptying, increased bladder capacity, and decreased bladder sensation. First introduced in 1976 by Moller and Olesen [1,2], DCP is defined as a progressive condition with a spectrum of clinical symptoms and urodynamic findings, with the condition of the bladder ranging from overactive to poorly contractile [3]. However, a lack of clarity and understanding remains regarding the etiology and pathophysiology of CDP. Initially, diabetic neuropathy was thought to be the cause of bladder dysfunction in DCP [4,5]. However, Malmgren et al. [6] suggested that the changed detrusor smooth muscle contractility may be associated with the density and distribution of the muscarinic receptor, intercellular connection and excitability changes, intracellular signal changes, and an abnormal number or function of cells, which may act as a pacemaker of smooth muscle contraction in the bladder.

Non-neuronal interstitial cells of Cajal (ICCs), known to form a network between the smooth muscle and neurons, have an important role in the activity of smooth muscle contraction as pacemakers in gut motility. The c-Kit receptor is known as a stem cell factor receptor [7] and belongs to the tyrosine kinase receptor type III family [8]. It is a transmembrane protein in ICCs [9,10] and has an important role in the maintenance and promotion of normal morphology, development, and function of ICCs [11]. The density of ICCs can be evaluated by determining the expression level of c-Kit. Although ICCs have traditionally been thought to occur in the gastrointestinal tract, ICC-like cells have also been detected in various urological organs, such as the bladder and ureter.

Moreover, the distribution of ICC has been found to be abnormal in several diseases in which urinary function is impaired, such as ureteropelvic junction obstruction (UPJO) [12], megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS) [13], and bladder outlet obstruction (BOO) [14]. This suggests that dysregulation of ICC density in the muscular layer may be associated with urinary dysfunction.

Luteolin (3',4',5,7-tetrahydroxyflavone) is a flavone contained in certain medicinal plants and certain vegetables and spices [15], and displays various biological activities, such as anti-neoplastic [16], pro-apoptotic [17], immune-regulatory [18], anti-metastatic [19], and anti-inflammatory actions [20]. It has been used clinically for the treatment of respiratory inflammatory diseases, cancer, and cardiovascular disease [21,22]. Luteolin has been reported to effectively protect against reactive oxygen species-mediated cell death by inhibiting the phosphoinisotide-3 kinase (PI3K) signaling pathway [23], which is a downstream pathway of c-Kit [24]. Previous studies have indicated that luteolin is a substrate for tyrosinase [15], and the biological and biochemical activities of luteolin may therefore be associated with the inhibition of tyrosine kinase. Solifenacin is now used clinically to improve overactive bladder by antagonizing the muscarinic receptor [25,26]. Due to having different targets, luteolin and solifenacin may have potential pharmacological synergy in the treatment of DCP.

The present study was performed to investigate whether luteolin is able to improve DCP in rats and investigated the underlying mechanism. The potential pharmacological synergy with solifenacin was also assessed.

Material and Methods

Chemicals

Luteolin was purchased from Shanghai Biyu Biotechnology Co., Ltd. (Shanghai, China) and streptozotocin (STZ) was obtained from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Solifenacin was purchased from Beijing Alliesyn Technology Co., Ltd. (Beijing, China). All other chemicals used in this study were of analytical grade and were commercially available.

Animals

A total of 24 male Wistar rats (weight, 180±20 g) were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002). The animals were housed at a controlled temperature of 22±2°C and relative humidity of 60±5% with a 12-h light/dark cycle and *ad libitum* access to water and food. The Ethics Committee of Dalian Medical University approved all animal experiments, which were performed in accordance with institutional guidelines and conducted according to National Research Council guidelines.

Rat model of DCP

Diabetes was induced in rats by intraperitoneal injection of STZ (50 mg/kg) after 12 h of fasting (27). Diabetes was confirmed by measuring tail blood glucose at 0 and 72 h and at 4 weeks after STZ administration. Normal rats were intraperitoneally injected with the same volume of vehicle buffer. After the model was successfully established, rats were randomly divided into 4 groups (6 rats in each group; n=6): i) Control group (normal rats were administered saline for 10 days by oral gavage); ii) DCP group (DCP rats were administered saline for 10 days by oral gavage); iii) Luteolin group [DCP rats were administered luteolin at 10 mg/kg (28) for 10 days by oral gavage]; iv) Combination group [DCP rats were administered luteolin at 10 mg/kg and solifenacin at 0.7 mg/kg (29) for 10 days by oral gavage].

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Bladder pressure and volume test in rats

Prior to surgery, rats were anesthetized by intraperitoneal injection of pentobarbital (35 mg/kg) and then fixed on the operating frame. The upper edge of the pubic symphysis skin was opened to expose the bladder, which was placed in the incision to avoid affecting the abdominal pressure and the detrusor pressure. The bladder was irrigated with saline (0.4 ml/min) via one 24G tube after two 24G tubes were inserted into the bladder and fixed. Furthermore, another tube was connected to a MedLab biological signal acquisition system (Beijing Zhongshi Dichuang Science and Technology Development Co., Ltd., Beijing, China) via a pressure transducer, and the maximum capacity of the bladder, residual urine volume, and maximum voiding pressure were measured.

Morphological changes

After the bladder pressure and volume tests, the rats were sacrificed by intraperitoneal injection of buffered and diluted barbiturates (3% sodium pentobarbital, 200 mg/kg). The bladder was removed and fixed in 10% (v/v) neutral formalin and processed by standard histological techniques, which were used to determine the expression of protein of c-Kit, stem cell factor (SCF), p110, and phosphorylated p110 (p-p110) by Western blot analysis, immunohistochemical staining, and immunofluorescence labeling.

Western blot analysis

Protein was extracted from rat bladder tissue by using a protein extraction kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's instructions, and the protein concentration was determined via the bicinchoninic acid method (BCA kit for protein determination, Solarbio, Beijing, China) with bovine serum albumin (BSA) as the standard. Twenty μg protein was separated by electrophoresis on a pre-cast 10% SDS-polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA), followed by electrotransfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). We used 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) to block membranes for 2 h at 37°C. β-Actin served as a loading control. Membranes were then incubated overnight at 4°C with respective polyclonal antibodies to c-Kit (sc-168), SCF (sc-9132), p110 (sc-7174), and p-p110 (sc-130211) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 1: 1000 dilution and with a 1: 1500 dilution of monoclonal antibody to β-actin (sc-130656) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After washing with TBST, the blots were incubated with a 1: 1000 dilution of goat anti-rabbit IgG-HRP (sc-2004) (Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. After another washing with TBST, enhanced chemiluminescence plus reagent (ECL) from Beyotime Institute of Biotechnology, Inc. was applied. Images were captured by detecting transmitted ultraviolet light using a BioSpectrum-410 multispectral imaging system with a Chemi HR camera 410 (UVP, LLC, Upland, CA, USA). Band densities were semi-quantitatively measured using Image Lab 4.0 (Bio-Rad Laboratories, Inc.).

Immunohistochemical staining

Histological sections (4-μm-thick) of rat bladder were mounted on poly-L-lysine-coated slides. Slides were deparaffinized in xylene and rehydrated in graded alcohols, and then pre-treated with citrate buffer (0.01 mol/l citric acid, pH 6.0) for 20 min at 95°C. Following immersion in PBS containing 3% H_2O_2 at room temperature for 10 min, samples were exposed to 10% normal goat serum in PBS for 30 min at room temperature. Subsequently, sections were incubated at 4°C overnight with rabbit polyclonal anti-c-Kit (dilution, 1: 100). After rinsing with PBS, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (SP-9001, ZSGB-BIO, Beijing, China) for 30 min at 37°C and treated with 3,30-diaminobenzidine chromogen (ZSGB-BIO) for 5 min at room temperature. Finally, sections were counterstained with hematoxylin for 2 min.

Immunofluorescence labeling

After rinsing with PBS, the slides (4-μm-thick) of rat bladder were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 2% BSA for 30 min. The specimen slides were incubated with primary anti-c-Kit antibody at 4°C overnight and then washed 3 times with PBS, followed by incubation with an appropriate Cy3-conjugated Affinipure goat anti-rabbit IgG (H+L) (1: 100; sa00009-4; ProteinTech Group, Inc., Chicago, IL, USA) at 37°C for 1 h. After additional washing, the immunofluorescent images were captured using a Nikon 80i microscope (Nikon, Tokyo, Japan).

Statistical analysis

Significance testing between groups was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard deviation. One-way analysis of variance was used to compare statistically significant differences of data between 2 sets. $P<0.05$ or $P<0.01$ was considered to indicate a statistically significant difference.

Results

Establishment of diabetic rat model

As shown in Table 1, on the 28th day after STZ administration, blood glucose levels in control rats were 6.20 mmol/L, and in the diabetic rats' blood glucose levels were significantly increased

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Table 1. Blood glucose levels in the normal and diabetic rats.

Group	Blood glucose levels (mmol/l)		
	0 h	72 h	4 weeks
Normal	6.47±0.93	6.75±1.42	6.20±1.23
Diabetic	6.48±1.02	23.24±3.98*	21.42±3.27*

[a] P<0.01 *vs.* normal group.

Table 2. Luteolin improves overactive bladder in DCP rats.

	Maximum capacity (ml)	Maximum voiding pressure (cmH_2O)	Residual urine volume (ml)
Control	1.00±0.12	32.42±1.40	0.38±0.08
DCP	1.43±0.17*	20.48±2.24*	1.25±0.45*
Luteolin	1.01±0.08**	27.96±1.88**	0.56±0.12**
Combination	1.10±0.07	31.14±2.12***	0.71±0.07#

* P<0.01 *vs.* Control group; ** P<0.01 *vs.* DCP group; # P<0.01 *vs.* Luteolin group. DCP – diabetic cystopathy.

to 21.42 mmol/L >16.7 mmol/l (30), which indicates that the model of diabetes was successfully established.

Luteolin ameliorates overactive bladder in DCP rats

As shown in Table 2, the maximum capacity of the bladder, maximum voiding pressure, and residual urine volume in the rats in the control group were 1.00 ml, 32.42 cmH_2O, and 0.38 ml, respectively. However, in the DCP rats, the maximum capacity of the bladder and the residual urine volume were 1.43- and 3.29-fold increased, respectively, and the maximum voiding pressure was decreased by 36.83% compared with that in the control group. These results also indicated that the model of DCP rats had been successfully established. After treatment with luteolin, the maximum capacity of the bladder and residual urine volume were reduced by 29.37% and 55.20%, respectively, and the maximum voiding pressure was increased to 1.37-fold of that in the DCP group. These results indicated that luteolin effectively ameliorated overactive bladder in DCP rats. Moreover, solifenacin enhanced the effects of luteolin on improving overactive bladder.

Effect of luteolin on the protein expression of SCF and c-Kit

As shown in Figure 1, the protein expression of SCF and c-Kit in the bladder of rats in the DCP group were increased to 5.75- and 2.79-fold, respectively, of those the control group. However, after treatment with luteolin, the protein expression of SCF and c-Kit was significantly decreased to 46.43% and 69.47%, respectively, of that in the DCP group. However, there were no significant differences in the SCF and c-Kit expression between the luteolin and the combination group.

As shown in Figures 2 and 3, the immunohistochemistry and immunofluorescence results were similar to the Western blot results. The protein expression of c-Kit in the bladder of rats in the DCP group was increased compared with that in the control group. After treatment with luteolin alone or in combination with solifenacin, the protein expression of c-Kit in the rats' bladders was decreased compared with that in the DCP group. However, there was no difference in c-Kit expression between the luteolin and the combination group. The density of ICCs can be evaluated by determining the expression level of c-Kit; thus, these results suggested that the increased density of ICCs in the DCP group could be suppressed by luteolin, and the effect could not be enhanced by combination with solifenacin.

Luteolin inhibits PI3K pathway activity in the bladders of CDP rats

The PI3K pathway is a downstream signaling pathway of SCF/c-Kit; therefore, the present study investigated PI3K pathway activity. As shown in Figure 4, the phosphorylation level of p110 in the DCP group was 5.09 times that in the control group. However, after treatment with luteolin alone or in combination with solifenacin, the phosphorylation level of p110 was significantly decreased to 60.32% and 55.73%, respectively, of that in the DCP group. These results suggested that luteolin significantly suppressed the activity of the PI3K/AKT pathway in DCP rats.

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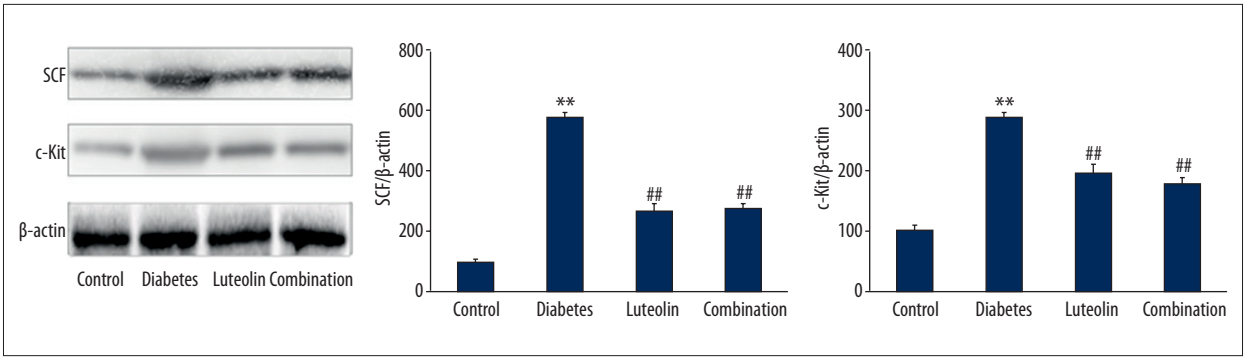

Figure 1. Luteolin suppressed the protein expression of SCF and c-Kit, which were increased in the bladders of rats with diabetic cystopathy. There was no significant difference in the SCF and c-Kit expression between the luteolin and the combination group. The relative expression ratios of each protein were calculated after normalization to β-actin. Values are expressed as the mean ± standard deviation (n=6). ** P<0.01 *vs.* Control group; ## P<0.01 *vs.* diabetes group. SCF – stem cell factor.

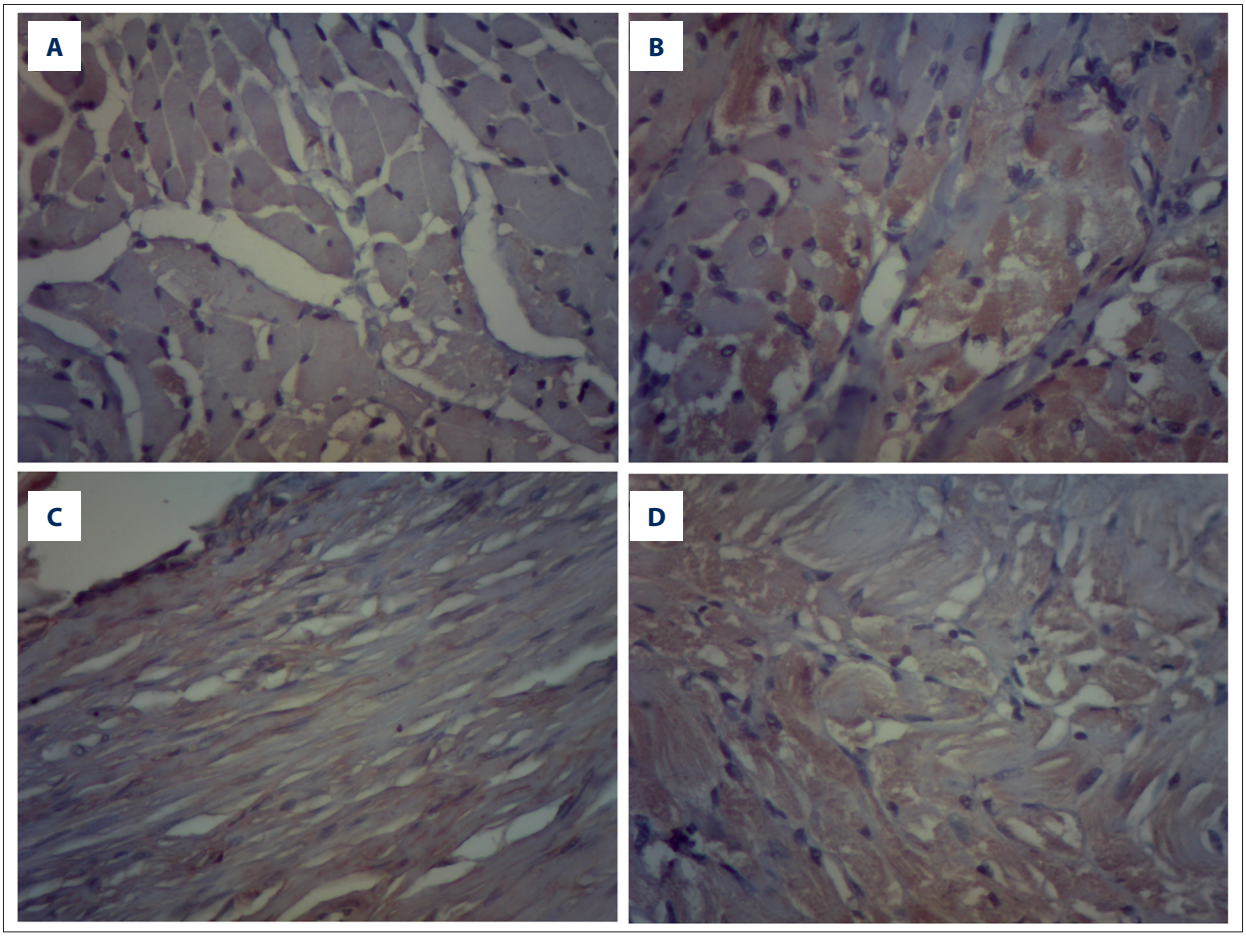

Figure 2. Immunohistochemical detection of the protein expression of c-Kit in the bladders of rats. Luteolin suppressed the protein expression of c-Kit, which were increased in DCP group. There was no significant difference between the luteolin and the combination group. (**A**) Control group, (**B**) DCP group, (**C**) luteolin group, and (**D**) combination group (magnification, ×400). DCP, diabetic cystopathy.

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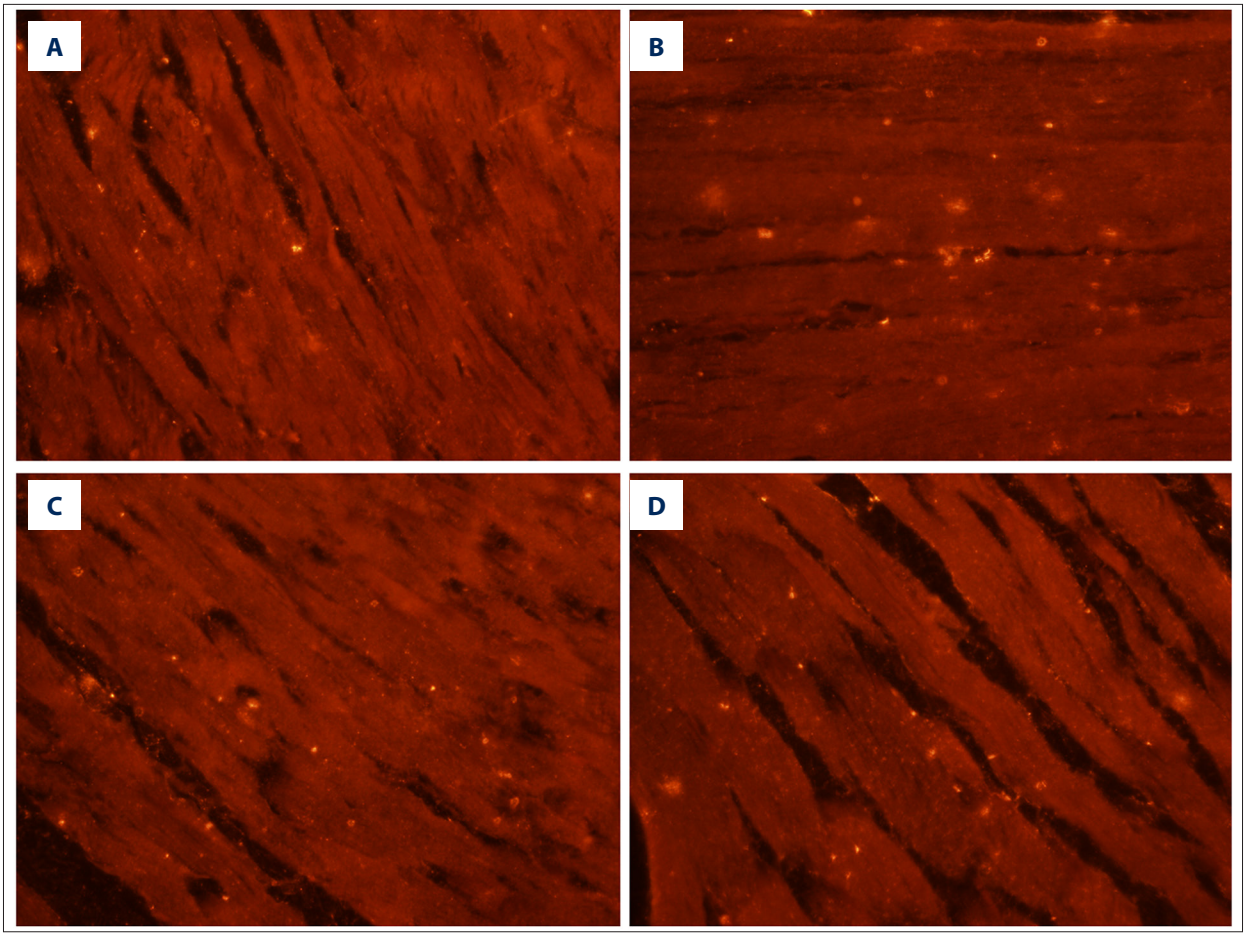

Figure 3. Immunofluorescence detection of the protein expression of c-Kit in the bladders of rats. Luteolin suppressed the protein expression of c-Kit, which were increased in the DCP group. There was no significant difference between the luteolin and the combination group. (**A**) Control group, (**B**) DCP group, (**C**) luteolin group and (**D**) combination group (magnification, ×400). DCP, diabetic cystopathy.

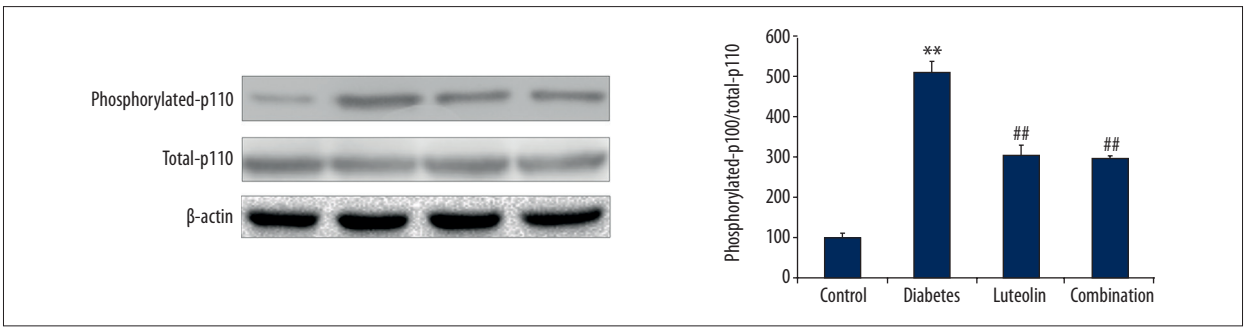

Figure 4. Luteolin inhibited the activity of the phosphoinositide-3 kinase signaling pathway, which was increased in the bladders of DCP rats. There was no significant difference between the luteolin and the combination group. The bar graph shows the relative expression ratio of p-p110 calculated after normalization to p110. Values are expressed as the mean ± standard deviation (n=6). ** P<0.01 *vs.* Control group; ## P<0.01 *vs.* DCP group. p-p110 – phosphorylated p110; DCP – diabetic cystopathy.

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Discussion

Diabetes mellitus, a chronic and systematic metabolic condition, has become a global health problem. The hyperglycemic condition is an important etiologic factor leading to numerous debilitating complications that also influence various cellular and organ functions [31,32]. In 1935, urological complications of diabetes were recognized, including DCP, which have a profound effect on the quality of life of diabetic patients [33]. DCP affects >50% of diabetic individuals with bladder dysfunction [34,35]. The classical symptoms of diabetic cystopathy are a triad of increased bladder capacity, decreased bladder sensation, and impaired bladder emptying, with resultant increased post-void residual volume [36]. However, certain symptoms associated with urine storage, such as overactive bladder, are encountered in the clinic. In the present study, the results of bladder pressure and volume tests showed that compared with those in the control group, the maximum capacity of the bladder and residual urine volume were increased and the maximum voiding pressure was decreased in the rats of the DCP group. These results also indicated that the rat model of DCP was successfully established.

At present, urodynamic examinations are necessary for diagnosing DCP as well as providing estimates of the prevalence rate of DCP. Moreover, the treatment choice for individual patients depends on their clinical symptoms and results of their urodynamic examinations. To date, few studies have evaluated the effect of diabetes mellitus on lower urinary tract symptoms, and some clinicians used to ignore the voiding complaints of patients with diabetes mellitus. Traditionally, diabetic neuropathy was thought to be the cause of DCP; however, the etiology and pathophysiology remain to be fully elucidated. Hence, the association between diabetes mellitus and DCP requires further investigation. A study by Malmgren et al. [6] reported that DCP may be induced by an abnormal number or function of cells acting as a pacemaker of smooth muscle contraction. Studies [12,13] have also suggested that urinary dysfunction, such as congenitally defective peristalsis of the ureter and overactive bladder, may be a result of a decrease or dysfunction of ICC-like cells. For instance, in patients with UPJO, ICCs were reduced at the ureteropelvic junction; ICCs were also reduced in bladders of patients with MMIHS, but in patients with detrusor overactivity induced by BOO, ICCs were increased in the bladder. Therefore, the present study investigated whether ICCs are associated with overactive bladder in DCP. The expression levels of c-Kit, which represents the density of ICCs, were assessed. c-Kit receptor is also known as SCF receptor and belongs to the tyrosine kinase receptor type III family [7,8]. Results of Western blot analysis showed that the protein levels of c-Kit were significantly increased in the bladders of DCP rats, which was inhibited by luteolin. We therefore hypothesized that luteolin improves DCP via reducing the number of ICCs.

SCF binds to c-Kit, leading to the activation of downstream signaling pathways such as the PI3K (23) Ras/extracellular signal-regulated kinase [36], phospholipase C-γ, Janus kinase/signal transducer, and activator of transcription signaling pathways [37,38]. To confirm this speculation, the present study investigated the activity of the PI3K signaling pathway. Phosphorylation of p110 is a crucial process in PI3K signal transduction. Therefore, the phosphorylation of p110 was examined by Western blot analysis, revealing that the phosphorylation level of p110 in the DCP group was significantly increased compared with that in the control group.

As mentioned above, luteolin is known to protect against reactive oxygen species-mediated cell death by inhibiting the PI3K signaling pathway, and based on previous studies, it was assumed that luteolin may ameliorate DCP. To the best of our knowledge, no previous study has assessed the effect of luteolin on ICC activities in DCP. Of note, luteolin significantly suppressed the increases in the expression levels of SCF and c-kit as well as the phosphorylation level of p110 in the DCP group compared with those in the control, suggesting that luteolin suppressed SCF/c-kit and PI3K pathway activity. Based on these findings, luteolin reduces SCF and c-Kit expression, and, at least to a certain extent, participates in suppressing the impairment of urinary function in DCP. Luteolin was able to control the increases in c-Kit expression during DCP.

In recent years, traditional Chinese medicine has attracted increasing attention and has provided a new direction for pharmacological research due to the lower side effects and excellent activity of certain natural products. Moreover, the combination of traditional medicine and medicinal chemistry has become a research focus.

Solifenacin is the selective antagonist of the muscarinic acetylcholine receptor and is now used to treat patients with overactive bladder. Therefore, the present study investigated the potential pharmacological synergy of luteolin and solifenacin in the treatment of DCP in rats. The results of bladder pressure and volume tests showed that the combination of luteolin and solifenacin led to an enhanced improvement in maximum voiding pressure and residual urine volume. However, the protein levels of SCF, c-Kit, and p-p110 determined by Western blot analysis showed no difference between the luteolin and the combination group. These results suggest that luteolin and solifenacin act via different targets, leading to an enhanced pharmacological effect.

Conclusions

Luteolin improves DCP in rats by downregulating the expression of SCF and c-Kit as well as the phosphorylation of p110

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in the bladder. Moreover, luteolin and solifenacin have potential pharmacological synergy in the treatment of DCP.

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