



Effects of flower extract from *Nelumbo nucifera* on high-K⁺ & Ca²⁺-induced colonic contraction in diabetic rats

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Abstract

Gastrointestinal (GI) disorder has been commonly found in diabetic patients. Diabetes can also affect GI motility through changes in intestinal smooth muscle or alterations in extrinsic neuronal control. The exact mechanism of the intestinal is not fully understood. The aim of this study was therefore to investigate the effect of *N. nucifera* flower extract (NNFE) on high K⁺ and Ca²⁺-ion induced colonic contraction in diabetic rats. Male albino Wistar rats were induced with 65 mg/kg b.w. streptozotocin. All groups of rats were daily once oral administered of NNFE (250 mg/kg b.w.) for 8 weeks. The rats were sacrificed by asphyxiation with CO₂. Longitudinal colonic smooth muscle strips of the rats were prepared for SNP-reduced contraction investigation. The results showed that NNFE significantly ($p<0.05$) reduced the contractile responses of colonic smooth muscle strips of the rats in the presence of K⁺ and Ca²⁺-ion. Also, NNFE significantly ($p<0.05$) reduced the responses of the strips in the presence of SNP. The changes in intracellular Ca²⁺ concentrations lead to fusion of insulin-containing vesicles to the plasma membrane and subsequent rapid exocytosis of insulin from β cells. The data indicated that *in vivo* study of the NNFE on hypoglycemic activity, producing 44% reduction in blood glucose levels and inhibited colonic contractile activity in diabetic rats.

Keywords: *Nelumbo nucifera*, Hypoglycemic activity, Diabetic rats, Colonic contraction

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1. Introduction

Diabetic gastrointestinal (GI) disorders are one of the complications, generally found in diabetic patients. These complications and their symptoms are often caused by abnormal GI motility, which is a consequence of diabetic autonomic neuropathy involving the GI tract. The disorders are occurred as autonomic neuropathy, which are usually happened in the patients who have been experiencing with diabetes for a long time [1], [2]. Diabetic GI disorders are reported to be related with severe small and large bowel motility as a result of intestinal nerve damages [3], [4]. It can cause diarrhea in diabetic patients frequently, known as diabetic diarrhea [5], [6], [7]. This diarrhea results from increasing of colonic muscle contraction frequency, induced by high concentrations of potassium and calcium ions in intestinal smooth muscles. High concentration of potassium depolarizes the smooth muscle cell membrane and opens voltage dependent calcium channels, resulting in an influx of extracellular calcium and an activation of contractile machinery [8]. In order to inhibit the mentioned mechanism, ATP-sensitive potassium channel, diabetic patients are usually treated with glibenclamide. The drug can inhibit opening of L-type calcium channels, leading to reduction of calcium influx and subsequently relaxation of smooth muscles [9]. It has been reported that the relaxation effects involve increasing of cGMP levels and activation of cGMP-dependent protein kinase (PKG) [11]. However, those drugs can cause several side

effects on the patients, including feeling or being sick, constipation, and signs of low blood sugar (glucose). Therefore, to overcome the problem, medicinal herbs and plant extracts have been suggested to use as a drug replacer. Nowadays, there are more than 1,200 plant species that can be used as alternative ways to treat diabetic patients and a number of plants were reported to have an effective hypoglycemic activity [12].

Nelumbo nucifera or lotus is one of the plants that have been used for its medicinal properties since ancient times. Lotus extract derived from its rhizome showed the hypoglycemic activity in diabetic rats [13]. In addition, it was found that methanolic extracts of the rhizome at doses of 200, 300 and 400 mg/kg could reduce spontaneous activity and muscle relaxant activity as well as inhibiting painful responses in mice. *N. nucifera* extract was also proved to have antioxidant activity [6], [14] sedative and antispasmodic effects in the intestine, resulting in diarrhea alleviation. Alcoholic extract of *N. nucifera* flowers was mentioned to have a potential to be used as a diabetic treatment due to its high levels of antioxidant activity [6], [13].

There are a number of diabetic rat studies reporting effects of plant extracts on alleviation of GI disorder symptoms. However, most research focuses on upper GI symptoms, especially gastroparesis [15], [16], [17]. Few studies were conducted to examine effects of plant extracts on colonic symptoms in diabetic rats and the results

were contradicted as well as unclear [6], [18]. Therefore, the aim of this present study was to investigate effects of lotus flower extract on colonic muscle contraction, induced by using KCl and CaCl_2 and subsequently inhibited by treating with SNP in 8-week old streptozotocin (STZ)-induced diabetic rats [3]. Hypoglycemic activity and plasma insulin levels were determined as they represented the presence of a low blood sugar [19], [20], [21], and resistant and mucosal absorption or decrease of secretion, respectively [22]. The results were compared with the use of glibenclamide, a drug commonly used in diabetic patients [23], [13].

2. Materials and Experiment

2.1 Chemicals

All chemicals were purchased from Sigma unless stated otherwise. STZ was purchased from Sigma-Aldrich Co Ltd, United Kingdom.

2.2 Plant material

The flowers of *N. nucifera* were collected from the local of fresh water in Khonkhean province, Thailand [13]. The identity of the plant materials was confirmed at Mahasarakham University, Thailand. A voucher specimen was maintained for the future reference in the department of Biology, faculty of Science, Mahasarakham University, Thailand, and the voucher number is code: MSU.SCI-BI-P001.

2.3 Preparation of *N. nucifera* flower extract (NNFE)

The corollas were spited from the flowers for washing and weighing fresh or dried specimens. The specimens were cut into small pieces and then dried in an oven at a 50°C for 72 h. Dried materials (100g) were coarsely powdered and macerated with 95% ethanol extract (600 mL; 1:6) for 7 days. The extract was dried using a rotary evaporator (Heidolph Laboratory 4000, Germany) followed by a freeze dryer (Christ Alpha 1-4, Germany) to get a powder of percent yield. The stock solution of the NNFE was stored at -20°C until use.

2.4 Animal

Male albino Wistar rats weighing 200-250 g purchased from the National Laboratory Animal Centre (NLAC), Mahidol University, Thailand, were used in this study. They were acclimatized in an air conditioned room at $25\pm2^\circ\text{C}$, 12-h light/12-h dark cycle and relative humidity 40-60%, and given a standard laboratory chow and watered ad libitum for 7 days prior to the commencing experiment.

The rats were maintained in accordance with the guidelines of the Committee Care and Use of Laboratory Animal Resource, National Research Council, Thailand and performed in accordance with the advice of the Institutional Animal Care and Use Committee, Mahasarakham University, Thailand (License No. 0005/2011).

2.5 Induction of diabetes

The rats were injected intra-peritoneally with a single dose of 65 mg/kg b.w. STZ. It was dissolved freshly in cold 20 mM citrate buffer adjusted to pH 4.5 [24], [25]. After injection, Rats were provided with 2% sucrose solution for 48 h to alleviate the discomfort after initiating the hypoglycemic phase [26]. The induction with STZ-induced diabetes was confirmed by determining the glucose level; FPG (fasting plasma glucose). The rats with FPG were higher than 126 mg/dL were used in the experiments [27].

2.6 Experimental design

The rats were randomly assigned into four groups with eight rats in each; Group I: normal controls, Group II: diabetic controls, Group III: diabetic rats treated with 0.25 mg/kg b.w. glibenclamide (GB), and Group IV: diabetic rats treated with 250 mg/kg b.w. NNFE. The normal controls and diabetic controls were administered with 0.05% ethanol orally and daily for eight weeks. NNFE and GB were suspended in 0.05% ethanol prior to the oral administration using an orogastric tube.

2.7 Measurement of fasting plasma glucose

The animals were fasted overnight before blood sample collecting. The blood was taken from tail vein of the rats. The FPG was measured once a week for 8 weeks) by using Glucometer (Accu-check Advantage II, Roche, Germany).

2.8 Measurement of serum insulin

After eight weeks of treatment, the rats were fasted overnight and sacrificed under by

cervical dislocation technique. The blood sample were drawn from the rat hearts and centrifuged with 3000 rpm for 10 min twice to separate blood serum. The serum insulin was estimated by using the radioimmunoassay kit (MP Biomedicals-Orangeburg, USA) and detected by an automatic gamma counter (Wallac, 1470 Wizard, Perkin Elmer instrument, Germany).

2.9 Measurement of Tension

The whole longitudinal colonic smooth muscles were prepared as the strips including mucosa, circular layer and neuronal plexus. The colonic smooth muscle strips ($2 \times 2 \times 15$ mm) were mounted vertically under resting tension of 1 g in a single bath chamber containing 25 mL (PowerLab system, ADInstruments Pty Ltd, Australia). The strips were attached and suspended in a chamber containing Kreb's solution with 5% CO_2 -95% O_2 at 37°C as described previously [21]. After an equilibrium period (spontaneous contraction) of 45 min, each strip was added agonists (KCl or CaCl_2) and antagonist (SNP) at different concentrations (0, 1, 10 and 100 μM) for 15 min followed by twice washing with Kreb's solution during 15 min period [22]. The recordings were analyzed by measuring the frequency (number of cycles/15 min), amplitude (average height in cm) which were taken as measures of the force of smooth muscle contraction [21].

2.10 Statistical analysis

FPG data and serum insulin were presented as mean \pm S.E.M. and "n" represents the number of samples, each one from a different

animal. Significance was tested using appropriate F test or One-way ANOVA followed by Scheffe's test and p values < 0.05 taken to be significant. Results were then express as percentages of control contractions (i.e. the control is 100%).

Colonic contraction data were recorded and analyzed using SPSS processing. Parameters were measured and presented as means \pm S.E.M. for tension recording studies. In this case of tension recordings, the amplitude and frequency of contraction were standardized as a percentage of the maximal contraction induced by KCl, CaCl_2 (agonists) but muscle reduced contraction by SNP (antagonist) in normal and diabetic rats (untreated,

treated with glibenclamide or NNFE). Pre-and post-drug/NNFE for eight weeks (*in vivo*) comparisons were computed using Student's paired *t* test, and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Results

3.1.1 Effect of NNFE on Fasting

Plasma Glucose (FPG)

Our investigation, NNFE possess a significantly ($p < 0.05$) hypoglycemic effects in the STZ-induced diabetic rats compared with diabetes control *in vivo* study for eight weeks (Fig.1).

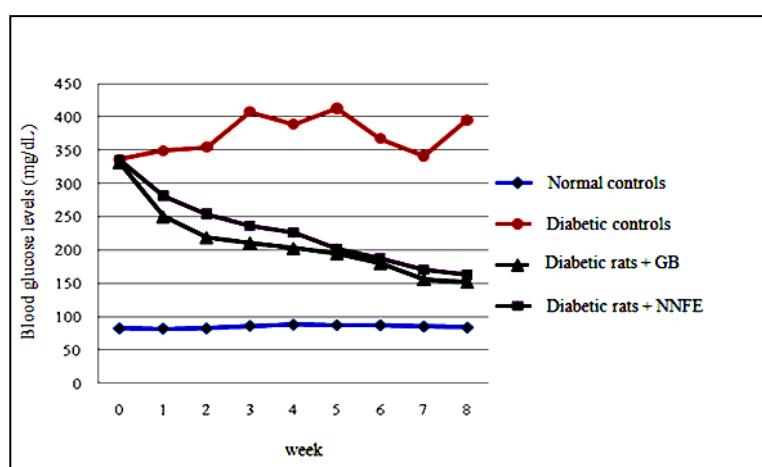


Fig. 1 The effect of nnfe on blood glucose levels in normal controls, diabetic controls, diabetic rats treated with glibenclamide (gb) and diabetic rats treated with nnfe (n=8).

As shown in Fig.1, an oral administration of NNFE at a dose of 250 mg/kg to the diabetic rats for eight weeks showed that NNFE had no effect on FPG of normal controls and diabetic control rats. However, they had significantly ($p < 0.05$) lowered

FPG in diabetic rats treated which was close to that in GB treated diabetic rats.

3.1.2 Effect of NNFE on Serum Insulin

The serum insulin affected by NNFE was depicted in Table 1.

Table 1: The effect of nnfe on serum insulin levels in normal controls, diabetic controls, diabetic rats treated with glibenclamide (gb) and diabetic rats treated with nnfe (n = 8)

| Groups | Insulin (μIU/ml) |
|---------------------------|-------------------------|
| Normal controls (I) | 21.46±1.87 ^b |
| Diabetic controls (II) | 10.10±0.99 ^a |
| Diabetic rats + GB (III) | 17.92±1.08 ^b |
| Diabetic rats + NNFE (IV) | 16.47±1.25 ^b |

The p-value for insulin levels of NNFE treated are significantly different from the control (p<0.05). Mean ± S.E.M. Within the same column are given by the different letter (a, and b).

As shown in Table 1, the effect of NNFE on the serum insulin levels in diabetic rats after eight weeks of treatment.

Serum insulin in the diabetic rats treated with NNFE showed significantly (p < 0.05) higher than in the diabetic controls. However, serum insulin level in NNFE was not significantly different from the normal control rats and GB treated diabetic rats.

3.1.3 Contractile response of colonic tissues

In vitro spontaneous of colonic contraction was observed before stimulating with KCl, CaCl₂-induced or SNP-inhibited contractility at a concentration of 100 μM (n=18). The frequency of contraction in normal controls (I), diabetes controls (II) diabetic rats treated with glibenclamide (GB), (III) and NNFE (IV) had been shown for 45 min as 1.42±0.10, 2.08±0.22, 1.57±0.13, and 1.38±0.16 time/min, respectively. The

tension of contraction was slightly decreased in group I, IV and II at 0.44±0.05, 0.82±0.18 and 0.94±0.27 g, but significantly enhanced in diabetic rats (group III) with 1.69±0.13 g (as shown in Table 2).

Table 2: Spontaneous colonic contraction of normal and diabetic rats kreb's solution

| Groups | Spontaneous contraction | | |
|---------------------------|-------------------------|------------------------|----|
| | Frequency (time/min) | Tension (g) | N |
| Normal controls (I) | 1.42±0.10 ^a | 0.44±0.05 ^a | 18 |
| Diabetic controls (II) | 2.08±0.22 ^b | 0.94±0.27 ^a | 18 |
| Diabetic rats + GB (III) | 1.57±0.13 ^{ab} | 0.69±0.13 ^b | 18 |
| Diabetic rats + NNFE (IV) | 1.38±0.16 ^a | 0.82±0.18 ^a | 18 |

The p-value for frequency and tension of NNFE treated are significantly different from the control (p<0.05). Mean ± S.E.M. Within the same column are given by the different letter (a, b and ab).

3.1.4 Effects of external KCl, CaCl₂ and SNP on colonic contractile activities in normal control rats

As shown in Fig.2A, after spontaneous for 60 min of smooth muscle contractions (100% of control), the strip of rat's colon was incubated in Krebs solution under 1 g of frequency and tension had equilibrium [21], [22]. In isolated tissues of normal rat, colon-dependent contraction induced by KCl (100 μM), and CaCl₂ (100 μM) were significantly (p<0.05) increased the frequencies to 132 and 137% and the tension was significantly increased from 143 to 149%, respectively compared with spontaneous contraction.

It had been shown that SNP (100 μM) significantly (p<0.05) inhibited both frequency and

tension contractile activities with $4.23\pm2.44\%$; $71.22\pm1.30\%$, respectively compared with control (100%).

3.1.5 Effects of external KCl, CaCl₂ and SNP on colonic contractile activities in normal-treated with NNFE rats

As shown in Fig.2B, the effects of KCl, CaCl₂ and SNP on colonic contraction were tested for spontaneous contractility (100%). The mean values of normal rats treated with NNFE (250 mg/kg)-induced contraction by KCl, CaCl₂ and SNP did not cause significant changes of contraction frequency with $98.95\pm1.98\%$, 99.81 ± 1.98 , 63.63 ± 2.36 and tension with $101.26\pm1.38\%$, $102.31\pm1.22\%$, $85.29\pm3.47\%$ respectively. However, in the presence of SNP at the concentration of 100 μM (n=18) in normal strips, contraction was slightly decreased the frequency to $89.43\pm1.56\%$ and tension to $86.49\pm1.42\%$ compared with spontaneous contraction (100%).

3.1.6 Effects of external KCl, CaCl₂ and SNP on colonic contractile activities in diabetic control rats

In case of the influence of STZ-induced diabetes study, in previous experiments, the strips of colon tissues were taken *in vivo*. An application of KCl, CaCl₂-induced and SNP-inhibited colonic contraction was tested for spontaneous contractility (100%). As shown in Fig.2C, the mean values of contraction frequency showed significant ($p<0.05$) changes to $113.34\pm1.04\%$, $129.21\pm1.48\%$, $58.442\pm1.56\%$ and contraction

tension to $123.42\pm1.05\%$, $131.21\pm1.67\%$, $64.62\pm1.98\%$ respectively. However, in the presence of SNP at the concentration of 100 μM (n=18) in normal and diabetes strips reduced contraction was slightly decreased the force lower than spontaneous contraction.

3.1.7 Effects of external KCl, CaCl₂ and SNP on colonic contractile activities in diabetic-treated with NNFE rats

The hypoglycemic activity *in vivo* was produced by the treatment of NNFE 250 mg/kg) of diabetic rats. In the external Ca²⁺ concentration mediated an increase in Ca²⁺-induced a transient contraction that was apparently due to Ca²⁺ release from the SR store [10]. As seen in Fig.2D, CaCl₂ produced concentration related contractile response of the colonic strips. The high- frequency and tension of extracellular KCl and CaCl₂-induced colon smooth muscle contraction were increased significantly ($p<0.05$) at the concentrations of 100 μM for NNFE (250 mg/kg; *in vivo*)-treated diabetes to $121.67\pm2.18\%$, $129.82\pm3.65\%$ and $127.37\pm5.20\%$, $139.53\pm12.18\%$, respectively. However, the mean values of frequency and tension contraction in the present of SNP was significantly reduced to $58.25\pm1.36\%$ and $67.71\pm1.36\%$, respectively compared with control.

3.1.8 Effects of external KCl, CaCl₂ and SNP on colonic contractile activities in diabetic-treated with glibenclamide rats

Glibenclamind inhibit the ability of the oral hypoglycemic agent in lowering the blood

glucose of diabetic rats [28], [29]. It works by binding to and activating the sulfonylurea receptor 1 (SUR1), the regulatory subunit of the ATP-sensitive potassium channels (KATP) in pancreatic beta cells. This inhibition causes cell membrane depolarization opening voltage-dependent calcium channel. This results in an increase in intracellular calcium in the beta cell and subsequent stimulation of insulin release [30].

As shown in Fig.2E, colonic contraction induced by KCl and CaCl_2 stimulated frequency and tension contractile ability. The influence effects of glibenclamide on KCl and CaCl_2 induced spontaneous contractions were significantly increased the contraction frequency and tension to 124 to 130% and from 126 to 136%, respectively compared with 100% of the control.

When 100 μM SNP was applied to spontaneous contraction in the present of KCl and CaCl_2 , it gradually decreased the frequency and tension contraction to $65.34 \pm 6.19\%$, $62.76 \pm 6.19\%$, respectively compared with control (100%).

3.2 Discussion

Under control conditions, the effects of NNFE (250 mg/kg. body weight) affected hypoglycemic activities *in vivo* and reduced spontaneous contraction of colonic smooth muscle in the diabetic rats compared with the control *in vitro* study. The decrease in activity was observed in diabetes colon disorder is directed towards inhibition of smooth muscle contraction [31]. The diabetes rats were additionally treated with NNFE

(250 mg/kg, $n = 6$), the flower extract was selected for their use to treat diabetes for eight weeks in earlier study. However, the stimulation with KCl-induced colonic contraction was produced by elevation of external KCl to 100 μM . This caused a rapid increase in force and depolarization [30], [31]. It showed similar effects to Ca^{2+} -induced on spontaneous, one of NNFE constituents, it can produce greater stimulated colonic contraction in diabetic rats than those induced by KCl. As a result showed its inhibitory effects of SNP were observed significantly reduce colonic contraction with higher (100 μM) concentration of the NNFE.

In addition, the colonic contraction induced by CaCl_2 and KCl, which play a vital role in agonist-induced contraction [32] was a direct affected on smooth muscle. It is well known that herbal medicines are traditionally used for their spasmolytic activities. Our data suggest that the decrease of colonic contractile activities in diabetes with NNFE-treated may act through NO pathway but not involving L-type calcium channels. Similarly, the Ca^{2+} -ATPase inhibitors, SNP (100 μM), produced contraction which were greater than KCl-induced contraction in diabetes tissues [32], [33], by increases in cGMP levels and activation of cGMP-dependent protein kinase [34], [35], [36]. The several smooth muscle studies support the supposition that K channels have an important role in regulating corporeal smooth muscle tone and thus [37], *N. nucifera* flower has considerable reputation as a potent act in the treatment of various ailments such as cancer,

hypertension, diarrhea, fever, weakness, infection and muscle relaxant activity [36], [38], [39]. The major constituents isolated from the lotus plant are alkaloids (liensinine, neferine, nuciferine, remrefidine and isoliensinine) and flavonoids (+)-1(R)-coclaurine, (-)-1(S)-norcoclaurine and

quercetin 3-O-*b*-D-glucuronide). Recently, the leaf of *N. nucifera* showed the hypotensive effect that was mediated by vasodilatation via nitric oxide [35], [36]. In Thai folk medicine, the decoction of *N. nucifera* leaves has been used for a treatment of hypertension.

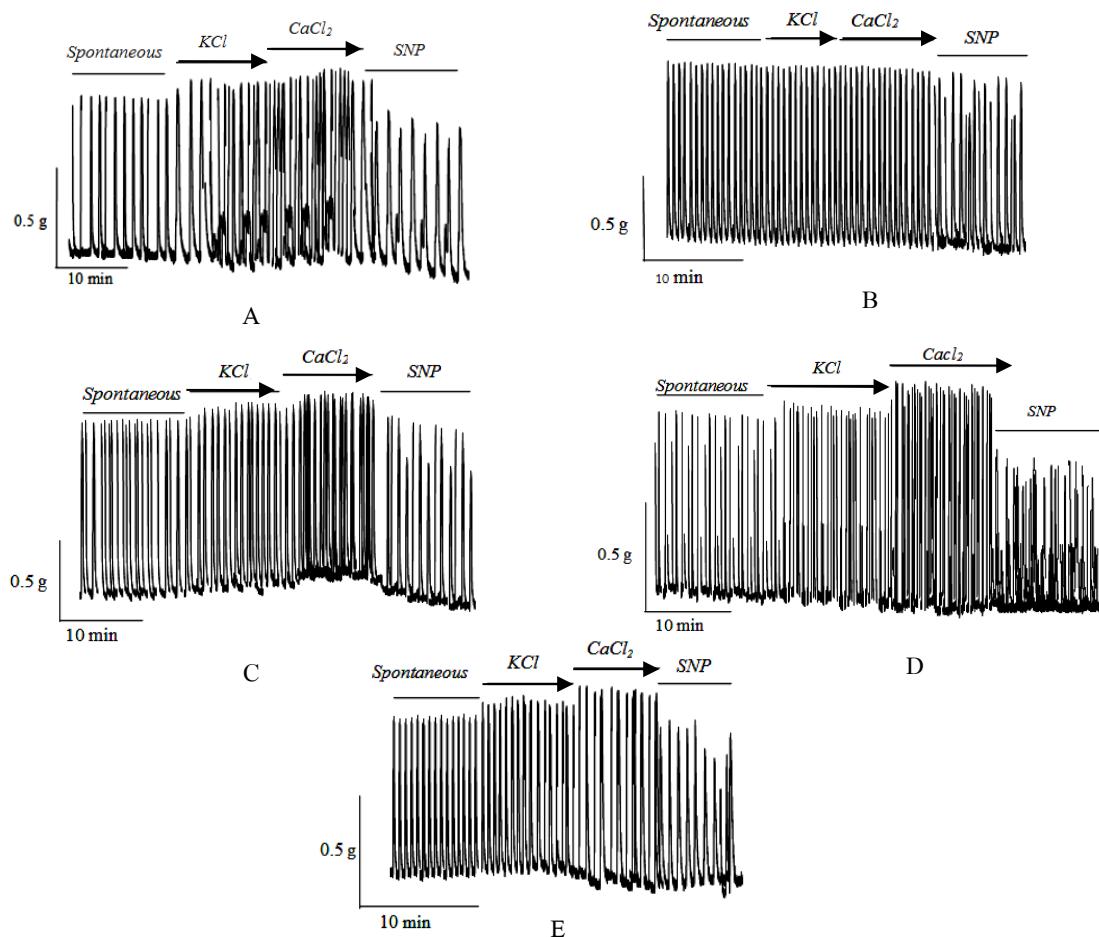


Fig.2 Effects of external KCl , $CaCl_2$ and SNP at $100 \mu\text{M}$ on spontaneous contractility in normal control rats (a), normal rats-treated with NNFE (b), diabetic control rats (c), diabetic rats-treated with NNFE (d), and diabetic rats-treated with glibenclamide (e)

The binding of agonists to the incretin receptors results in the production of cAMP via adenylyl cyclase (AC) activation and subsequent activation of PKA [33], [35], [37] and the Epac family of cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), which leads to the elevation of intracellular Ca^{2+} levels via a depolarization of plasma membrane by inhibition of KATP and KV channels after ATP generation from glucose and consequent opening of voltage-gated L-type Ca^{2+} channels. Intracellular Ca^{2+} levels are further increased via stimulation of IP3R and RyR on the ER. Long-term GLP-1 treatment also stimulates the expression of GLUT2 transporter and glucokinase (the β -cell glucose sensor), which lead to an increased in mitochondrial ATP synthesis. In addition, L-type Ca^{2+} channels are phosphorylated by PKA, resulting in an increase in their open probability and thus facilitation of enhanced Ca^{2+} influx [38]. The changes in intracellular Ca^{2+} concentrations lead to the fusion of insulin-containing vesicles to the plasma membrane and subsequent rapid exocytosis of insulin from β cells.

4. Conclusion

In summary, this study indicated and presented evidence that rapid increases $[\text{Ca}^{2+}]_i$ in smooth muscle [39], [40] induced by activating (high- K^+ depolarization [41]) stimulus with two temporally distinct separated events: 1) the well-established activation of myosin light chain kinase (MLC) kinase causing rapid, strong increases in MLC phosphorylation and cross-bridge cycling,

producing a peak contraction [39], [40], [41], and 2) Ca^{2+} -calmodulin-activated increases in ROK translocation to caveolae, leading to maintenance of tonic force (shown here). In conclusion, these data support the hypothesis that any stimulus increases $[\text{Ca}^{2+}]_i$ in colonic smooth muscle will cause increased ROK translocation to caveolae at the cell periphery, where additional signaling events like activation of ZIP-like kinase.

The results are clearly indicative of the beneficial effects of NNFE in reducing lateral side effects of hyperglycemic and reduction of smooth muscle contraction in diabetic rats. These data demonstrate that NNFE has protective effects on K^+ and Ca^{2+} -induced colonic contraction in rats by 250 mg/kg.bw intake. Therefore, it could be applied for reducing risk of mild *diarrhea* and constipation.

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