



Evaluation of spasmolytic effects of naringenin on ileum contraction and intestinal charcoal meal transit: Involvement of ATP-sensitive K⁺ channels

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ABSTRACT

Introduction: Naringenin is a flavonoid constituent of many herbal plants, including citrus fruits. Biological studies have suggested various therapeutic effects for naringenin, including protective effects on gastrointestinal (GI) motility. The present study was performed to investigate the involvement of ATP-sensitive K⁺ channels on the effect of naringenin in rat ileum motility.

Methods: Ileum contractions were induced by either KCl or acetylcholine (ACh) *in vitro*. Inhibitory concentration-response curves were constructed for naringenin and diazoxide after exposure of rat isolated ileum to KCl (20mM) or ACh (500nM). The relaxant effects of naringenin and diazoxide were also examined in the presence of glibenclamide. Furthermore, oral effects of diazoxide (25 mg/kg) and naringenin (25, 50 mg/kg) were also assessed on the intestinal charcoal meal transit in mice (n=10) in the absence and presence of glibenclamide (50 mg/kg).

Results: Diazoxide and naringenin in a concentration-dependent manner inhibited ileum contractions induced by low bath concentration of KCl (20mM). However, both drugs had no effect on contractions induced by a high concentration of KCl (160mM). The inhibitory effects of diazoxide and naringenin were blocked by glibenclamide. Oral administration of diazoxide and naringenin significantly reduced the intestinal transit of charcoal meal. The delay in the intestinal transit was blocked by the oral dose of glibenclamide. The effect of naringenin on the rat intestinal strip pre-contracted with the KCl was relatively similar to that of ATP-sensitive K⁺ channel opener (diazoxide).

Conclusion: This research supports that ATP-sensitive K⁺ channels are involved in the rat small intestinal smooth muscles relaxation induced by naringenin.

Implication for health policy/practice/research/medical education:

This paper provides pharmacological evidence for the involvement of ATP-sensitive K⁺ channels in spasmolytic action of naringenin on rat intestinal tissue. Therefore, naringenin as a smooth muscle relaxant may have similar clinical indication as other ATP-sensitive K⁺ channel openers.

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Introduction

Polyphenols containing plants are a major source of antioxidants (1). Flavonoids are present in most polyphenolic compounds and comprise a wide range of natural products (2,3). Naringenin [5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one] as a predominant flavanone is found in citrus fruits, especially the

grapefruit, bitter orange, and tomatoes (4). Naringenin has been reported to exhibit various pharmacological effects, such as anti-inflammatory, antiatherogenic, hepatoprotective, anti-mutagenic, and anticancer effects (5). Naringenin reportedly exerts potential therapeutic effects on diabetes (6), hyperlipidemia (7), cardiovascular diseases (8), and inflammatory pain (9). Furthermore,

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naringenin is able to affect gastrointestinal (GI) motility and intestinal transit time by reducing the smooth muscle contractility (10). It has been reported that naringenin reduces the potential amplitudes of interstitial cells of Cajal and smooth muscle excitability resulting in the reduction of gastric tone (11), intestinal peristalsis, and intestinal transit (12). The contractile inhibitory effects of naringenin may be explained by regulating the GI hormones and neurotransmitter levels, such as motilin, gastrin, endothelin, substance P, and vasoactive intestinal peptide (13).

Ion channels play an important role in the regulation of GI motility (14). Thus, agents regulating ion channel activities may affect the GI motility function (15). It is generally accepted that K⁺ channel activation prevents the development of action potentials in smooth muscles, so allowing graded changes in membrane potential to regulate their tone (16). Several K⁺ channels, with different molecular bases, contribute to the regulatory basal K⁺ conductance in smooth muscle cells, including ATP-sensitive K⁺ channel channels (16)

Studies have indicated that increased ATP-sensitive K⁺ channel expression in inflammatory conditions is responsible for decreased colonic motility due to hyperpolarized colon smooth muscle (10,17). Moreover, in an animal model of colitis, it has been shown that the sensitivity of inflamed smooth muscle cells of the colon to ATP-sensitive K⁺ channel openers is significantly increased (18). Several endogenous agonists, such as calcitonin gene-related peptide, adenosine, etc, activate ATP-sensitive K⁺ channels leading to hyperpolarization, a response that is mimicked by treatment with ATP-sensitive K⁺ channel openers (18). In contrast, various neurotransmitters (noradrenaline, 5-HT, neuropeptide Y) and vasoconstrictors (angiotensin II, endothelin-1) indirectly inhibit ATP-sensitive K⁺ channels leading to depolarization and contraction (18). Importantly, Wang and co-workers (19) showed that ATP-sensitive K⁺ channels are involved in setting the resting membrane potential and basic muscle tone in mouse small intestinal smooth muscles (19). In addition, activation of muscarinic M₃-receptors inhibits the ATP-sensitive K⁺ channels independently of intracellular Ca²⁺ ions concentration, which can further contribute to cell membrane depolarization (19). Thus, it has been suggested that modulation of ATP-sensitive K⁺ channels has a contribution in the regulation of contractility of smooth muscle (20).

Several studies have shown that naringenin modulates the activation of ion channels (21). For example, it has been suggested that naringenin interacts with ATP-sensitive K⁺ channels (21). Naringenin has also been reported to directly activate large conductance Ca²⁺-activated K⁺ channels, which consequently hyperpolarizes the intestinal smooth muscles, decreases Ca²⁺ influx and therefore reduces intestinal motility (10,21). Naringenin as a natural flavonoid is potassium channel modulator

(10). It has been proposed that naringenin reduces inflammatory pain by activating the plasma membrane ATP-sensitive K⁺ channel in mice (9). Given that there are several reports of naringenin action as muscle relaxant in the GI tract, one possible mechanism may be through K⁺ channels. By examining the effect of naringenin on ileum contraction *in vitro* and *in vivo*, we intend to find out if naringenin action is associated with the activation of the ATP-sensitive K⁺ channels.

Material and Methods

Drugs and solutions

Naringenin (Sigma-Aldrich, China) and glibenclamide (Sigma, China) were dissolved in dimethylsulfoxide (DMSO) as 20mM and 10mM stock solutions, respectively. Acetylcholine (ACh, Sigma, China) was dissolved in distilled water as 100mM stock solution and acidified with a drop of acetic acid. Further serial dilution was made in distilled water. Diazoxide ampule (Hyperstat, Schering-Plough, USA) was diluted with distilled water. KCl was dissolved in distilled water as 2M and 4M stock solutions. All dilutions were made freshly every day. Tyrode's physiological solution was made up in distilled water with the following composition (mM): NaCl 136.9, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaHCO₃ 11.9, NaH₂PO₄ 0.42, and glucose 5.6, and saturated with oxygen. Charcoal meal was prepared daily by mixing an equal volume of 3% charcoal with 5% tragacanth suspension. Unless stated, chemicals were purchased from Merck (China).

In vitro experimental protocols

Adult Wistar male rats (180-225 g) bred in the School of Pharmacy and Pharmaceutical Sciences animal house were anesthetized with carbon dioxide and sacrificed. The abdominal cavity was opened up, and a section of ileum was dissected out and placed in oxygenated Tyrode's solution at room temperature. The tissue was cut into 1-1.5 cm long pieces and trimmed off the excess fat and connective tissues. Both ends of the tissue were tied with two separate cotton threads. One end of the tissue was tied up to the tissue hook and set up longitudinally in an organ bath (Palmer, England) containing 20 mL of Tyrode solution at 37°C and gassed continuously with oxygen. The other thread was tied up into the lever of Harvard isotonic transducer. Tissue contractions under 1 g tension were recorded on a calibrated Harvard Oscillograph (England) pen recorded device.

The preparations were washed regularly with Tyrode's solution and allowed to settle down in the organ bath for a period of 30 minutes. When stable baseline recording was established, KCl (20 or 160mM) was added into the organ bath, and contractions were recorded continuously. After 15 minutes equilibration time, naringenin or diazoxide was added cumulatively into the organ bath at 5 minutes intervals. Similar experiments were performed in the presence of glibenclamide (10μM). The effects of

the above drugs were also examined on ileum contraction induced by ACh (500nM). ACh was in contact with the tissue for 30 s before it was washed away. Parallel time-matched vehicle-treated control tissues were treated with an equal volume of DMSO. Each drug was tested on at least six tissues taken from 6 different animals. The added volumes of drugs into the bath did not exceed 5% of the bath volume. Appropriate drug concentrations were selected following a series of pilot experiments.

In vivo experimental protocol

Adult white male mice (20-25 g) bred in the School of Pharmacy and Pharmaceutical Sciences animal house were used for this set of experiments. In this experiment charcoal meal transit in the small intestine was assessed following oral administration of the investigating compounds. Animals fasted for 12 hours before the experiment with free excess to water. The charcoal meal and drugs were given orally by intragastric administration. Diazoxide and naringenin were given 30 minutes prior to the administration of charcoal suspension. Glibenclamide was administered 10 minutes prior to the oral gavage of the above drugs.

The animals were randomly divided into nine groups (n=10): One group received oral diazoxide solution (25 mg/kg). Another group was treated with oral diazoxide + glibenclamide (50 mg/kg) solutions. Three groups were treated with naringenin solutions (25, 50, 100 mg/kg). Two groups were treated with naringenin + glibenclamide solutions. In a control group, the mice were given saline solution. The vehicle-treated group received DMSO + dextrose (5%).

Forty-five minutes after the charcoal meal administration, the animals were sacrificed and the whole length of the small intestine tissue was taken out carefully for the assessment of intestinal transit. The whole length of the small intestine and the distance traveled by charcoal meal from pylorus toward caecum were measured.

Data and statistical analysis

Isolated ileum contractions were measured as the peak amplitude of recorded contractions from the baseline and expressed as a percentage of initial response prior to the addition of the test drugs. The concentration-response curve was plotted for each tissue, and when appropriate, the drug concentration causing 50% of maximum inhibitory response (IC_{50}), was calculated. Data are presented as mean \pm standard error of the mean (SEM) for each drug concentration (n=6).

The intestinal transit was expressed as a percentage of the distance traveled by charcoal meal relative to the total length of the intestine for each mice. Data are given as mean \pm SEM (n=10). For statistical analyses, unpaired Student's t-test was used for intergroup comparison and one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc tests was used for intragroup

comparison. A *P* value less than 0.05 was considered statistically significant.

Results

Rat-isolated ileum suspended under 1 g tension in the organ bath was gradually relaxed into a relatively stable baseline within 30 minutes and exhibited irregular spontaneous spike activities. The addition of KCl (20mM) produced a small tonic contraction with some rhythmic contraction imposed on it. High extracellular concentration of KCl (160mM) produced a sustained tonic contraction, which was maintained during the course of the experiment. The addition of ACh (500nM) into the organ bath produced a sharp contractile response within 30 seconds of the contact time. This concentration of ACh produced 60% to 80% of the maximum ACh response. Following washing the tissue with fresh Tyrode's solution, the ileum quickly relaxed back to the baseline.

In vitro experiments

The addition of diazoxide into the organ bath exhibited concentration-dependent relaxation of rat-ileum contraction induced by a low concentration of KCl (20mM), while it had no effect on tonic contraction induced by the high extracellular concentration of KCl (160mM) (Figure 1). The inhibitory effect of diazoxide was observed with 40nM bath concentration, and the ileum contraction was reduced by 73% with 2.56 μ M diazoxide in the bath. The inhibitory concentration causing 50% of maximum response (IC_{50}) was calculated to be $1.1 \pm 0.09 \mu$ M (Figure 1). In the presence of glibenclamide (10 μ M), the inhibitory effect of diazoxide on the KCl (20mM) was blocked. In the presence of glibenclamide at bath concentration of 2.56 μ M diazoxide only reduced the KCl response by 11% (Figure 1). There was no statistically significant change in the tissue response treated with the vehicle (ANOVA). Furthermore, with 160mM KCl in the bath, there was no statically significant difference between the tissues treated with the vehicle or diazoxide.

Naringenin in a concentration-dependent manner inhibited low KCl (20mM) induced contractions in rat-isolated ileum (Figure 2). The inhibitory effect of naringenin on rat ileum was started at 2.5 μ M bath concentration, and total inhibition was achieved with 160 μ M naringenin in the bath. The calculated IC_{50} value of naringenin was $59 \pm 2.3 \mu$ M (Figure 2). The inhibitory effect of naringenin on rat ileum was also blocked by glibenclamide (10 μ M). With 160 μ M naringenin in the bath, the KCl (20mM) response was reduced only by 36% (Figure 2). Naringenin had no effect on contractions induced by high extracellular KCl (160mM) concentration. There was no statistically significant change in the control group treated with the naringenin vehicle over the course of the experiment. Furthermore, there was no significant difference between the control group and the naringenin-treated group in the ileum contraction induced by the

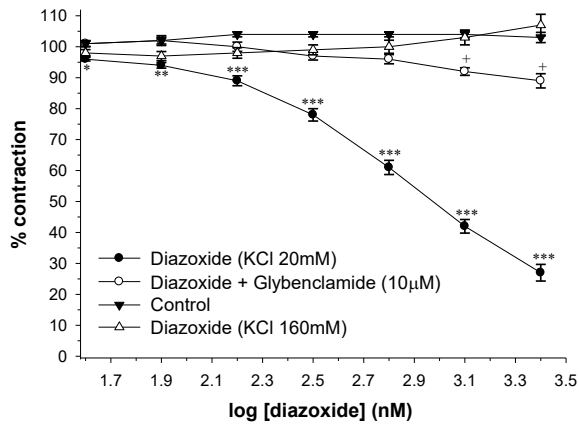


Figure 1. Inhibitory effect of diazoxide on KCl induced contractions in the rat-isolated ileum in the absence and presence of glibenclamide. Ileum contractions were induced by low (20mM) and high (160mM) extracellular concentrations of KCl. Data are expressed as a percentage of initial contraction recorded prior to addition of diazoxide using two-fold increment in concentration. Results are presented as mean \pm SEM (n=6). Stars show the statistically significant differences between the diazoxide and its corresponding control group using unpaired Student's t-test (* P < 0.05, ** P < 0.01, *** P < 0.001). Cross sign shows the statistically significant differences between the diazoxide in the presence of glibenclamide with the vehicle-treated control group (+ P < 0.05). There were no statistically significant changes in the KCl (160mM) induced response before and after the addition of successive concentration of diazoxide over the course of the study (ANOVA).

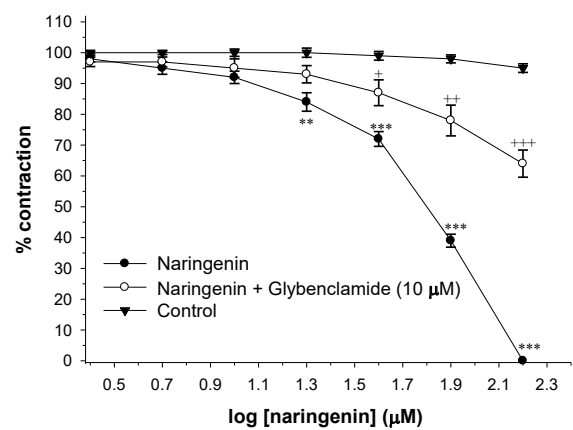


Figure 2. Inhibitory effect of naringenin on KCl (20 mM) induced contractions in the rat-isolated ileum in the absence and presence of glibenclamide. Data are expressed as a percentage of initial contraction recorded prior to the addition of naringenin using two fold increment in concentration. Results are presented as mean \pm SEM (n=6). Stars show the statistically significant differences between the naringenin and its corresponding control group using unpaired Student's t-test (** P < 0.01, *** P < 0.001). Cross sign shows the statistically significant differences between the naringenin in the presence of glibenclamide with the vehicle treated control group (+ P < 0.05, ++ P < 0.01, +++ P < 0.001). There were no statistically significant changes in the tissues treated with the equal volume of the vehicle (ANOVA). The maximum used concentration of DMSO in the bath was 0.16%.

high extracellular KCl (160mM) in the bath.

Diazoxide at its highest used concentration (2.56 μ M) reduced ACh contractile response only by 22% relative to the control response (Figure 3). On the other hand, naringenin (160 μ M) potentiated rat-ileum contractile response to ACh. A similar increase in ACh response was seen with the naringenin-vehicle-treated control group (DMSO) (Figure 3). Therefore, this effect on ACh should be DMSO-related.

In vivo experiments

The intestinal charcoal meal transit technique was used to investigate natural intestinal motility activities. In the normal saline-treated control group, within 45 minutes of administration, the charcoal meal traveled about two-third of the total length of the small intestine (Figure 4). Diazoxide with the oral dose of 25 mg/kg, significantly delayed charcoal meal transit. The charcoal meal transit was reduced by about 46% in comparison with the control group. The retarded effect of diazoxide on charcoal meal transit was blocked with oral administration of glibenclamide (Figure 4). Glibenclamide vehicle had no effect on the inhibitory effect of diazoxide on intestinal meal transit (Figure 4).

To investigate the effect of naringenin on intestinal meal transit, three oral doses of naringenin (25, 50, and 100 mg/kg) were used. As shown in Figure 5, in the control group (DMSO), the charcoal meal has moved up to 72%

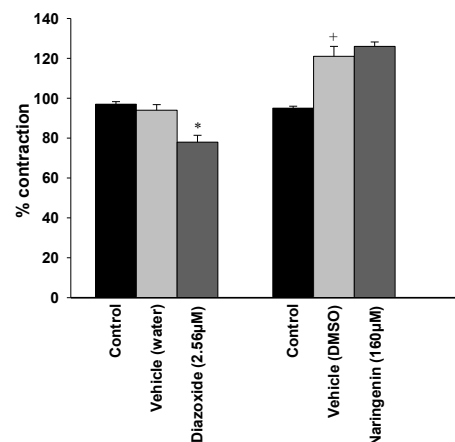


Figure 3. Effect of diazoxide and naringenin on acetylcholine (ACh, 500nM) induced contractions of the longitudinal muscle of rat ileum. Values are expressed as a percentage of the corresponding control contraction for each tissue. Results are given as mean \pm ESM (n=6). Significant differences from the respective control group were compared with unpaired Student's t-test (* P < 0.05). Naringenin vehicle (dimethylsulfoxide, 0.16%) significantly potentiated ACh responses (+ P < 0.05).

of the total length of the small intestine. All three doses of naringenin, compared to the vehicle treated control group, significantly inhibited the passage of charcoal meal by 16%, 33%, and 41%, respectively (Figure 5). An oral dose of glibenclamide (50 mg/kg) blocked the inhibitory effect of naringenin within 45 minutes, so there was no

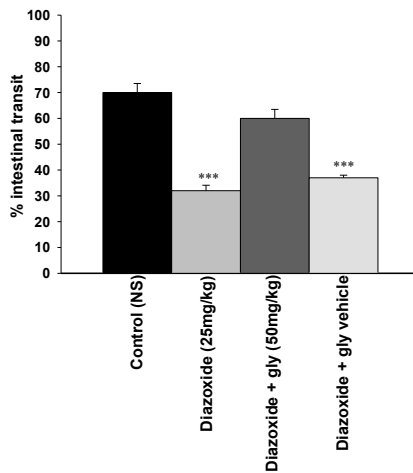


Figure 4. Effect of diazoxide on intestinal transit in the mice in the absence and presence of glibenclamide (gly, 50 mg/kg). Drugs were administered orally 30 minutes and 45 minutes before the charcoal meal. Results are expressed as a percentage of the distance traveled by the charcoal meal from pyloric sphincter to caecum for each animal. Each value represents the mean \pm SEM (n=10). Significant differences from the respective control group were compared with unpaired Student's *t* test ($***P < 0.001$). Glibenclamide vehicle (dimethylsulfoxide + dextrose) did not significantly alter the inhibitory effect of diazoxide on intestinal transit. (NS = normal saline).

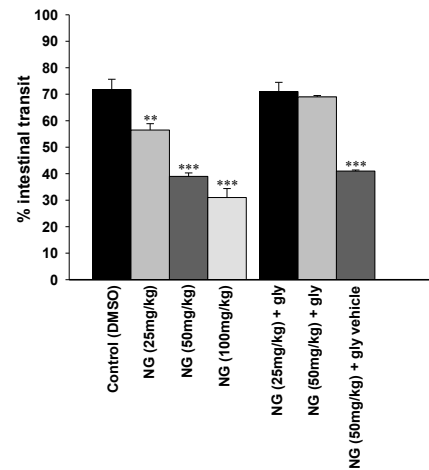


Figure 5. Effect of naringenin (NG) on intestinal transit in mice in the absence and presence of glibenclamide (gly, 50 mg/kg). Drugs were administered orally 30 minutes and 45 minutes before the charcoal meal. Results are expressed as a percentage of the distance traveled by the charcoal meal from pyloric sphincter to caecum for each animal. Each value represents the mean \pm SEM (n = 10). Significant differences from the respective control group were compared with unpaired Student's *t* test ($***P < 0.001$). Glibenclamide vehicle (dimethylsulfoxide + dextrose) did not significantly alter the inhibitory effect of naringenin on intestinal transit.

significant difference with the vehicle-treated control group. Glibenclamide vehicle (DMSO + dextrose) also had no significant effect on the inhibitory action of naringenin (Figure 5).

Discussion

The ileum comprises a large section of the small intestine (22). Two layers of smooth muscle cells lie within the walls of the ileum and are responsible for its contractile activities (23,24). The spontaneous contractions of isolated ileum indicate that ileum smooth muscle possessed involuntary contractions. The ileum smooth muscle cells receive neural innervation from the enteric nervous system which regulates GI motility and secretions (25,26). Contractile activity in smooth muscle is initiated by a Ca^{2+} -calmodulin interaction to stimulate phosphorylation of the light chain of myosin (15,24). Removal of Ca^{2+} from the cytosol initiates the process of smooth muscle relaxation (24). In smooth muscles, contraction can be produced either by Ca^{2+} ion entry through voltage gated or ligand gated calcium channels or by inositol triphosphate (IP_3) mediated Ca^{2+} release from the sarcoplasmic reticulum (27,28). Adding KCl into the organ bath results in depolarization of smooth muscle cells, thereby activating voltage gated calcium channels (29). ACh, on the other hand, activates muscarinic receptors on the smooth muscle, which in turn activates phospholipase C and enhances the production of IP_3 (30). Stimulation of muscarinic receptors has also been reported to inhibit ATP-sensitive K^+ channels in the ileal smooth muscles (31), thereby contributing to membrane

depolarization and contraction of smooth muscles.

The driving force for the movement of ions across the cell membrane is governed by the concentration gradient of the ion and cell resting membrane potential (32). That's why diazoxide inhibited ileum contraction induced by low extracellular concentration of KCl (20mM) but not high extracellular concentration of KCl (160mM) (33). Diazoxide opens a certain class of K^+ channels in the smooth muscle cell membrane, known as ATP-sensitive K^+ channels (33). This leads to the hyperpolarization of the cell membrane and relaxation of the tissue (34,35). Hyperpolarization induced by diazoxide prevents Ca^{2+} entry through voltage activated calcium channels (34,35). Furthermore, hyperpolarization of the cell membrane inhibits agonist induced increase in IP_3 production and, therefore, reduces Ca^{2+} mobilization from intracellular stores (34, 35). This can explain the inhibitory effect of diazoxide on ACh induced contraction of rat ileum. Some K^+ channel opener may also affect Ca^{2+} sensitivity of the contractile apparatus (36). Therefore, it was suggested that K^+ channel opener may involve other mechanisms of smooth muscle relaxation not linked to the opening of cytoplasmic membrane K^+ channels. For example, nicorandil, in addition to its K^+ channel opening action, it has nitro-vasodilator activity (37).

The relaxant effect of the K^+ channel openers is abolished in media containing a high concentration of K^+ ions (>50mM) (38). For example, cromakalim inhibited contractions induced by low KCl (20-30 mM) concentrations, but it had no effect on those elicited by

60-100 mM KCl (39). In our experiment, naringenin concentration-dependently inhibited the contractions induced by 20mM KCl in the bath but not with the high concentration of KCl (160mM), indicating similar characteristics with ATP sensitive K⁺ channel openers. Another characteristic feature of ATP-sensitive K⁺ channels is their inhibition by sulfonylureas such as glibenclamide (40,41). The inhibitory effects of both diazoxide and naringenin on rat-isolated ileum were blocked by glibenclamide, confirming the involvement of ATP-sensitive K⁺ channels. Therefore, other types of K⁺ channels may also be involved in the relaxant effect of naringenin. For instance, it has been shown that in whole-cell patch-clamp recording from rat colonic smooth muscle cell, outward K⁺ current was potentiated in the presence of naringenin. This current was partially blocked by tetraethylammonium, indicating the involvement of Ca²⁺ activated K⁺ channels (10).

The transit of food through the intestine may be hastened or inhibited by drugs. Ligand-gated channels are regarded as drug-target. ATP-sensitive K channels are known to be expressed in GI smooth muscles (42). Under our experimental condition, both diazoxide and naringenin inhibited the intestinal propulsion of charcoal meal. The inhibitory effect of naringenin was dose dependent and statistically significant with all three used doses (Figure 4). *In vitro* study showed that naringenin has direct relaxant action on ileal smooth muscle. Blockade of the inhibitory effect of naringenin on intestinal transit by glibenclamide supports the involvement of ATP- sensitive K⁺ channels. Drugs that reduce spasm in the gut could have valuable effect in the treatment of irritable bowel syndrome and diverticular disease.

Conclusion

In this experiment, naringenin exhibited similar pharmacological properties as diazoxide on the rat ileum. Thus, it can be concluded that, like diazoxide, the relaxant effect of naringenin on the ileum smooth muscle involved the activation of ATP-sensitive K⁺ channels. However, as a useful therapeutic drug, naringenin should have sufficient tissue selectivity. Therefore, before it can be introduced as an alternative therapeutic agent, proper evaluation of pharmacological actions of naringenin is needed.

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Authors' contributions

HS was the project manager and supervised the pharmacological studies. SS was responsible for the experimental works and analysis of the data. MG supervised the experimental works. All authors approved the final manuscript for publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Isfahan University of Medical Sciences. The project was confirmed by the ethical committee of the university (IR.MUI.RESEARCH.REC.1399.360).

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