

RESEARCH ARTICLE



Spasmolytic Activity of *Piper Nigrum* Fruit Aqueous Extract on Rat Non-Pregnant Uterus

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ABSTRACT

Black pepper (*Piper nigrum*) from Piperaceae and its main ingredient (piperine) reduces the gastric emptying in rats. In Iranian traditional medicine, black pepper is used to relieve menorrhalgia in women. The aim of this study was to investigate the effect of black pepper fruit aqueous extract on rat non-pregnant uterus contractions and the mechanism(s) of its action. To prepare the extract, black pepper powder was added to boiling distilled water and then, solvent was evaporated. Uterus was dissected from non-pregnant adult rat (Wistar) and in De Jalon solution the tissue contractions were recorded isometrically under 1 g tension. The extract (0.125-2 mg/ml) reduced the uterus contractions induced by KCI (60 mM) and oxytocin (10 mU/ml) dose dependently (p<0.0001). The spasmolytic effect of extract on the KCI-induced contractions was not reduced by L-NAME (100 µM), phentolamine (1 µM) and naloxone (1 µM). However, propranolol (1 µM) reduced the extract activity (p<0.01–p<0.0001). In Ca²⁺-free De Jalon solution with high potassium (60 mM), extract (0.0312-0.25 mg/ml) reduced the contractions induced by cumulative concentrations of CaCl₂ (0.1-0.5 mM) dose dependently (p<0.05– p<0.0001). Our results suggest that the spasmolytic effect of the extract on rat uterus was mediated via voltage dependent calcium channels and β -adrenoceptors could also be involved in this action. Our results may support the use of black pepper in traditional medicine to relief the menorrhalgia.

Keywords: Piper nigrum fruit, Rat, Uterus, Spasmolytic

Black pepper (*Piper nigrum*) from Piperaceae is the main spice food stuff and piperine is a pungent alkaloid of black pepper [1]. Black pepper increases gastric acid secretion in rat [2] and piperine has the same effect [3]. Piperine reduces mice small intestine secretions stimulated by castor oil [4] and inhibits gastric emptying in rat [5]. Black pepper stimulates bile flow [6] and increases pancreatic enzymes activities in rat [7]. Black pepper has antioxidant activity [8] and reduces oxidative stress induced by high fat diet in rat [9]. However, the effect of black pepper on smooth muscle has not been investigated. It has been reported that menorrhalgia is caused by hyperactivity of the myometrium and uterus ischemia [10] and in the Iranian traditional medicine, black pepper is used to relieve menorrhalgia in women but, this effect has not been studied scientifically till now. The aim of the present study was to investigate the effect of black pepper fruit aqueous extract on the non-pregnant rat uterus contractions induced by some spasmogens and the possible mechanism(s) involved.

MATERIALS AND METHODS

Plant Material

Black pepper fruit was purchased from local herbal shops in Ahwaz and identified by botanists in the Department of Horticulture, Ahwaz Ramin University of Agriculture and Natural Resources.

Extraction

Black pepper fruit was powdered by an electrical grinder and the powder (10 g) was added to 200 ml boiling distilled water for 15 min. The mixture was then filtered through very fine cloth and thereafter the filtrate was centrifuged at 3500 rpm for 20 min. The supernatant was dried by expending the extract on a flat glass in front of a hair dryer at a temperature of 30 °C to obtain a solid mass (1.3 g). The black pepper extract powder (BPE) was stored at 4 °C until being used and the diluted concentration of the extract were made up with De Jalon solution.



Fig. 1. The spasmolytic effect of black pepper fruit aqueous extract on the rat uterus contractions induced by KCl (60 mM, n=10) or oxytocin (10 mU/ml, n=8). The extract spasmolytic effect on KClinduced contractions are greater than that of the oxytocin-induced contractions (* p<0.05, ** p<0.01, *** p<0.001).

Drugs

Propranolol and N^{ω} -nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (USA) and phentolamine from Novartis (USA). Naloxone, oxytocin and estradiol valerate were purchased from Tolidaru, Minoo and Aboraihan Companies respectively (Iran) and other chemicals from Merck (Germany). To prevent changes in electrolyte composition of the organ bath solution, all chemicals were dissolved in the De Jalon solution and the total volume of all solutions were added to the organ bath did not exceed more than 5% of the bath volume.

Animals and uterus tissue preparation

All of the animals used in this study were treated in accordance with principals and guidelines on animals care of Ahwaz Jundishapur University of Medical Sciences. Adult female Wistar rats (240-320 g, 267±3.4 g) were obtained from Ahwaz Jundishapur University of Medical Sciences animal house and kept at 12-h light/dark cycle and at 20-24 °C with free access to food and water.

On the day of experiment the rats were sacrificed by a sharp blow on the neck. After laparotomy, from the cervical portion of each uterus horn a piece (1-1.5 cm) was dissected and mounted in an organ bath containing De Jalon solution (10 ml) between two stainless steel hooks vertically. The lower hook was fixed at the bottom of the organ bath and upper one was connected to an isometric transducer (UF1 Harvard transducer, UK) connected to an ink-writing curvilinear recorder (Harvard Universal Oscillograph, UK). The De Jalon solution composition (pH 7.4 and 29 °C) was (in mM): NaCl, 154; KCl, 5.6; CaCl₂, 0.3; NaHCO₃, 1.7; MgCl₂, 1.4 and glucose, 5.55 [11] which continuously was bubbled with air.



Fig. 2. The spasmolytic effect of black pepper fruit aqueous extract on the rat uterus contractions induced by KCl (60 mM, n=10) in presence and absence of phentolamine (1 μ M, n=8) or propranolol (1 μ M, n=8). The effect of the extract in the presence of antagonists are compared with contractions in the absence of antagonist (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

The initial tension was 1 g throughout the experiment and equilibrium period was 60 min in which, the bath solution was refreshed every 15 min. After equilibrium period, the uterus was contracted by 60 mM of KCl [12] and once the plateau was achieved for KCl-induced contraction, the extract (0.125, 0.25, 0.5, 1 and 2 mg/ml) was added cumulatively to the organ bath.

A group of animals were received estradiol valerate (0.5 mg/kg, SC) 24 hours prior the experiment [11]. The uterus of these rats was pretreated with the extract (0.125, 0.25, 0.5, 1 and 2 mg/ml) for 3 min and then tissue preparation was contracted by 10 mU/ml of oxytocin [13]. The extract spasmolytic effect was also studied on separate tissues after 30 min incubations with 1 μ M of phentolamine [14], propranolol [15] or naloxone [16] as non-selective α - and β -adrenoceptors and opioid receptors antagonists respectively. In addition, the spasmolytic effect of the extract was studied after 20 min tissue incubation with 100 μ M of L-NAME [17] as a nitric oxide synthase inhibitor.

To study the role of extracellular calcium, in Ca^{2+} -free and rich KCl (60 mM) De Jalon solution, the tissue was depolarized but the contraction occurs only in the presence of extracellular calcium [18], therefore, calcium chloride was added to the organ bath cumulatively (0.1, 0.2, 0.3, 0.4 and 0.5 mM) before and after tissue incubation with different concentration of the extract (0.0312, 0.0625, 0.125, 0.25 mg/ml). Each uterus preparation was used only for one of the spasmogens and antagonists.

Statistical analysis

Values (changes in contraction recorded in comparison with 100% contractions induced by the spasmogens in the absence of the extract) are expressed as mean±SEM. Statistical comparisons were made by Student's t-test and one-way ANOVA and P values less than 0.05 were considered significant.



Fig. 3. The spasmolytic effect of black pepper fruit aqueous extract on the rat uterus contractions induced by KCl (60 mM, n=10) in presence and absence naloxone (1 μ M, n=7). The extract inhibitory effect at 0.125, 0.25 and 0.5 mg/ml was potentiated by naloxone (* p<0.05).



Fig. 5. The spasmogenic effects of cumulative concentrations of the CaCl₂ on the rat uterus before and after 3 min incubation uterus preparation with different concentrations of the black pepper fruit aqueous extract. The De Jalon solution was Ca²⁺-free but with high K⁺ (60 mM). The statistical comparisons were carried out only for extract at 0.0312 mg/ml (n=7-9, * p<0.05, ** p<0.01, **** p<0.001, **** p<0.0001).

RESULTS

Effect of BPE on the KCI- and oxytocin- induced uterus contractions

Black pepper fruit aqueous extract (BPE) reduced the uterus contractions induced by KCl (60 mM, n=10) and oxytocin (10 mU/ml, n=7) significantly (ANOVA, p<0.0001) and in a dose dependent manner. The com-



Fig. 4. The inhibitory effect of black pepper fruit aqueous extract on the rat uterus contractions induced by KCl (60 mM, n=10) in presence and absence of L-NAME (100 μ M, n=7).

parison of these inhibitory effects indicates that the spasmolytic effect of the extract on KCl-induced contractions is greater than for oxytocin-induced contractions (p<0.05 - p< 0.001) as shown in Fig 1. Representative trace of the extract spasmolytic effect on KClinduced uterus contraction is shown in Fig. 6 A.

Effect of BPE on KCI-induced contractions in the presence of adrenergic antagonists

Fig 2 shows that the spasmolytic effects of BPE on KCl-induced uterus contractions was not reduced by phentolamine, a α -adrenoceptor antagonist, but rather increased. However, the presence of propranolol, a β -adrenoceptor antagonist, the extract spasmolytic effect was reduced (n=8, p < 0.01-p < 0.0001). Representative trace of the extract spasmolytic effect on KCl-induced uterus contraction in the presence of propranolol is shown in Fig. 6 B.

Effect of BPE on KCI-induced contractions in the presence of opioid receptor antagonists

As the Fig 3 shows, the spasmolytic effect of BPE on the KCl-induced uterus contractions was not reduced in the presence of naloxone, as a non-selective opioid receptor antagonist, but rather the inhibitory effect of extract at 0.125, 0.25 and 0.5 mg/ml has been potentiated by naloxone (n=7, p<0.05).

Effect of BPE on KCI-induced contractions in the presence of L-NAME

The spasmolytic effect of BPE on the KCl- induced uterus contraction was unaffected by L-NAME (as a nitric oxide synthase inhibitor) as shown in Fig 4.



Fig. 6. Representative traces of the black pepper fruit aqueous extract on the rat uterus contractions induced by KCl (60 mM) in the absence (A) and in the presence (1 μ M) of propranolol (B). Representative trace (C) shows the uterus contractions induced by the cumulative concentrations of CaCl2 (0.1-0.5 mM) in the absence of the extract (*) and in the presence of two different extract concentrations (**, ***)

Effect of BPE on the depolarized rat uterus contraction induced by CaCl₂

Calcium chloride (0.1, 0.2, 0.3, 0.4 and 0.5 mM) induced contractions in rat uterus in a dose dependent manner (p<0.0001). Pretreatment (3 min) of tissue preparations with BPE (0.0312, 0.0625, 0.125 or 0.25 mg/ml) reduced the calcium chloride-induced contractions dose-dependently (at 0.0312 mg/ml, p<0.05p<0.0001). Representative traces of the extract spasmolytic effect on CaCl₂-induced uterus contractions in the absence and in the presence of two extract concentrations are shown in Fig. 6 C.

DISCUSSION

This study showed that black pepper fruit aqueous extract (BPE) induced spasmolytic effect on the rat uterus contraction caused by KCl and oxytocin. In this study, De Jalon solution with low calcium and potassium concentration and low temperature (29 °C) were used to reduce the spontaneous uterus contractions [19]. All uterus tissue preparations were also dissected from the cervical segment since, it has been reported that different segments of the uterus vary in their responsiveness to stimulants [19]. The observed spasmolytic effect of the BPE was reversible since washing and refreshing the organ bath solution was accompany with disappearance of the extract spasmolytic effect.

Extracellular high potassium concentration, as a non receptor spasmogen, depolarizes the smooth muscle followed by contraction [20]. It has been reported that in the KCl-induced contractions, the voltage dependent calcium channels (VDCCs) are involved [21] and the existence of L-type VDCCs in rat uterus has been documented [22, 23]. It has been suggested that those substances that inhibit the KCl-induced contractions act through blocking the VDCCs [24]. On the other hand, oxytocin elevates $[Ca^{2+}]_i$ by activating the L-type VDCCs [25] and also by activation of phospholipase C and increasing inositol triphosphate (IP₃) production [26, 27] followed by promotion of calcium release from intracellular calcium pools such as sarcoplasmic reticulum [27]. In the absence of external Ca^{2+} , however, oxytocin is able to release Ca²⁺ from the sarcoplasmic reticulum (SR) through IP₃ but produces only a small increase in force, demonstrating a requirement for Ca²⁺ entry as part of the mechanism of agonist action [28]. IP_3 stimulates Ca^{2+} release from SR which further triggers Ca2+ influx from extracellular stores. Increased cytosolic Ca²⁺ binds with calmodulin to activate myosin light chain kinase which phosphorylates myosin light chain to trigger contractile machinery of the myocytes [29].

As it was mentioned in the results section, the spasmolytic effect of PBE on the KCl-induced uterus contractions was greater than this effect on the oxytocininduced contraction. It may suggest that the extract inhibits the influx of calcium without affecting on the releasing calcium from intracellular pool as mentioned above. Adrenoceptors are important in uterus contractility [30] but as mentioned before, propranolol reduced the BPE spasmolytic effect by antagonizing the β adrenoceptors. This result indicates that some part of inhibitory effect of extract has been through activation of these receptors, since, it has been reported that activation of β -adrenoceptors causes uterus relaxation [30].

On the other hand, phentolamine enhanced the BPE spasmolytic effect. It is documented that aadrenoceptors activation induces contraction in uterine smooth muscle but the potentiation of BPE effect by phentolamine could be due to abolishing the remaining of α -adrenergic tone and therefore, by antagonizing this tone by phentolamine the BPE spasmolytic effect has been potentiated. However, the ineffectiveness of phentolamine in lowering the BPE activity indicates that at least, these receptors were not involved. Opioid receptors activation inhibits uterus contractions [3131]. However, naloxone as a non-selective opioid receptors antagonist was unable to reduce the BPE spasmolytic effect. This result suggests that these receptors were not involved. Nitric oxide (NO) relaxes rat uterus via increasing cGMP synthesis [3232] but the relaxatory effect of BPE was unaffected by L-NAME, as a nitric oxide synthase inhibitor, which indicates the NO synthesis was not involved.

Uterus relaxation induced by black pepper fruit extract

In order to clarify the involvement of VDCCs in the BPE spasmolytic effect on the uterus contraction, Ca^{2+} -free with high K⁺ De Jalon solution was used to depolarize tissue preparation but applying calcium was necessary to induce contraction [20]. As it was mentioned in result section, applying the cumulative concentrations of Ca^{2+} induced uterine contractions dose-dependently which were inhibited by BPE in a dose dependent manner. It is known that after depolarization, the main route of increasing $[Ca^{2+}]_i$ is influx of Ca^{2+} from extracellular fluid [20], therefore, it seems that BPE has inhibited the Ca^{2+} influx. Our results are consistent with findings concerning the inhibitory effects of black pepper on rat gastric emptying [5].

To our best knowledge, this is the first report on the relaxant effect of black pepper on the isolated smooth muscle, therefore, the comparison our results with other studies was impossible. In conclusion, these results indicate that black pepper fruit aqueous extract induces spasmolytic effect on rat uterus mainly through blockage of the VDCCs and by inhibiting the β -adrenoceptors. It is believed that women menorrhalgia is caused by elevated uterus contractility, uterus blood supply reduction and ischemia [10] and traditional consumption of black pepper to relief menorrhalgia may be explained by the present study. The precise mechanism of extract activity can be the study of $[Ca^{2+}]_i$ alterations in the presence of the extract in animals or human myometrium.

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