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Effects of aqueous ginger extract on smooth muscle contraction in bovine cecum: *in vitro* study

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Abstract

Intestinal hypomotility cause health risks and economic losses and is considered as an important digestive disorder that efforts to find prokinetic drugs can solve this major problem. This study investigated the effects of *Zingiber officinale* aqueous extract (ZOAE) on caecal smooth muscle contractions in healthy cows. To perform *in vitro* tests, cecum strips connected to the organ bath. Ginger aqueous extract caused concentration-dependent contraction in caecal smooth muscle with an effective threshold concentration of 6.00 mg L^{-1}. The strongest contraction was caused at a concentration of 100 mg L^{-1} with an average contraction of 141%. To evaluate the possible mechanisms underlying the contractile effect on cecum strips, atropine, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) and verapamil completely inhibited aqueous extract induced smooth muscle contractions, while addition of hexamethonium had no effect on the contraction process. The lack of reduction of contractions caused by the extract in the presence of hexamethonium indicates that presence of acetylcholine-like constituents independent of nicotinic receptors. The inhibitory properties of atropine and 4-DAMP indicate that at least part of the prokinetic effect of the extract is due to stimulating the muscarinic receptors, especially M3 receptors. Also, verapamil inhibitory function proves that the extract acting by L-type calcium channels. The results suggest that the ZOAE has a potential prokinetic effect which may provide a pharmacological base to its medicinal or prophylactic use in caecal motility disorders.

Introduction

Gastrointestinal tract of ruminants is directly related to its contraction movements, for its proper physiological function. Atony or hypomotility of the cecum leads to gas and intestinal impaction followed by dilatation and secondary displacement.[1,2] The caecal dilatation may also be accompanied by distension of the spiral colon.[1] The etiology of caecal dilatation and volvulus has not been determined definitively, and it is likely multifactorial but it seems as mentioned atony of the cecum is the most important factor in the occurrence of dilatation .[3,4]

There are several studies about the effect of chemical drugs such as erythromycin, levamisole, bethanechol, neostigmine, metoclopramide, and propranolol on myoelectric activity of the cecum.[5-7] However, chemical drugs often have limitations such as serious adverse side effects.[8]

Therefore, herbal products appear to be significant alternatives due to their lower risk of prokinetic effects.[9-11] *Zingiber officinale Rosc.* (ginger), as the member of *Zingiberaceae* family comprises some 80 - 90 species which are aromatic plants with fleshy rhizomes and tuberous roots.[12] Ginger is used as a medicine to treat a number of diseases, including gastrointestinal dis-oerders.[13-16] Ginger is universally famous for its usage as a prokinetic, antidiarrheal, anti-dysenteric, antispasmodic and anti-colic aid.[13,14] Ginger extract causes prokinetic effects due to its spasmogenic components, in addition, due to its spasmolytic components, it causes relaxation effects in the smooth muscles of the specimens.[17,18]

Numerous studies have proven the prokinetic function of ginger in animals and humans.[14,17] Several studies have been performed on the effects of herbal remedies on GI smooth muscle contractions of different species of animals.[13,19] To the best of our knowledge, there

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are few studies about prokinetic effects of plant extracts in various gastrointestinal tissues of ruminants.[18,20-22] Thus, the objective of present study was to investigate the prokinetic effect and probably contractile mechanisms of aqueous ginger extract on bovine caecal smooth muscle.

Materials and Methods

Drugs and chemicals. Acetylcholine chloride (ACh), atropine sulfate, verapamil hydrochloride, hexamethonium, and 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) were purchased from Sigma (St. Louis, USA). Other chemicals including calcium chloride, potassium chloride, magnesium chloride, sodium chloride, sodium bicarbonate, sodium dihydrogen orthophosphate and D-glucose were obtained from Merck (Darmstadt, Germany).

Extraction procedure. *Zingiber officinale* rhizomes were purchased from the local market of Urmia, Iran. The plant material was cleaned and washed properly with distilled water and coarsely grounded. The grounded material was extracted with 70.00% ethanol by cold maceration for three days with occasional shaking. It was filtered through a muslin cloth and after that through a Whatman qualitative grade one filter paper (Sigma). This procedure was repeated twice and the combined filtrate was evaporated in a rotary evaporator to obtain hydro-alcoholic extract of *Z. officinale.* The extract was poured into distilled water and extracted with chloroform. The mixture was allowed to separate into two layers. The upper layer (aqueous fraction) was again taken into a separating funnel; ethyl acetate was added to it, separated and evaporated with the rotary evaporator (Model: RE-201D, ZZKD, Canada) to get the ethyl acetate fraction. The remaining lower layer was collected and evaporated to obtain the *Z. officinale* aqueous extract (ZOAE).[21]

Collection and preparation of tissue and data acquisition. The cecum samples were taken immediately after clinically healthy cows were slaughtered in Urmia Industrial Slaughterhouse. The cows and their carcasses were clinically examined both before and after entering the slaughter line. Tissue samples were taken from Holstein crossbred dairy cows (four to eight years old; n = 21) with no previous history of caecal disorders. The contents of the cecum samples were purified. Different parts of the cecum, including the apex, body, and base, were sampled and evaluated for their spontaneous contraction. The results showed that the middle of base and apex of cecum with moderate muscle thickness had the lowest rate of spontaneous contractions, and the same tissue was used for the experiment. The tissues were immediately rinsed with cooled (4.00 ˚C) Tyrode's solution (composition (mM): NaCl (136.90), $CaCl_2$ (1.80), KCl (2.70), $MgCl_2$ (1.10), $NaHCO_3$ (11.90), NaH_2PO_4 (0.40), and glucose (5.60). They were stored in Tyrode's solution that had been aerated previously (95.00% O_2 and 5.00% CO_2) at least for 2 hr. Then samples were transferred to the laboratory in the shortest possible time for further testing. The tissue was placed in a petri dish filled with Tyrode's solution at room temperature and the mucosa was carefully removed from the muscle layers and tissue strips (20.00 × 2.00 mm) prepared from muscle fibers. The muscle strips were placed vertically in a 25.00 mL chambers, maintained at 37.00 ˚C in Tyrode's solution, and aerated continuously with a mixture of 95.00% O_2 and 5.00% CO_2. One end of each strip was fixed to the bottom of the chamber, and the other end was attached to an isometric muscle transducer (model TRI 202P; PanLab, Barcelona, Spain) coupled to bridge amplifier (model ML224; AD Instruments, Castle Hill, Australia) and data acquisition PowerLab system (model ML870; AD Instruments) using Labchart software (version 8.00, AD instruments). Specimens were allowed to equilibrate for 1 hr in the Tyrode's solution in the organ bath to reduce tissue contractions to approximately zero at the start of the experiment. Then muscle tension was adjusted in two stages at intervals of 10 min to 2.00 g (1.00 g each). During this time Tyrode's solution was replaced every 15 min with fresh solution. To evaluate tissue functional viability, acetylcholine was added to the organ bath before and after of all tests. The dose-response curves to determine the effect of acetylcholine were obtained by exposing the preparation to increasing concentrations added to the bath (2 min to each concentration). The submaximal (inducing responses approximately 70.00% of the maximum) concentration was determined for each of the strips. The strips producing three consistent repeatable responses to submaximal concentration of acetylcholine were used. After removing acetylcholine by rinsing, the cecum responses were recorded in the presence of increasing cumulative concentrations of *Z. officinale* aqueous extract (ZOAE; 1.00 to 100 mg L^{-1}). The experiment was performed in 8 replicates (n = 8). To evaluate the possible mechanisms underlying the contractile effect of the aqueous fraction on cecum strips, atropine, a muscarinic receptor blocker (10.00 µM); hexamethonium, a nicotinic receptor antagonist (10.00 µM); 4-DAMP, a muscarinic M3 receptor antagonist (10.00 µM) and verapamil, a calcium channel blocker (0.10 µM) was added to the organ bath 10 min prior to the addition of the extract (1.00 to 100 mg L^{-1}).

Statistical analysis. Data were analyzed graphically for the assumptions of normal distribution and homogeneity of variation. For this purpose, The Shapiro-Wilks test and inspection of histograms and residual plots was performed. Non-parametric statistical test was used for analysis because the assumptions did not have a normal distribution. The Friedman repeated measures analysis of variance on ranks was used to compare results in strength of contractions between different concentrations of the extract. A $p < 0.05$ was considered significant. Pair-wise

comparisons between each treatment group (concentration) versus control group were made using Dunnett's Method. Results are expressed as medians and interquartile ranges (25^{th} - 75^{th} percentiles). Data were analyzed using SigmaPlot for windows (version 12.3; Systat Software Inc., San Jose, USA).

Results

Before using different concentrations of aqueous extract on tissue samples, acetylcholine chloride was used to evaluate the functional viability of tissues. The experiment was conducted on 84 specimens and only seven did not respond to the acetylcholine chloride stimulation, so they were removed from further testing. All 77 specimens were tested again in the presence Ach at the end of the recording period and indicated that the muscle has not been damaged by non-specific action.

The distilled water, as a control, did not exert an effect on basal tonus of any preparations of cattle cecum. The *Z. officinale* aqueous extract (1.00 to 100 mg L^{-1}) contracted the isolated strips of cattle cecum smooth muscle in a concentration-dependent manner with an effective threshold concentration of 6.00 mg L^{-1} ($p < 0.05$, Fig. 1). However, treatment of tissue strips with 1.00 and 3.00 mg L^{-1} of *Z. officinale* extracts did not show any significant difference with the control. A typical trace indicating the effect of the ZOAE extract (1.00 to 100 mg L^{-1}) is covered in Figure 2. The ZOAE increased basal tone of muscles at 6.00, 12.00, 25.00, 50.00 and 100 mg L^{-1} concentrations and showed a significant different ($p < 0.05$) with the control.

The mechanism of prokinetic effects of the ZOAE was investigated using different antagonists. For this purpose, 10 min before adding the different concentrations of extract to the organ bath, the desired antagonist was added to the bath.

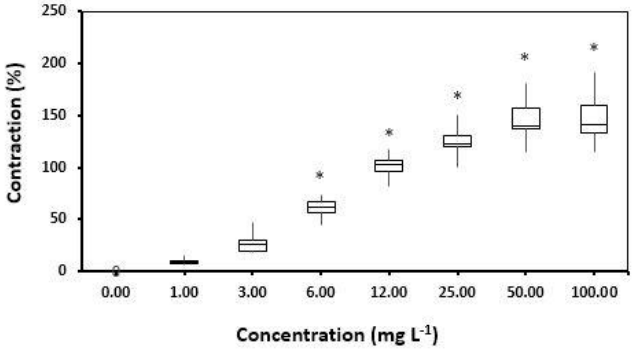

Fig. 1. Box plots for effect of *Z. officinale* aqueous extract (n = 8) on basal tonus of healthy cattle cecum preparations. Each box represents the central 50.00% of the values, the horizontal line within each box represents the median value, and the whiskers indicate the range of values that are within the inner boundary.
* Indicates significant differences compared to the control at $p < 0.05$.

Addition of hexamethonium (10.00 µM) had no effect on the contraction process of the extract while verapamil (0.10 µM), atropine (10.00 µM) and 4-DAMP (10.00 µM) completely inhibited ZOAE induced smooth muscle contractions (Fig. 3).

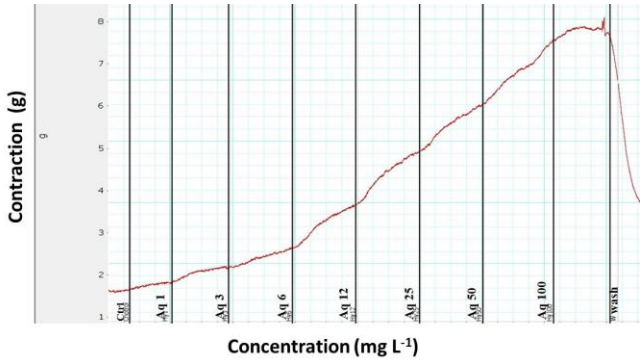

Fig. 2. Basal contractions (g) and dose dependent response (mg L^{-1}) of cecal smooth muscle to ZOAE.

Discussion

In the present study, it was shown that *Z. officinale* aqueous extract can have significant prokinetic effect on bovine smooth muscle preparations of cecum. Various studies have shown the effects of ginger extracts on gastrointestinal contractions.[13,14,17,18]

Our findings were in agreement with a previous study conducted by Najafzadeh, which reported that the aqueous extract of *Zingiber officinale* had spasmogenic effects on muscles of broiler's ileum.[23]

In another study, the prokinetic effect of ginger aqueous extract in different laboratory models (ileum and fundus tissue of rat stomach, jejunum and ileum of rabbit, guinea-pig ileum) has been studied. The results of this study showed that guinea pig's ileum contracted eight to fifty times more intensely than rabbit's jejunum and ileum or rat's gastric ileum and fundus in contact with the aqueous extract of ginger. According to these results, the researchers of this study suggested a species difference in response to aqueous ginger extract.[24]

The next aim of the present study was to identify the possible mechanisms of spasmogenic effect of ginger aqueous extract. Acetylcholine, the major endogenous neurotransmitter in the cholinergic system, causes a contraction of the smooth muscle layers in the gastrointestinal through activation of muscarinic receptors[25,26] Muscarinic receptors present in various tissues (such as smooth muscle, heart, mucous cells and brain) perform their functions by binding to acetylcholine. .[27] The muscarinic receptors are divided into five subtypes (M1 - M5).[28,29] Muscarinic acetylcholine receptors (mAChRs) have been shown to regulate gastrointestinal smooth muscle function and play a significant role in mediating contraction in smooth muscle preparations.[30]

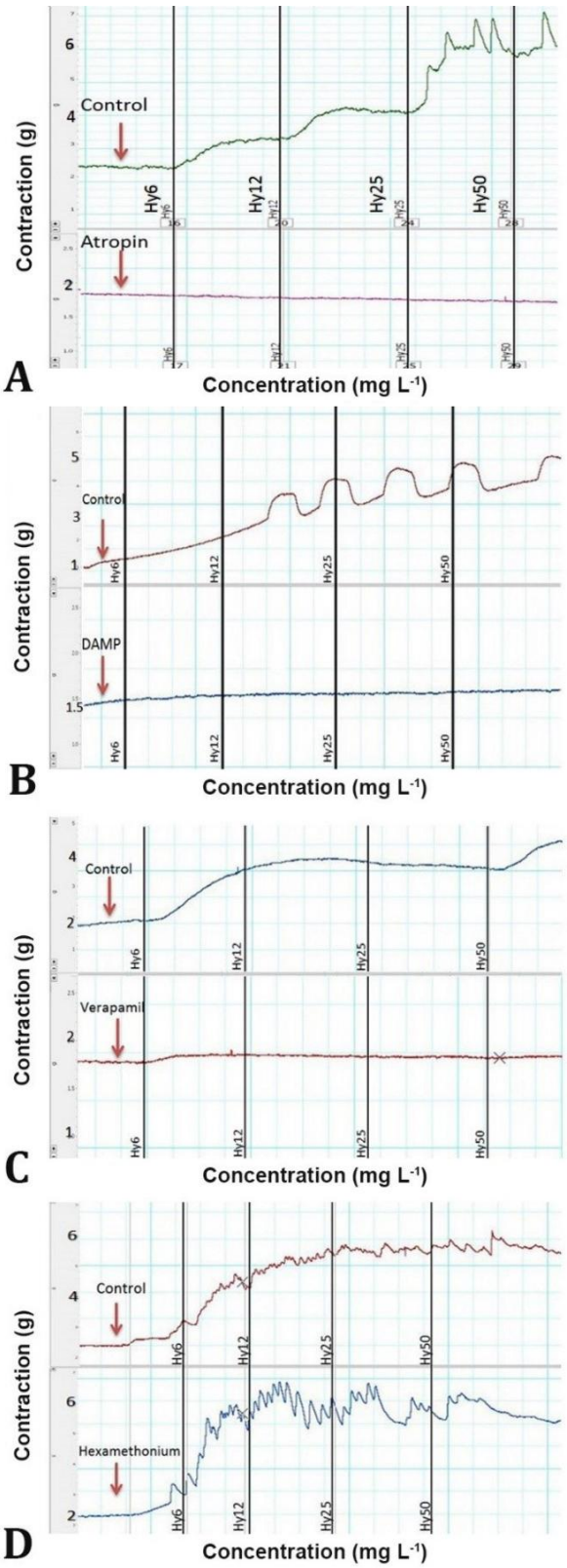

Fig. 3. Inhibitory effects of **A)** atropine, **B)** 4-DAMP and **C)** verapamil on the ZOAE-induced contractions of cecal smooth muscle. **D)** Lack of effect of hexamethonium on the ZOAE-induced contractions of cecal smooth muscle.

Atropine, an antagonist of all muscarinic receptors, eliminates the prokinetic effects of the aqueous extract. (Fig. 3A) This indicates that the aqueous extract exerts its contractile effects by stimulating muscarinic receptors.

The results of the present study on the mechanism of aqueous extract for spasmogenic effects through muscarinic receptors are in consistent with a study conducted by Najafzadeh .[23] Due to the importance of the M3 receptor in regulating gastrointestinal motility, the muscarinic M3 receptor-preferring antagonist, 4-DAMP, was used in this study. It was revealed that 4-DAMP completely blocked the ZOAE-induced contractions (Fig. 3B). Therefore, it can be said that the ZOAE stimulates the smooth muscles of the cecum by stimulating the M3 receptor.

In smooth muscle, an increase in cytoplasmic calcium concentration is the first stimulus for contraction, which occurs through the release of intracellular calcium and the penetration of extracellular calcium. The most important way for calcium to enter the cell is through L-type channels.[31,32]

Addition of verapamil, a calcium channel blocker, completely eliminates the prokinetic effect of the aqueous extract (Fig. 3C). This action proves that the ZOAE makes calcium enter the cytoplasm and causes contraction of caecal smooth muscle by acting on L-type calcium channels.

Hexamethonium is a nicotinic receptor antagonist and is often used to eliminate nicotine receptor interactions in muscarinic agonists.[33] When the effect of hexamethonium addition with the extracts was studied, the contractile effects of aqueous extract were not reduced showing that presence of Acetylcholine-like constituents independent of nicotinic receptors (Fig. 3D).

As the interest in using plant extracts and essential oils to treat animal diseases has been increasing,[34-36] we conclude that *Z. officinale* aqueous extract can be recommended as a potential agent to replace chemical prokinetic drugs to prevent or treat caecal disorders.

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Conflict of interest

There are no conflicts of interest of any kind.

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