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Calcium channel blocking activity as a mechanism of the spasmolytic effect of menthol on isolated bovine ileum

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Abstract

Menthol is an organic compound commonly derived from mint oils, known for its cooling and soothing properties, often used in various pharmaceutical, cosmetic and therapeutic applications. It exerts beneficial effects on the digestive system by relaxing smooth muscles, reducing spasms and improving gastrointestinal motility. The present study aimed to evaluate the effects of menthol on bovine ileal smooth muscle contractions *in vitro*. Ileal tissue segments were collected from adult cattle at an abattoir and subsequently immersed in 25.00 mL organ baths containing Tyrode's solution. The baths were maintained at 37.00 ˚C and continuously aerated with a gas mixture of 95.00% O_2 and 5.00% CO_2. The tissues were subjected to various contractile agents, including potassium chloride at concentrations of 30.00 and 80.00 mM, carbachol chloride at 1.00 and 4.00 µM and barium chloride at 30.00 mM. Menthol was cumulatively applied in incremental concentrations to assess its modulatory effects on contraction amplitude. Results demonstrated that menthol elicited a dose-dependent inhibition of smooth muscle contractions across most stimulatory conditions with the extent of inhibition varying among different stimuli. The Ca^{2+} channel blocking activity was further confirmed when pre-treatment of isolated ileums with menthol (23.00 and 200 µg mL^{-1}) caused a rightward shift in the Ca^{2+} concentration-response curves, similar to verapamil. These findings suggested that menthol spasmolytic action might be mediated through the modulation and inhibition of calcium channels. In conclusion, menthol effectively attenuated bovine ileal smooth muscle contractions *in vitro*, indicating its potential as a natural therapeutic agent for controlling gastrointestinal hyperactivity in cattle.

Introduction

The ileum constitutes a critical segment of the digestive tract in ruminants and plays a pivotal role in the overall process of digestion.[1] It is primarily responsible for nutrient absorption and the transfer of digested materials into the large intestine.[1] The regular peristaltic contractions of the intestinal smooth muscle, which are typically rhythmic and well-coordinated, are fundamental to maintaining this delicate physiological process.[1] Disturbances in these movements-whether manifested as hypotonia (reduced muscle tone) or hypermotility (excessive contractions)-can result in common gastrointestinal disorders such as bloating, constipation and in severe cases, intestinal obstruction.[2] These pathological conditions directly compromise animal health and consequently reduce production efficiency, for instance, milk yield in dairy cattle which is of considerable economic importance.[3] Therefore, identifying effective strategies to regulate intestinal motility, particularly through the application of medicinal plants, is both a practical and urgent necessity.[4]

In the present study, menthol -a naturally occurring compound in plant essential oils- was selected due to its diverse pharmacological properties and biological activities.[5] Menthol is a cyclic monoterpene alcohol extracted from peppermint oil (*Mentha piperita*), widely recognized for its cooling and soothing effects on smooth muscle tissue.[6,7] Menthol shows potential for improving feed efficiency, mitigating chronic inflammation and oxidative stress, inhibiting environmental and gastro-intestinal pathogens and enhancing calcium absorption.[8,9] Moreover, it has demonstrated efficacy in alleviating intestinal spasms across various animal species.[10-12] These attributes highlight menthol as a promising candidate for both research and practical application. Given the current

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challenges in livestock production, including the emergence of antibiotic resistance, fluctuating consumer demands and the growing need for healthier meat and dairy products, the exploration of menthol potential is crucial not only for advancing veterinary science but also for ensuring the economic sustainability of farming systems.[13] Accordingly, there is an urgent need to investigate its safety, efficacy and mechanisms of action in modulating gastrointestinal function, which may pave the way for innovative approaches in animal husbandry and veterinary medicine.

It has been demonstrated that menthol influences smooth muscle contractions.[14,15] Nevertheless, the precise mechanisms underlying its action as well as the magnitude of its effects on bovine ileum, remain insufficiently clarified often resulting in speculative interpretations or inconsistent conclusions within the literature. Accordingly, the present investigation was designed to examine the *in vitro* effects of menthol on bovine ileal smooth muscle contractions. By systematically assessing these effects, this study sought to contribute a novel perspective on the potential application of natural compounds in promoting gastrointestinal health in livestock.

Materials and Methods

Chemicals. Menthol, acetylcholine chloride, verapamil hydrochloride and carbachol chloride (CCh) were purchased from Sigma Chemical Co. (St. Louis, USA). Calcium chloride, potassium chloride (KCl), sodium chloride (NaCl), glucose, sodium dihydrogen phosphate (NaH_2PO_4), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), barium chloride ($BaCl_2$), ethylene-diaminetetraacetic acid (EDTA) and dimethyl sulfoxide were obtained from Merck (Darmstadt, Germany).

Tissue sample collection. Ileal tissue samples were collected from slaughtered bulls aged 2 - 4 years at the Urmia Industrial Slaughterhouse. Prior to sampling, animals were clinically evaluated, and those with a history or signs of gastrointestinal disorders were excluded. The ileum was excised from the gastrointestinal tract within 20 min postmortem. A 10.00-cm segment of the ileum was isolated, and a longitudinal incision was made to expose the mucosal surface.[16] Tyrode's solution, containing NaCl (136.90 mM), $NaHCO_3$ (11.90 mM), glucose (5.60 mM), KCl (2.70 mM), calcium chloride (1.80 mM), $MgCl_2$ (1.10 mM), and NaH_2PO_4 (0.40 mM) was used for rinsing, transport and incubation of the tissues. Both mucosal and serosal surfaces were immediately rinsed with cooled (4.00 ˚C) Tyrode's solution to remove luminal contents. The specimens were then immersed in fresh Tyrode's solution at 4.00 ˚C and maintained at this temperature during transport to the laboratory. The solution was replaced 10 min after collection to further remove residual contents and maintain tissue viability. Upon arrival at the laboratory, ileal segments were placed in Tyrode's solution on a dissecting board. The mucosa and submucosa were carefully removed and the remaining smooth muscle layer was isolated. Tissue strips measuring 20.00 × 5.00 mm were prepared parallel to the circular muscle layer.

Recording of smooth muscle activity. Each tissue strip was mounted vertically in an organ bath chamber, with one end attached to a fixed hook at the bottom of the chamber and the other end connected to an isometric force transducer (TRI 202P; PanLab, Barcelona, Spain) using a suture thread. Six transducers were connected to an amplifier (ML224; AD Instruments, Bella Vista, Australia) and a PowerLab data acquisition system (ML870; AD Instruments). Data were visualized and recorded using LabChartPro software (AD Instruments).[17] Organ bath chambers were filled with 25.00 mL of Tyrode's solution, maintained at 37.00 ˚C and continuously aerated with a gas mixture of 95.00% O_2 and 5.00% CO_2.

Experimental design. Initially, ileal smooth muscle strips were allowed to equilibrate in Tyrode's solution for 1 hr to adapt to the experimental conditions. During this period, the bathing solution was replaced every 15 min, and a resting tension of 2.00 g was applied twice at 15-min intervals. Tissue viability and contractile responsiveness were assessed prior to the main experiments by the addition of acetylcholine (10.00 µM). Following acetyl-choline-induced contraction, tissues were washed until basal tone was restored. This procedure was repeated three times and tissues exhibiting consistent responses were considered suitable for experimentation. The effects of menthol on bovine ileal circular smooth muscle contractions were evaluated in four experimental groups. These groups were designed to investigate the effects of menthol on spontaneous contractions as well as contractions induced by CCh, KCl, and $BaCl_2$. In the first group, cumulative concentrations of menthol were added to the organ bath after the tissue had reached a stable spontaneous contractile pattern. In the remaining groups, menthol was cumulatively administered following the establishment of stable contractions induced by CCh (0.30 and 1.00 µM), KCl (30.00 and 80.00 mM) or $BaCl_2$ (30.00 mM), and the resulting changes in contractile activity were recorded. Menthol was first dissolved in a solvent mixture containing 2.00% dimethyl sulfoxide and 2.00% ethanol. Then, serial concentrations of 0.00, 1.00, 3.00, 8.00, 24.00, 70.00, 100, 200, and 600 µg mL^{-1} were prepared by dilution with Tyrode's solution. Each concentration was added cumulatively to the organ bath at 2-min intervals. At the end of each experiment, tissues were washed with Tyrode's solution and muscle viability was reconfirmed by the addition of acetylcholine (10.00 µM).

Mechanism of action. The involvement of calcium channels in the effects of menthol was investigated using bovine ileal smooth muscle preparations. Tissue strips were separately pretreated with menthol (23.00 and 200

μg mL^{-1}) and calcium concentration-response curves (CRCs) were subsequently generated in a calcium-free medium. For comparison, CRCs were also obtained for verapamil (0.10 and 0.30 μM), used as a standard calcium channel blocker, in separate experimental groups.[18] For these experiments, ileal tissues were incubated in calcium-free Tyrode's solution containing NaCl (136.90 mM), $NaHCO_3$ (11.90 mM), glucose (5.60 mM), KCl (2.70 mM), $MgCl_2$ (1.10 mM), NaH_2PO_4 (0.40 mM) and EDTA (0.10 mM) for 30 min. The solution was then replaced with a depolarizing, potassium-rich calcium-free solution containing NaCl (91.04 mM), KCl (60 mM), $NaHCO_3$ (11.90 mM), glucose (5.55 mM), $MgCl_2$ (1.05 mM), NaH_2PO_4 (0.42 mM) and EDTA (0.10 mM) for 45 min. Prior to these procedures, tissues were allowed a 1 hr equilibration period in Tyrode's solution and their viability was confirmed using acetylcholine. Solutions were refreshed every 15 min throughout the protocol. Following incubation in the depolarizing solution, two control calcium CRCs were recorded by cumulative addition of calcium. After each recording, tissues were washed with the depolarizing solution to restore baseline conditions. In the third cycle, calcium CRCs were recorded 10 min after pretreatment with menthol or verapamil.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 10.6; GraphPad Software Inc., San Diego, USA). Normality of the data distribution was evaluated using the Shapiro-Wilk test, which indicated that the data were not normally distributed. Outliers were identified but were not removed in order to maintain the original structure and variability of the dataset. Due to the violation of the normality assumption and the repeated measures design of the study, the Friedman Repeated Measures Analysis of Variance on Ranks was utilized to assess differences across conditions. Post hoc pairwise comparisons between each extract concentration and the control group were conducted using the Dunn's test. A significance level of $p < 0.05$ was considered statistically significant.

Results

This study demonstrated that menthol, across a range of concentrations, significantly reduced both basal (spontaneous) and stimulated (spasmogenic) contractions in bovine ileal smooth muscle. Mechanistic analysis revealed that, similar to verapamil, menthol exerted its relaxant effect primarily through blockade of calcium channels. Importantly, the solvent used for menthol (*i.e.* dimethyl sulfoxide) had no measurable impact on ileal smooth muscle contractility. The inhibitory effects of menthol were reversible, as tissue function returned to baseline following rinsing. Also, at the conclusion of the experiments, tissues exhibited normal responsiveness to acetylcholine, confirming that menthol exposure did not induce structural or functional damage.

Effects on ileal contraction. Spontaneous contractions were significantly inhibited at concentrations of 24.00 - 600 μg mL^{-1} of menthol ($p < 0.05$; Fig. 1A). Carbachol chloride (0.30 and 1.00 μM) induced contractions were markedly attenuated at concentrations of 24.00 - 600 μg mL^{-1} of menthol ($p < 0.05$; Figs. 1B and C). Barium chloride induced contractions were significantly inhibited at concentrations of 24.00 - 600 μg mL^{-1} of menthol ($p < 0.05$; Fig. 1D). Menthol also relaxed contractions induced by KCl at 30 mM and 80 mM, with significant effects observed at 70.00 - 600 μg mL^{-1} of menthol ($p < 0.05$; Figs. 1E and 1F).

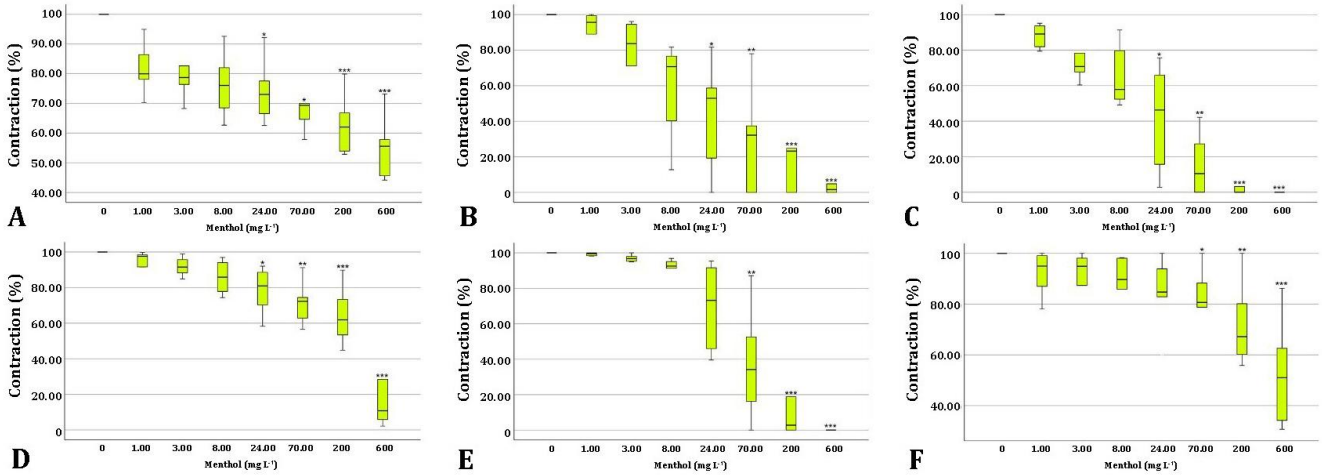

Fig. 1. Box plots illustrating the spasmolytic effect of menthol on **A)** spontaneous, and induced contractions by **B)** carbachol chloride (CCh) 1.00 μM, **C)** CCh 4.00 μM, **D)** Barium chloride 30.00 mM, **E)** Potassium chloride (KCl) 30.00 mM, and **F)** KCl 80.00 mM on isolated bovine ileum. For each concentration, the central horizontal line within the box represents the median. The lower and upper boundaries of the box correspond to the first and third quartiles, respectively. The box represents the interquartile range (IQR). The whiskers extend to the most extreme data points that fall within 1.50 × IQR of the lower and upper quartiles. Statistical significance is indicated relative to the control group (Menthol 0): *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Calcium channel blockade. To confirm the calcium ion (Ca^{2+}) inhibitory effect, menthol pre-incubated ileum tissues shifted the Ca^{2+} CRCs curves at 23.00 and 200 µg mL^{-1} towards the right, similar to that caused by verapamil at concentrations of 0.10 and 0.30 µM (Fig. 2).

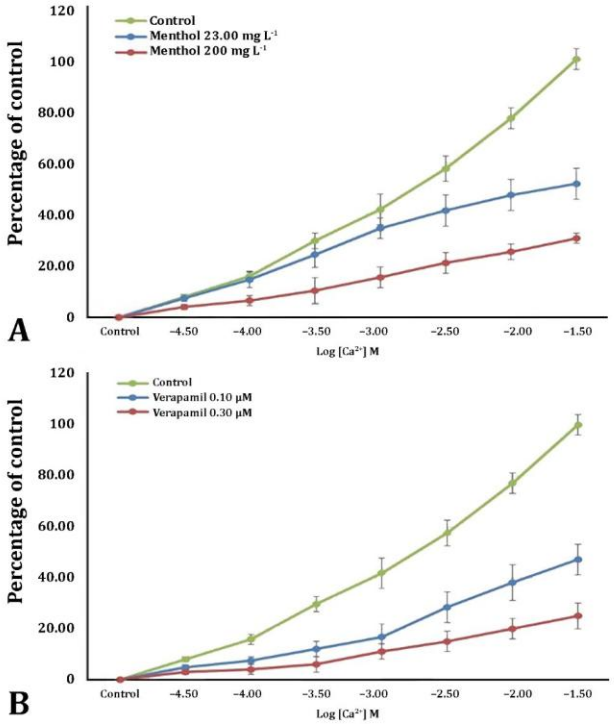

Fig. 2. Concentration-response curves of calcium ion (Ca^{2+}) in the absence and presence of the increasing concentrations of **A)** menthol and **B)** verapamil in isolated bovine ileum preparations. Values shown are mean ± SEM, n = 6.

Discussion

The results of the present study confirmed that menthol directly inhibited ileal smooth muscle contractility. This inhibitory effect was fully reversible following tissue washing indicating that menthol did not induce structural or functional damage to the intestinal tissue. Notably, this study was the first to demonstrate, under *in vitro* conditions, that menthol significantly reduced the mechanical activity of the circular smooth muscle of the bovine ileum.

Extracts of medicinal plants and their essential oils are widely reported to exert antispasmodic effects on various smooth muscles including those of the gastrointestinal tract. These effects are thought to be mediated through multiple mechanisms such as anticholinergic activity,[19] calcium channel blockade,[20] phosphodiesterase inhibition,[21] and/or activation of potassium channels.[22,23]

To elucidate the pharmacodynamic mechanisms underlying the observed antispasmodic activity, menthol was evaluated in the presence of different spasmogenic agents on isolated bovine ileum, as previously described.[24] Given that many antispasmodic agents exert their inhibitory effects on gastrointestinal smooth muscle primarily through calcium channel blockade,[25,26] additional experiments were performed to investigate the effect of menthol on ileal contractions induced by CCh and high potassium concentrations.[27]

Critical analysis of the inhibitory CRCs of menthol against both CCh- and high K^+-induced contractions revealed a verapamil-like pharmacological profile. Menthol exhibited significantly greater inhibitory potency against high K^+- and CCh-induced contractions, suggesting the involvement of calcium channel blocking -like activity.[28,29]

To further substantiate this hypothesis, ileal tissues were rendered calcium-free by replacing the bathing medium with Ca^{2+}-free Tyrode's solution containing the chelating agent EDTA. The calcium-depleted tissues were subsequently preincubated with two concentrations of menthol. Concentration–response curves for exogenously administered Ca^{2+} were then constructed in the absence and presence of menthol. Preincubation with menthol produced a rightward shift in the Ca^{2+}-CRCs along with a marked suppression of the maximal contractile response, comparable to the effects observed with verapamil, a standard calcium channel blocker.[30,31] These findings further confirmed the verapamil-like calcium channel blocking action of menthol.

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Conflict of interest

There are no conflicts of interest of any kind.

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