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Original Scientific Paper

EFFECTS OF MENTHOL AND MONEPANTEL ON THE CONTRACTIONS OF THE ISOLATED NEUROMUSCULAR PREPARATION OF *ASCARIS SUUM*

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Summary
Parasitic nematodes in animals lead to serious health disorders, high economic losses in animal husbandry, and often are zoonoses, which represent a direct danger to human health. Prevention and treatment of parasitic infections involves the use of medication, but modern antiparasitic pharmacotherapy is faced with several major challenges. The development of parasite resistance, the limited possibility of increasing the doses of existing drugs due to potential toxicity, the lack of new antiparasitics and the long withdrawal period are the reasons why the possibility of antiparasitic application of substances of plant origin is increasingly being researched. In this paper, we present a study of the anthelmintic effect of the menthol terpenoid in the model of contractions of the neuromuscular preparation of the parasitic nematode *Ascaris suum*. All cholinergic agonist drugs (levamisole, pyrantel, morantel, etc.) lead to the death of parasites by causing strong contraction of their muscles. In our research, menthol by itself did not cause changes in the tone of the neuromuscular preparation of *Ascaris suum*, but it significantly potentiated the contractions caused by acetylcholine and pyrantel, thus exhibiting the characteristics of an allosteric modulator of nAChR, especially of the L type. This increase in contractility is sensitive to monepantel, an nAChR antagonist. The potentiating effect of menthol on the contractions of *Ascaris suum* could be used in combined therapy with cholinergic agonists pyrantel and levamisole, which would increase their effectiveness without increasing doses and reduce the possibility of developing parasite resistance.
Keywords: menthol, monepantel, A. suum, nAChR

INTRODUCTION

Infections of humans and animals with parasitic nematodes are a growing health problem in veterinary and human medicine. Roundworms or nematodes are the most diverse of all animal species and over 80,000 species have been described so far, of which more than 15,000 are parasitic (Hugot et al., 2001). Based on data from the

World Health Organization in 2018, more than 1.5 billion people, or 24% of the world's population, are infected with at least one type of intestinal parasite, which mainly includes roundworms (*Ascaris lumbricoides* and *Trichuris trichiura*) and tapeworms (*Necator americanus* and *Ancylostoma duodenale*). Animal infections with nematodes lead to serious health disorders, high economic losses in animal husbandry, and are often zoonoses, i.e. they pose a direct threat to human health (Paterson and Barber, 2007). The primary way of preventing and suppressing parasitic infections is the use of anthelmintic drugs, but modern antiparasitic pharmacotherapy is faced with several major challenges. An increasing number of reports refer to the resistance of parasitic nematodes to classical antiparasitic drugs. On the other hand, in contrast to antimicrobial drugs, whose doses are safe even after increasing in order to suppress resistance, increasing the doses of antiparasitic drugs often results in the occurrence of toxicity. Particularly important for veterinary medicine is the fact that most antiparasitic drugs require a long withdrawal period after administration in animals whose tissues are intended for human consumption (Martin et al., 2004). We have only mentioned some of the most important reasons that directed pharmacological research towards the potential application of herbal products, instead of conventional synthetic antiparasitic drugs. Plants produce etheric or essential oils as organic products of secondary metabolism. Based on their pharmacological characteristics (antimicrobial, antiparasitic, etc.), essential oils (or their active ingredients) are potentially the most serious alternative medicines and biocides. Their active ingredients are able to effectively and safely replace classic antiparasitic drugs or significantly enhance their effect, without the need to increase their dosage.

Menthol is a well-known compound, a monoterpenoid that is found in nature in a high percentage in the essential oil of mint (*Mentha piperita*) with a characteristic taste and smell. The properties of menthol as a local anesthetic and positive analgesic effects on the skin and mucous membranes are described in the literature (Patel et al., 2007). In our earlier research, we showed that menthol is a positive allosteric modulator of the nicotinic acetylcholine receptor (nAChR) of parasitic nematodes (*Ascaris suum* and *Oesophagostomum dentatum*) (Choudhary et al., 2019).

More than 35 years have passed since the discovery of avermectins, the last major group of effective antiparasitic drugs. All other, later discovered potential drugs, generally did not meet the criteria for mass use. Monepantel is a representative of the aminoacetonitrile group (AAD), one of the newest classes of anthelmintics, which are considered, based on the manufacturer's research, to act as allosteric modulator of nematode nAChR. Studies published later described monepantel as an nAChR antagonist (Abongwa et al., 2018). The specific mechanism of action indicated that monepantel could be a very important new drug in the pharmacotherapy of parasitic infections.

In this paper, we present research on the effect of menthol on the contractions of an isolated neuromuscular preparation of *Ascaris suum* (*A. suum*), induced by ACh or pyrantel, as well as the influence of monepantel on the effect of menthol.

MATERIALS AND METHODS

The parasitic pig nematode *A. suum* was delivered in temperature-controlled containers from a slaughterhouse in the vicinity of Belgrade (Ambar) immediately after gut cleaning and collection in a specially prepared - Lockes solution. The composition of Lockes solution was standardized (mM): NaCl 155, KCl 5, $CaCl_2$ 2, $NaHCO_3$ 1.5 and glucose 5. In the laboratory the nematodes were kept in the same solution, which was maintained at a temperature of 32-36ºC in a water bath. Lockes solution was changed every 12 hours for 5 days. Only adult female Ascaris were used in the experiments because they are larger and more numerous in the parasite population. The neuromuscular preparation for testing contractions was prepared by dissection of the front part of the parasite's body 2 to 3 cm caudal to the head. By further cutting the tissue between the two red lines longitudinally, a preparation (segment) of always the same dimension (1 cm) was obtained, which was further used.

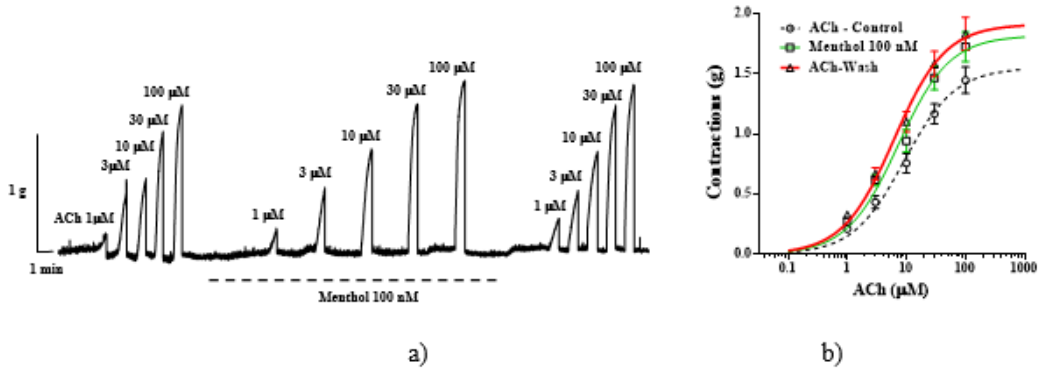
The prepared ascaris segment was placed in a chamber (bath) with a volume of 20 ml, which was filled with Ascaris Perienteric Fluid Ringer/APF of standard composition (mM): NaCl 23, Na-acetate 110, KCl 24, $CaCl_2$ 6, $MgCl_2$ 5, glucose 11, HEPES 5, with a pH value of 7.6. One end of the segment is attached to a hook on the bottom of the bath, while the other part is attached to an isometric transducer connected to a PC computer via an interface and amplifier. Continuous mixing of the applied substances in the bath was ensured by constant insertion of ambient air with an electric air pump, whereby the movement of the bubbles did not affect the contractions. After being placed in the bath and connected to the isometric transducer, the preparation is exposed to an initial tension of 0.5-1.0 g for 15-20 minutes with a micrometer regulator on the transducer support. When the basic tone stabilized at around 0.5 g, the tested substances were applied and then the contractions were monitored and measured in real time. Contractions were monitored and recorded with eLAB 44 software (ElUnit, Belgrade). A protocol of application of increasing concentrations of tested substances, nicotinic receptor agonists (acetylcholine and pyrantel) and substances in certain concentrations that we assumed would modulate the effects of these substances (menthol and monepantel) was used. After each maximal contraction, the preparation was washed off.

Acetylcholine chloride (Sigma-Aldrich, St. Louis, MO, USA), monepantel (Zolvix®, Novartis, USA), pyrantel citrate (Sigma-Aldrich, St. Louis, MO, USA) and menthol (Sigma-Aldrich, St. Louis, MO, USA) were used in the trials.

Statistical analysis of the obtained results was performed in the program Graph Pad Prism, Version 6.0 (San Diego, Ca, USA). Differences between series of results were compared by analysis of variance (ANOVA), i.e. Tukey-test, while the dose dependence of the obtained effects was analyzed by nonlinear regression. All results are expressed as means ±SE.

RESULTS

Based on data from our previous research (Choudhary et al., 2019), we tested the effect of menthol 100nM on contractions of *A. suum* neuromuscular preparations induced by increasing concentrations of ACh (1, 3, 10, 30 and 100mM). Figure 1a shows the original recording of contractions without and in the presence of 100nM menthol. The obtained results of contractions were processed by non-linear regression, based on which the value of the mean effective concentration (EC_{50}) of ACh was determined. The control value was 9.15±1.22mM, in the presence of menthol the EC_{50} was reduced to 7.29±1.21mM, while after the removal of menthol from the bath it was even lower, 6.19±1.20mM. At the same time, the value of the maximal effect (E_{max}) increased. The control E_{max} was 1.55±0.09g, in the presence of menthol it increased to 1.82±0.09g and continued to increase after washing to 1.91±0.09g (Figure 1b).



a) b)

Figure 1

a) Representative recording of isometric contractions of *A. suum* neuromuscular preparation induced by increasing concentrations of ACh and the effect menthol 100nM on these contractions

b) Dose-dependent sigmoid curves of the contractile effect of ACh without and in the presence of menthol 100nM (n=6)

We also examined the effect of menthol on the contractions of *A. suum* caused by pyrantel (Figure 2a and 2b). The control EC_{50} of pyrantel was 8.42±1.14nM, in the presence of menthol, it was significantly reduced to 4.51±1.11nM (p=0.0483), while after washing it returned to the control level (8.42±1.18nM). On the other hand, the maximal effect was changed very little, from 1.98±0.09g in control series to 2.02±0.05g in the presence of menthol and 1.82±0.08g after washing.

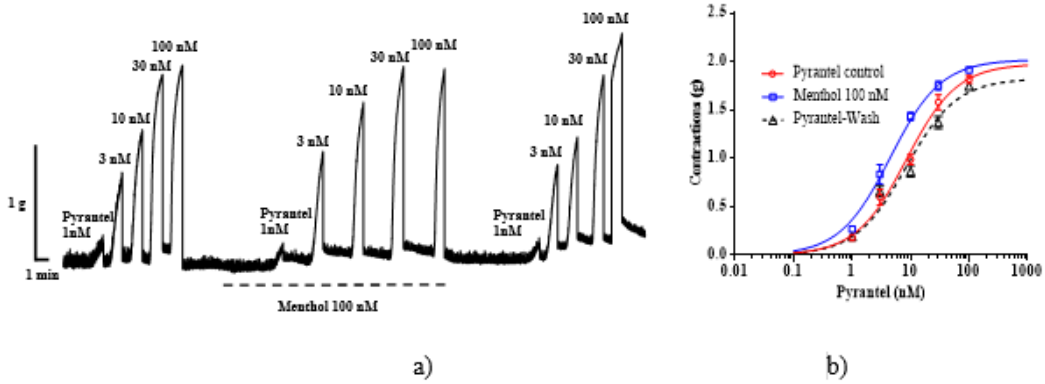


Figure 2

a) Representative recording of isometric contractions of the A. suum neuromuscular preparation induced by increasing concentrations of pyrantel and the effect of menthol 100nM on these contractions

b) Dose-dependent sigmoid curves of the contractile effect of pyrantel without and in the presence of menthol 100nM (n=5)

In the third part of the study, we tested the effect of monepantel on the potentiating effect of menthol in ACh-induced contractions (Figure 3a and 3b). The control EC_{50} value for ACh was 13.30±1.25mM, but incubation with 100nM of menthol resulted in a decrease in EC_{50} to 9.04±1.24mM. However, after the addition of 1mM monepantel, the value of ACh EC_{50} compared to the control increased significantly ($p=0.0245$) to 18.48±1.33mM. This value was significantly higher than the EC_{50} of ACh in the presence of menthol ($p< 0.0001$), but after the removal of monepantel from the bath solution it increased even slightly above the control value (15.30±1.27mM). Incubation of the neuromuscular preparation of *A. suum* with 100nM of menthol caused an increase in the maximal effect (E_{max}) from 2.07±0.14g to 2.32±0.14g, but incubation with monepantel (1mM) significantly reduced ($p=0.0418$ compared to control and $p=0.0011$ compared to contractions in the presence of menthol) E_{max} to 1.50±0.15g. It is interesting that after washing the preparation, i.e. removing menthol and monepantel, the E_{max} value was higher than the control, reaching 2.18±0.17g.

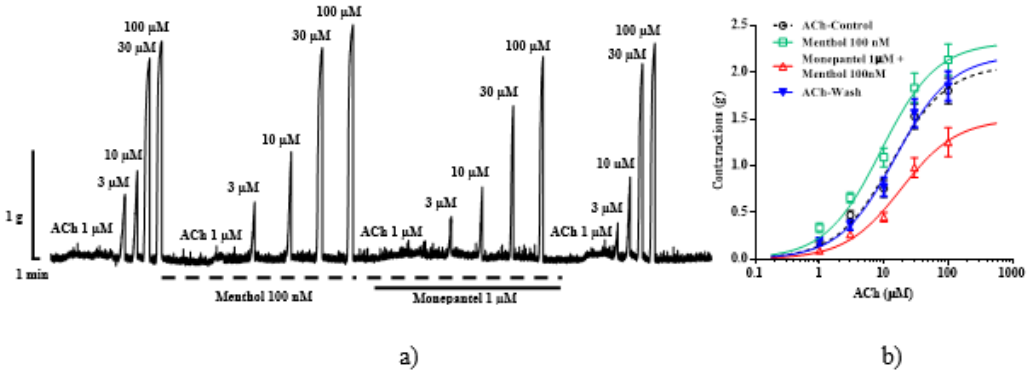


Figure 3

a) Representative recording of isometric contractions of *A. suum* neuromuscular preparation induced by increasing concentrations of ACh, stimulation of contractions with 100nM of menthol and inhibition with 1µM monepantel (dashed line: incubation with 100nM menthol; solid line: incubation with 1µM monepantel)
b) Dose-dependent sigmoidal curves of the contractile effect of ACh without and in the presence of 100nM menthol or menthol and 1mM monepantel (n=6)

DISCUSSION

The obtained results showed that menthol enhances ACh-induced contractions of the neuromuscular preparation of *A. suum*. Incubation with 100nM of menthol decreases the EC_{50} value of ACh and increases the maximal contractile effect (E_{max}) for about 20%. However, menthol itself did not cause changes in basal tone of preparation. These results confirm our previously published research where we obtained a similar effect of menthol on the nAChR of *A. suum* and *O. dentatum* expressed in *Xenopus laevis* oocytes and showed that it is most likely an allosteric modulator of the nAChR of parasitic nematodes (Choudhary et al., 2019).

Based on pharmacological characteristics, the nematode muscle nAChR has similarities to the neuronal type of vertebrate nAChR, as it is relatively insensitive to blockade by alpha-bungarotoxin (Tornøe et al., 1995). Unlike vertebrates, however, nematodes possess more than one type of muscle nAChR found in the neuromuscular synapses of the adult nematode. *A. suum* has at least three types of nAChRs that differ in their sensitivity to different agonists and the characteristics of the ion channel they regulate. Each of the three subtypes is named for the agonist to which it is predominantly sensitive, N type (preferring nicotine and oxanthel), L type (preferring levamisole and pyrantel), and B type (preferring bephenium) (Martin et al., 2004; Robertson et al., 2002). In our earlier research with menthol, it was shown that it primarily stimulates the effects of ACh, which is understandable because the ACh binding site is well conserved in all types of nACh receptors. However, menthol had a effective stimulatory effect on L-type but very weak on N-type of nAChR. Precisely for this reason, we tested the effect of menthol on pyrantel-induced contractions.

Menthol potentiated the action of pyrantel, significantly reducing its EC_{50} value with a minimal increase in E_{max}. Given that pyrantel is primarily an L-type nAChR agonist, these results are in agreement with our statement previously published (Choudhary et al., 2019). It was particularly interesting to examine the effect of monepantel, which is a negative allosteric modulator of the nematode nAChR (Abongwa et al., 2018). In our study, monepantel completely neutralized the stimulatory effect of menthol and significantly inhibited the contractile effect of ACh, increasing its EC_{50} value and decreasing E_{max}.

CONCLUSION

The research presented shows that herbal anthelmintics can relieve pressure on a limited group of available anthelmintics. Positive allosteric modulation of nAChR exhibited by menthol would be useful in combined therapy with cholinergic anthelmintics such as pyrantel or levamisole, which would increase their effectiveness without increasing the dose and reduce the possibility of development of parasite resistance.

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Conflict of interest statement: The authors declare that there is no conflict of interest.

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