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Blood-brain barrier breakdown, memory impairment and neurotoxicity caused in mice submitted to orally treatment with thymol

Matheus D. Baldissera[a,*], Carine F. Souza[b], Antônio Francisco Igor M. De Matos[a], Pedro Henrique Doleski[a], Bernardo Baldisserotto[b], Aleksandro S. Da Silva[c], Silvia G. Monteiro[a,*]

[a] Department of Microbiology and Parasitology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Department of Physiology and Pharmacology, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Department of Animal Science, Universidade do Estado de Santa Catarina, Chapecó, RS, Brazil

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ABSTRACT

Several evidences have related the biochemical and pharmacological properties of thymol, but the possible neurotoxic effects of this compound remain unknown and not evaluated. Thus, the purpose of this study was to evaluate whether intake of thymol in different doses (10, 20 and 40 mg/kg) induce neurotoxicity and behavioral alterations using mice as experimental model, as well as the involvement of blood-brain barrier (BBB) and brain neurotransmitters in these alterations. Thymol (20 and 40 mg/kg) significantly decrease latency time to inhibitory avoidance task when compared to control group, indicating a memory loss after 30 days of oral treatment. Also, thymol (20 and 40 mg/kg) induced a significant increase on BBB permeability to Evan's blue dye when compared to control group, which is an indicative of BBB breakdown. Moreover, a significant increase of brain acetylcholinesterase (AChE) was observed in mice treated with 40 mg/kg of thymol, while the activity of sodium-potassium pump (Na^+, K^+-ATPase) was inhibited in mice treated with 20 and 40 mg/kg thymol when compared to control group. Finally, mice that received 20 and 40 mg/kg thymol showed a significant increase on cerebral reactive oxygen species (ROS) levels and cerebral xanthine oxidase (XO) activity compared to control group. Based on these evidences, the rupture of BBB can be considered an important pathway linked in thymol-induced memory loss. Also, the augmentation of brain ROS levels elicited by increase on XO activity may be a via involved in the damage to BBB, and an oxidative pathway that impairs the activity of brain neurotransmitters, as AChE and Na^+, K^+-ATPase. In summary, the dose of 10 mg/kg thymol can be safe and without neurotoxic effects in a period of 30 days of intake.

1. Introduction

Monoterpenes ($C_{10}H_{16}$), the main constituents of essential oils, are extensively used due to their several biological and pharmacological properties, representing an interesting and promising alternative for development of new therapeutic agents (Barreto et al., 2014; Maroccia et al., 2018). Also, many monoterpenes and other secondary metabolites of essential oils have been included in clinical trials in order to obtain new drug candidates for treatment of diseases that affect animals and/or humans (Maroccia et al., 2018). It is important to highlight that monoterpenes referred as "natural" and commonly considered safe and without side effects contributed to boost their consumption in animal and human medicine (Mujezinovic et al., 2018), but very little research has been performed to study the possible toxic effects of monoterpenes.

Recently, some evidence have demonstrated that morphological and functional hepatic alterations associated with necrosis, steatosis, vascular lesions and augmentation on biomarkers of hepatic alterations, as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are elicited by monoterpenes as citral (Fandohan et al., 2008), limonene (Lei et al., 2012), pulegone and menthol (Lassila et al., 2016), but the toxic effects of thymol, a monoterpene extensively used in fish and poultry nutrition remains poorly investigated. Thymol (2-isopropyl-5-methylphenol) is a phenolic crystalline monoterpene found in the essential oils of *Thymus vulgaris* (Lamiaceae) (Hegazy et al., 2018) and *Origanum vulgare* (Lamiaceae) (Aybastier et al., 2018), commonly used as active ingredients in food flavorings, perfumes, deodorants, cosmetics, and pharmaceutical products (Aboelwafa and Yousef, 2015). Additionally, thymol is known to exert several pharmacological properties such as antimicrobial (Wang and Yam, 2018), antioxidant (Luna et al., 2018), antiparasitic (Silva Lima et al., 2018), growth promoter

* Corresponding authors.
E-mail addresses: mdbaldissera@mail.ufsm.br (M.D. Baldissera), sgmonteiro@uol.com.br (S.G. Monteiro).

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(El-Hack et al., 2016), hypocholesterolemic, and hypoglycemic (Saranavan and Pari, 2015). Although many studies have been performed in order to determine biochemical and pharmacological properties of thymol, very little information is known about its toxic effects, as negative impact on superoxide dismutase (SOD) activity (Suzuki et al., 1987), genotoxicity for human lymphocytes (Aydin et al., 2005) and cytotoxicity for murine macrophages (Belato et al., 2018), but possible neurotoxic effects remain unknown. In this sense, Baldissera et al. (2016) reported that monoterpene alpha-terpinene induced potent neurotoxicity and behavioral abnormalities through alteration on brain neurotransmitters, as acetilcholinesterase (AChE) and sodium-potassium pump (Na^+, K^+-ATPase). AChE is an enzyme of cholinergic pathway responsible by the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetate, where decrease of ACh content in brain tissue is linked to cognitive deficits and memory impairment (Kaundal et al., 2018). Moreover, Na^+, K^+-ATPase is an enzyme that has a key role in intracellular ion and membrane potential homeostasis, contributing to neuronal excitability, and inhibition of its activity has been associated with impaired synaptic response and consequently memory loss (Zhang et al., 2013; Pacheco et al., 2018). Also, some evidence have suggested the involvement of reactive oxygen species (ROS) with neurotoxicity induced by natural products, including their contribution on memory impairment affecting the activity of the brain neurotransmitters above cited (Muthulakshmi et al., 2018; Maharajan et al., 2018).

Thus, the purpose of this study was to determine whether intake of thymol in different doses induce neurotoxicity and behavioral alterations using mice as experimental model, as well as the involvement of blood-brain barrier (BBB) and brain neurotransmitters in these alterations.

2. Material and methods

2.1. Chemical, animals and study design

Thymol (molecular weight: 150.22 g/mol) was commercially acquired at ≥98.5% of purity from Sigma-Aldrich (St. Louis, USA).

Twenty-four adult male Swiss mice (outbred strain, heterogenic, conventional weighing) approximately 12 weeks old (30–35 g) were used as experimental model. All mice were maintained at 21 ± 1 °C and 70% of humidity with free access to water and food, under a 12-hour light-dark cycle with lights being turned on at 7:00 A.M. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Resources of the Universidade do Estado de Santa Catarina (protocol number 7061210318). These animals were divided into four groups (n = 6 per group), as follows: control group (received water via oral) and groups that received 10, 20, or 40 mg/kg of thymol orally (dissolved in water at 23 °C) for 30 consecutive days (once a day in intervals of 24 h) based on the protocol stablished by Saranavan and Pari (2015), where all tested doses presented potent hypoglycemic and hypocholesterolemic activities. Beyond the doses, the time and via of treatment follow the protocol stablished by Saranavan and Pari (2015).

2.2. Behavioral tests

2.2.1. Inhibitory avoidance task

Non-spatial long-term memory was evaluated using an inhibitory avoidance task following the protocol stablished by Sakaguchi et al. (2006), and recently published in details by Baldissera et al. (2016). A step-down latency was assumed as a measure of acquisition considering a cut-off time of 300 s.

2.2.2. Open field

The open field was used to observe possible alterations on locomotor and exploratory activities using the method proposed by Walsh and Cummins (1976). After the inhibitory avoidance acquisition test, the mice were transferred to a 40 × 40-cm open field, with the floor divided into 9 squares. Each mouse was gently placed in the center of apparatus and observed for 4 min in order to record the locomotor and exploratory activities. The number of segments crossed with the four paws was used to evaluate the locomotor activity, the number of times it reared on hind limbs was used to determine the exploratory activity.

2.3. BBB permeability to Evan's blue

The BBB integrity was determined using Evan's blue dye extravasation. The mice were injected intraperitoneally with 1 mL of Evan's blue (1%) 1 h before euthanasia according to the protocol stablished by Barichello et al. (2012).

2.4. Tissue collection

Thirty days after the beginning of treatment, all mice were anesthetized with isoflurane in an anesthetic chamber followed by decapitation to access the brain tissue, that was removed in a glass dish over ice and longitudinally divided into two hemispheres (left and right): the left hemisphere was used to evaluate the BBB permeability, and the right hemisphere to determine the variables mentioned below.

2.5. Evan's blue quantification

The brain was homogenized (1:5 w/v) with trichloroacetic solution and centrifuged (1000 × g, 10 min at 4 °C), the supernatant was diluted in ethanol (1:3 v/v), and its fluorescence was measured (excitation at 620 nm and emission at 680 nm) using a luminescence spectrophotometer, as preconized by Barichello et al. (2012). The results were expressed as ng of Evan's blue/mg of tissue.

2.6. Brain AChE and Na^+, K^+-ATPase activities

AChE enzymatic activity was measured spectrophotometric using the protocol stablished by Ellman et al. (1961), and previously described in detail by Rocha et al. (1993). Enzymatic activity was expressed as μmol AcSCh/h/mg of protein.

Na^+, K^+-ATPase activity was colorimetrically evaluated at 650 nm using the protocol preconized by Fiske and Subbarow (1925) and recently described in details by Wilhelm et al. (2013). Enzymatic activity was expressed as nmol Pi released/min/mg of protein.

2.7. Brain ROS levels and XO activity

ROS levels were evaluated by the DCFH oxidation method described by LeBel et al. (1992) using fluorescence method (excitation at 485 nm; emission at 528 nm), recently published in detail by Biazus et al. (2017). Results were expressed as U DCF/mg of protein.

XO activity was evaluated following the method preconized by Westerfeld and Richert (1949) and cited in detail by Baldissera et al. (2017). Enzymatic activity was expressed as UI/mg of protein.

2.8. Brain acetylcholine (ACh) levels

Brain ACh levels were evaluated using a commercial ACh assay kit (Thermo Fisher Scientific®, Massachusetts, USA) according to the manufacturer's protocols. The color intensity at 570 nm is directly proportional to the ACh concentration in the sample. The ACh levels were reported as ng/mg of protein.

2.9. Brain protein content

Protein content was evaluated by the method of Coomassie blue G dye (Read and Northcote, 1981), using serum bovine albumin as the standard.