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Menthol does not Affect NSAIDs Effects in Behavioral Tests on Rats

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(Presented by Academy Member Nodar Mitagvaria)

ABSTRACT. Temperature poignancy is a finely tuned part of mammalian somatosensory system, allowing animals and humans avoid thermal conditions in nature that may be potentially harmful, as well as attract organisms to the thermal clime that is the most amenable to survival. Of different somatosensory modalities, cold is one of the more ambiguous percepts, evoking the pleasant sensation of cooling, the stinging bite of cold pain, and welcome relief from chronic pain. It is widely accepted that for temperature, cation channels of the transient receptor potential (TRP) family function as molecular thermometers, providing the receptor potential that initiates signaling to the central nervous system. For the last decades menthol is widely used in food and oral hygiene products for its fresh cooling sensation. Moreover, it is well established that menthol enhances cooling by interacting with the cold-sensitive thermo TRP channel TRPM8, but its effect on pain is less well understood. We have recently found that menthol dose-dependently increases the latency for noxious heat-evoked withdrawal of the treated hindpaw of rats indicating antinociception. Moreover, menthol has a biphasic effect on thermal avoidance. We are currently engaged in the study of non-steroidal anti-inflammatory drugs (NSAIDs) influence on the actions of agonists of TRP channels. Here we report that menthol does not affect thermo TRPM8 channel after treatment with widely used NSAIDs as diclofenac, ketorolac and xefocam. *© 2016 Bull. Georg. Natl. Acad. Sci.*

Key words: antinociception, cold pain, hypoalgesia, mechanical pain, paw withdrawal reflex

Temperature sensation like tactile and pain is mediated by peripheral sensory neurons through activation of modality-specific sensory receptors. For temperature, cation channels of the transient receptor potential (TRP) family function as molecular thermodetectors, providing the receptor potential that initiates signaling to the central nervous system (CNS). Recent data established that thermo-TRP melastatin subfamily number 8 (TRPM8) channel, the receptor for menthol functions as the primary mammalian detector of cold sensation [1-8]. Menthol is derived from plants of the mint family and imparts their distinctive odor. For the last decades menthol is commonly used in food additives and has broad industrial use in oral hygiene, medicinal and other applications [9].

In electrophysiological *in vitro* experiments, the TRPM8 channel is activated at temperatures below

~25°C, with currents increasing in magnitude as temperatures decrease in trigeminal ganglion cells [10]. Behaviorally, mice lacking TRPM8 channels fail to distinguish warm from cool and poorly avoid noxious cold [1,11-13]. Furthermore, cold pain caused by innocuous stimuli (allodynia) associated with inflammatory and neuropathic injury is diminished in these animals [11,14]. Thus, because of its prominent role in cold sensation, TRPM8 channel and the neurons expressing this receptor are attractive targets in the study of cold and cold pain transduction [3, 11].

We have recently found that menthol dose-dependently increased the latency for noxious heat-evoked withdrawal of the treated hindpaw indicating antinociception. Moreover, menthol at the highest concentration (40%) weakly but significantly reduced mechanical withdrawal thresholds, with no effect at lower (0.1, 1, and 10%) concentrations. In addition, menthol had a biphasic effect on thermal avoidance. At high concentrations (10 and 40%) menthol reduced avoidance of colder temperatures (15 and 20°C) compared to 30°C , while at lower (0.01, 0.1 and 1%) concentrations menthol enhanced cold avoidance. Finally, in a –5° C cold-plate test, 40% menthol significantly increased the nocifensive response latency (cold hypoalgesia) while lower concentrations were not different from vehicle controls. This effect was lost using a 0° C cold plate test [9].

We are currently engaged in the study of non-steroidal anti-inflammatory drugs (NSAIDs) influence on the actions of agonists of TRP channels. We have found that after pretreatment with the three widely used NSAIDs such as diclofenac, ketorolac and xefocam in the ipsilateral (injected) hindpaw, allyl isothiocyanate (AITC) (main compound of mustard oil), cinnamonaldehyde (CA), and capsaicin resulted in significant decreases in latency of the thermal withdrawal reflex compared with vehicle or the contralateral hindpaw. The same findings were observed for the mechanical paw withdrawal threshold. In approximately 30 min the effects of AITC, CA, and capsaicin returned to baseline. The data are different from our previous evidence, where TRPA1 agonists AITC and CA and TRPV1 agonist capsaicin produced hyperalgesia for nearly 2 h and resulted in facilitation of these withdrawal reflexes [15,16].

The apparently opposing or at least different effects of menthol on the perception of cold and mechanical pain after pretreatment with NSAIDs prompted the present study. We wished to systemically investigate and compare the modulatory effects of topical menthol on cold and mechanical sensitivity in rats using two behavioral assays.

Materials and Methods

Animals. Behavioral studies using adult male Wistar rats (350-450 g) were singly housed and given rodent chow and water *ad libitum*. The Beritashvili Experimental BMC Animal Care and Use Committee approved the study protocol. Every effort was made to minimize both the number of animals used and their suffering. Guidelines of the International Association for the Study of Pain regarding animal experimentation were followed throughout [17].

Application of chemicals. L-Menthol dissolved in ethanol and Tween-80 (Fisher Scientific, Fair Lawn, NJ, USA) at doses 10 and 40%, (640 mM and 6.4 M, respectively) or vehicle control (10% ethanol with 5% Tween-80) was topically applied by cotton tip applicator to both ventral hind paws, allowed to dry for 2 min, and the paw was cleaned with an ethanol wipe prior to placing the animal into the test arena. Vehicles were applied in the same manner separately as controls. For the cold plate test, menthol or vehicle was applied to both paws. The rationale for bilateral application was to ensure that at least one hind paw would contact the thermal surface even if the animal guarded the other paw. Twenty minutes prior to apply menthol, 2 μl of NSAIDs, diclofenac (2.5%), ketorolac (3%), and xefocam (0.4%) were injected in both hindpaws and animals were examined by the cold plate and mechanical paw tests. Different animal groups were used for the experiments and they were only tested with one concentration of menthol or

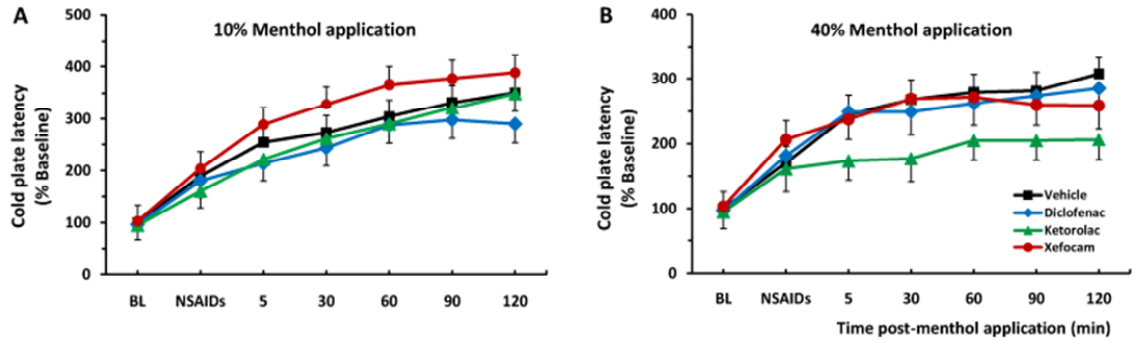

Fig. 1. Intraplantar NSAIDs pretreatments result in significant increase of the cold plate latency. Subsequent topical application of 10 % menthol does not change the latency. Increased values of the cold plate latency should be due to developing of NSAIDs action (A). 40 % menthol some attenuate a development of NSAIDs action, especially for ketorolac and xefocam (B). Graph plots change in cold plate latency (% of intact animal baseline) *vs.* time after NSAIDs and topical menthol application.

vehicle and were not repeatedly used. Six rats were used for each group.

Cold plate test. To test sensitivity to cold temperatures, rats received topical application of menthol or vehicle bilaterally to the ventral hindpaws, and 2 min later were placed onto the thermoelectric surface that was set at –5°C (AHP-1200DCP, Teca Thermoelectric, Chicago, IL, USA). The latency for nocifensive behavior (lifting and licking one hind paw, or jumping) was measured, at which point the rat was immediately removed and returned to its home cage. A cut of time 150 s was imposed to prevent tissue damage. The cold plate surface was cleaned, and ice scraped off in between tests. All animals were tested at 5, 30, 60, 90 and 120 min post-applications of menthol.

Mechanical paw withdrawal threshold (von Frey) test. Rats were habituated over 3 successive days to standing on a wire mesh screen surface. Baseline mechanical withdrawal thresholds were assessed using an electronic von Frey filament (1601C, IITC, Woodland Hills, CA, USA) pressed against the plantar surface of one hindpaw. This device registered the force (g) at the moment that the hind paw was withdrawn away from the filament. Following application of menthol or vehicle, mechanical paw withdrawal thresholds were measured at 5, 15, 30, 45, 60 and 120 min.

Data analysis. The time spent on the cold plate and the mechanical pressure withdrawal threshold were normalized to baseline averages and subjected to repeated-measures analysis of variance (ANOVA) with *post-hoc* Tukey-Kramer multiple comparison test using software InStat 3.05 (GraphPad Software Inc., USA). A 95 % confidence interval was used for all statistical comparisons, and the error reported is the standard error of the mean (s.e.m.).

Results

Cold plate test. Bilateral intraplantar injections of NSAIDs induced a significant increase of the cold pain latency compared to the intact control group for diclofenac ($t = 8.682$, $P < 0.001$, $n = 30$), ketorolac ($t = 6.523$, $P < 0.001$, $n = 30$), and xefocam ($t = 11.218$, $P < 0.001$, $n = 30$), respectively in 10% menthol groups (Fig. 1A). Similar results we found in 40% menthol groups where differences between experimental and intact control groups were for diclofenac ($t = 7.725$, $P < 0.001$, $n = 60$), ketorolac ($t = 5.821$, $P < 0.001$, $n = 60$), and xefocam ($t = 10.106$, $P < 0.001$, $n = 60$), respectively (Fig. 1B). These effects cannot be attributed to the antinociceptive action of NSAIDs as the control vehicles resulted in the same effect for the first ($t = 9.854$, $P < 0.001$, $n = 30$), and the second session ($t = 6.974$, $P < 0.001$, $n = 30$), respectively. In either case, it should be a certain protective effect of these solutions. On the other hand, it is well known that NSAIDs act principally on opioid midbrain structures [18].

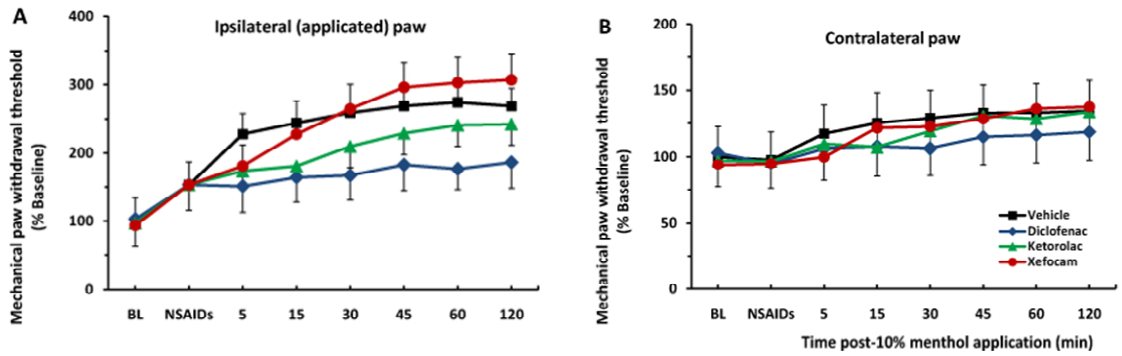

Fig. 2. Intraplantar NSAIDs pretreatments result in increase mechanical paw withdrawal threshold (A). However, subsequent application of 10% menthol does not change the process as the control group shows the same trend except diclofenac treated group of rats. (B) As for contralateral paw. Graph plots change in mechanical pressure withdrawal threshold (% of intact animal baseline) *vs.* time after NSAIDs injections and 10% topical menthol application.

Subsequent applications of menthol did not change the time-course of the process as it did not differ from the control at both concentrations of menthol. The cold pain latency in menthol treated groups did not differ from vehicle groups except for ketorolac in the high concentration of menthol (Fig. 1B). By comparing two doses of menthol it is evident that 40% menthol somewhat attenuates the effect of NSAIDs, especially for xefocam and ketorolac. Two-tailed test showed a statistically significant difference between 40 and 10% menthol for xefocam ($t = 7.085$, $P < 0.0001$, $df = 58$), and ketorolac ($t = 7.479$, $P < 0.0001$, $df = 58$), respectively.

Von Frey paw withdrawal. After injection of NSAIDs, the hindpaw receiving topical 10 % menthol exhibited increase in mechanical pressure threshold for xefocam and slightly for ketorolac, but they were not significantly different from vehicle (Fig. 2A). Although xefocam and ketorolac groups showed an increase in the threshold, diclofenac group remained at the same level after the application of 10% menthol. For the contralateral, non-treated hindpaw none of NSAIDs groups were significantly different from vehicle group (Fig. 2B). The ipsilateral (treated) and contralateral (untreated) paw groups confirmed some protective effects of NSAIDs and of the vehicle solution as well.

Almost the same results were obtained for 40% menthol, with one difference that xefocam more greatly increased mechanical pressure withdrawal threshold than ketorolac and diclofenac (Fig. 3). Comparison of 10% and 40% menthol groups showed significant differences for xefocam ($t = 3.86$, $P = 0.0008$, $df = 22$); i.e. 40% menthol significantly increased mechanical paw withdrawal threshold compared to 10% of menthol.

Discussion

The therapeutic effects of NSAIDs are based on their inhibitory actions on cyclooxygenase enzymes and subsequent interference with metabolites of the arachidonic acid pathway [18-20]. Previous reports have also described NSAIDs as blockers of TRP channels and the latter are considered as targets for analgesic drug discovery [21-25]. In our study we did not find significant influences of menthol on NSAIDs effects by behavioral assays probably through TRPM8 channel. Here menthol and NSAIDs act in the same direction without changing the overall picture of the action of NSAIDs, which confirms by the control group of rats with vehicle. The data of the control group with vehicle did not significantly differ from the experimental groups except for the group treated with 40% menthol after being treated with ketorolac in the cold plate test (Fig. 1B), and the group treated with 10% menthol after being treated with diclofenac (Fig. 2A).

The mechanism of cold transduction has been subtle, and despite the discovery of the thermo-TRP channels it remains a complex issue [1,2,5]. We have

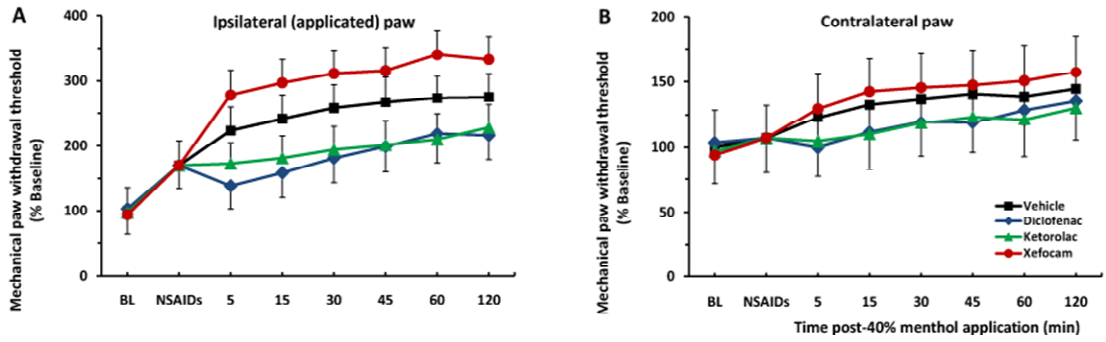

Fig. 3. Intraplantar NSAIDs pretreatments result in increase mechanical paw withdrawal threshold (A). However, subsequent application of 40% menthol does not change the process as the control group shows the same trend. (B) As for contralateral paw. Graph plots change in mechanical pressure withdrawal threshold (% of intact animal baseline) *vs.* time after NSAIDs injections and 40% topical menthol application.

recently found that the behavioral effects of different concentrations of topical menthol are consistent with effects on TRPM8 channel [6,9]. An important role for TRPM8 becomes apparent when this channel is missing. TRPM8 null mice exhibit a deficit in cold avoidance and lower incidence of cold-sensitive afferent fibers [1,11,12]. However, the authors cannot rule out the possibility that menthol may interact with other channels expressed in sensory neurons [26-28]. Recent observations suggest that cold sensation is likely to involve other channels, including potassium channels [29-31], in transducing and modulating the transmission of cold temperature information [2,5,32].

Based on our results, we cannot confirm the interaction of NSAIDs and menthol on TRPM8 channel, although our preliminary data have showed that NSAIDs attenuate thermal and mechanical hyperalgesia (increased pain sensitivity) following TRPA1 activation by its agonists AITC and CA, and TRPV1 activation by capsaicin [33]. Moreover, intraplantar AITC and capsaicin have produced a dose dependent cold hyperalgesia at –5°C, 0°C and +5°C temperatures [34].

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ადამიანისა და ცხოველთა ფიზიოლოგია

მენთოლი არ უნდა ზემოქმედებდეს არასტეროიდული ანთების საწინააღმდეგო პრეპარატების ეფექტებზე, ვირთაგვებზე ჩატარებულ ქცევით ტესტებში

ი. ნოზაძე*, ნ. წიკლაური*, გ. ღურწკაია*, ე. აბზიანიძე*, მ. ცაგარელი*

** ივ. ბერიტაშვილის ექსპერიმენტული ბიომედიცინის ცენტრი, ტკივილისა და ანალგეზიის ლაბორატორია, თბილისი*

(წარმოდგენილია აკადემიის წევრის ნ. მითაგვარიას მიერ)

უკანასკნელი წლების განმავლობაში მენთოლი ფართოდ გამოიყენება საკვებ პროდუქტებსა და პირის ღრუს ჰიგიენურ საშუალებებში მისი გამაჯანსაღებელ-გამაგრილებელი შეგრძნების გამო. დღეისათვის კარგად არის ცნობილი, რომ მენთოლი ზრდის სიცივის შეგრძნებას სიცივისადმი მგრძნობიარე თერმულ ტრანზიტორულ რეცეპტორულ პოტენციალთა (ტრპ) არხებზე ზემოქმედების შედეგად, მაგრამ მისი ეფექტი ტკივილზე ნაკლებად არის შესწავლილი. ჩვენ ახლახანს გამოვიკვლიეთ, რომ მენთოლი დოზა-დამოკიდებულად ზრდის მტკივნეული სითბური გაღიზიანებით გამოწვეულ თათის მოცილების რეფლექსის ფარულ პერიოდს ვირთაგვებში. ეს ფაქტი მიუთითებს მენთოლის ტკივილსაწინააღმდეგო, ანტინოციცეპტურ მოქმედებაზე. ჩვენ ასევე ვნახეთ, რომ მენთოლი ავლენს ბიფაზურ მოქმედებას სითბური განრიდების რეაქციებში. ამჟამად ჩვენ შევისწავლეთ არასტეროიდული, ანთების საწინააღმდეგო პრეპარატების (ასასპ) გავლენა ტრპ არხების აგონისტებზე. წარმოდგენილ შრომაში ვიკვლევდით ტრპმ8 არხის აგონისტ მენთოლის მოქმედებაზე ფართოდ გამოყენებად ასასპ-ის, დიკლოფენაკის, კეტოროლაკისა და ქსეფოკამის გავლენას. მიღებული მონაცემების თანახმად ასასპ-ების წინასწარი ინექციები ვირთაგვების უკანა თათებში არ იწვევენ სტატისტიკურად სარწმუნო ცვლილებებს მენთოლის აპლიკაციისას, საკონტროლო ჯგუფის ცხოველებთან შედარებით. ჩვენ ვფიქრობთ, რომ ამ შემთხვევაში ასასპ-ები არ უნდა მოქმედებდნენ ტრპმ8 არხზე.

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