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Menthol Induces Surgical Anesthesia and Rapid Movement in Fishes

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Abstract: To determine whether fishes respond to menthol, Japanese medaka *Oryzias latipes,* goldfish *Carassius auratus,* and zebrafish *Danio rerio* were exposed to various types of menthol receptors agonists and the behavioral responses to these drugs were observed. Waterbone application of *dl*-menthol (0.5 mM) induced surgical anesthesia in 100% of medaka, 90% of goldfish, and 100% of zebrafish. The percentage of response increased dose-dependently from 0.2 mM to 0.5 mM. There were no differences in either percentage or latency of the response in surgical anesthesia among *dl*-, *d*-, and *l*- types of menthol. A high (3.0 mM) concentration of any of the three types of menthol induced rapid movement followed by the anesthetic response. Rapid movement was observed with allyl isothiocyanate, a cold nociceptor agonist, but not with icilin, a cold receptor agonist, in medaka and goldfish. Both allyl isothiocyanate and icilin failed to induce surgical anesthesia. To determine the involvement of γ-aminobutyric acid (GABA) system in menthol-induced surgical anesthesia, the effect of the receptors antagonist for the $GABA_A$ was tested. Pretreatment with a specific $GABA_A$ receptor antagonist prolonged the latency of the anesthetic response to menthol, but not to cold-water stimulation, in medaka and goldfish. These results demonstrate that menthol can play a role in the induction of surgical anesthesia in fishes, related at least in part to the activation of $GABA_A$ receptors, and of rapid movement possibly via cold nociceptors.

Keywords: $GABA_A$ receptors, Transient receptor potential melastatin-8 (TRPM8), Transient receptor potential cation channel, subfamily A, member 1 (TPRA1).

INTRODUCTION

It is generally accepted that animal's respond to stress, such as capture, handling and crowding, influences their physiological functions. Anesthetic agents are important for the reduction of the physiological effects caused by these stresses. Administration of anesthesia in fishes has become a routine practice in aquatic animal medicine and immobilization of fish with fully innocuous substances for their effortless transportation. Tricane methanesulfonate (MS222), an anesthetic agent widely used in fish, is reported to cause occupational hazard (retinopathy) in the users and has a 21-day withdrawal period before the product can be consumed [1]. Therefore, it needs to find new drug to be used for reduction of stress in fish with safety.

Menthol is a widely used product in food, cigarettes, and in the pharmaceutical industry. The U.S. Food and Drug Administration has classified menthol as a topical analgesic material. Systemic exposure to menthol is reported to induce mild anesthetic effect in flat oyster *Ostrea edulis* [2] and surgical anesthetic effect in sea cucumber *Aspostichopus japonicas* [3], prawns *Macrobrachium rosenbergii* [4]. It is well known that menthol induces cold and pain sensations in subjects [5]. Application of a given dose of menthol or icilin is associated with cooling perception or pain relief, whereas treatment with higher doses of menthol causes burning, irritation, and pain [5-8]. In mammals, cold perception is generally believed to be mediated by a small subpopulation of unmyelinated C and thinly myelinated Aδ primary afferent fibers that discharge in the innocuous temperature range of 15°C – 28°C. The cold- and menthol-sensitive receptor 1 (CMR1) or transient receptor potential melastatin-8 (TRPM8) has been recognized as a member of transient receptor potential (TRP) family of excitatory ion channels and functions as a transducer of cold stimuli in the somatosensory systems [9, 10]. TRPM8 receptors are widely expressed and are considered to function specifically as cold receptors in tyrosine kinase receptor A ($TrkA^+$) affected small-diameter primary sensory neurons [9, 11-14]. At relatively high concentrations, topical application of menthol induces cold pain and hyperalgesia, possibly via activation of both cold-sensitive C-type nociceptors and Aδ-fibers [5,15]. In mammals, transient receptor potential cation channel subfamily A member 1 (TRPA1) is activated by noxious cold [9, 11, 16, 17]. Deletion of the TRPA1 gene suggests the functional importance of the TRPA1 protein in perception of noxious cold [18] and its involvement in chemical nociceptive signaling [18, 19].

In addition, menthol is known to be a potent positive modulator of the γ-aminobutyric acid type A ($GABA_A$) receptor [20] and acts as a potent positive allosteric modulator of $GABA_A$ receptors via sites similar to propofol, an intravenous anesthetic agent [21]. These finding suggest a possibility that menthol has an anesthetic effect on fish as well as sea cucumber and prawns [3, 4]. However, it has not been elucidated whether menthol produces systemic surgical anesthesia in fish.

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In the present study, the effects of menthol on medaka, goldfish, and zebrafish were examined with the aim to determine the surgical anesthetic activity of menthol on fishes and to determine effective and safe concentrations for use in surgical anesthesia. With the objective of identifying the receptor type by which menthol induces a response, the effects of icilin (TRPM8 receptor agonists), allyl isothiocyanate (TRPA1 receptor agonists), and GABA, along with the effect of a $GABA_A$-receptor antagonist on the menthol-induced anesthesia, were examined in medaka and goldfish.

MATERIALS AND METHODS

Animals

Japanese medaka fish *Oryzias latipes*, goldfish *Carassius auratus*, *and* zebrafish *Danio rerio* were used in this study and were obtained from an aquatic shop in Kagoshima city, Kagoshima, Japan. Fishes were kept under laboratory conditions and acclimated to the laboratory environment for several weeks. For acclimation, 5–8 fishes were housed in a tank (20 × 25 × 40 cm height × width × length) in which water was introduced at the aquarium bottom, flowed upward, and was recirculated by a pump after charcoal filtration. The room temperature of the laboratory was maintained at 25 ± 1°C. In a 12:12 light/dark cycle (light onset, 07:00 local time), light was provided by a fluorescent lamp that produced a mean light intensity of 500 lx at the water surface. A diet (Hikari Chappy, KYORIN FOOD IND. LTD, Japan) was supplied several times per day in the tank. For experiments, a single fish (standard length 1.6 ± 0.2 cm for medaka, 3.2 ± 0.4 cm for goldfish, and 1.7 ± 0.2 cm for zebrafish, mean ± SD) was placed in an experimental tank (8 × 8 × 8 cm) with 300 ml dechlorinated water. Experiments were performed from 10:00 to 16:00 during the daytime.

Waterborne Application

Three types of menthol (0.1–3.0 mM) (*dl*-menthol and *l*-menthol from Nacalai Tesque Inc., Japan, *d*-menthol from Tokyo Chemical Industry Co. Ltd., Japan), icilin (25–75 μM, Sigma-Aldrich Co., USA), and allyl isothiocyanate (0.5–1.0 μM, Sigma-Aldrich Co. USA) were used as chemical stimuli (Fig. **1**). These were dissolved in molybdoenzyme dimethylsulfoxide (DMSO; 50 μl) and 300 ml deionized water was added. This concentration of DMSO never produced an anesthetic response in medaka (n=6), goldfish (n=7) and zebrafish (n=6). Administration routes for these drugs are waterborne, with the drug added to the water. To investigate whether GABA is involved in the mechanisms of anesthetic responses, GABA (Sigma-Aldrich Co., USA) was applied to medaka fish. In addition to, medaka and goldfish were pretreated with a specific $GABA_A$-receptor antagonist, SR-95531 (Sigma-Aldrich Co., USA) for 30 or 120 min with or without prior menthol administration.

Local Application

To identify the action site of menthol, 10 mM of *dl*-menthol was locally applied to one side of a gill (50 μl), oral cavity (50 μl), and one side of the body surface (100 μl) of medaka fish and goldfish (100 μl, 100 μl and 300 μl, respectively) by injection with a 1-ml syringe, and the peritoneal cavity (10 μl for medaka fish and 30 μl for goldfish) was injected with a 100-μl Hamilton microsyringe. The fishes were placed on a stage and covered with paper on the region outside the injection site to avoid spreading of the solution. The solution was gently applied within 10 s to avoid stress and an anesthetic effect by hypoxia and then the fishes were placed back gently into the experimental tank.

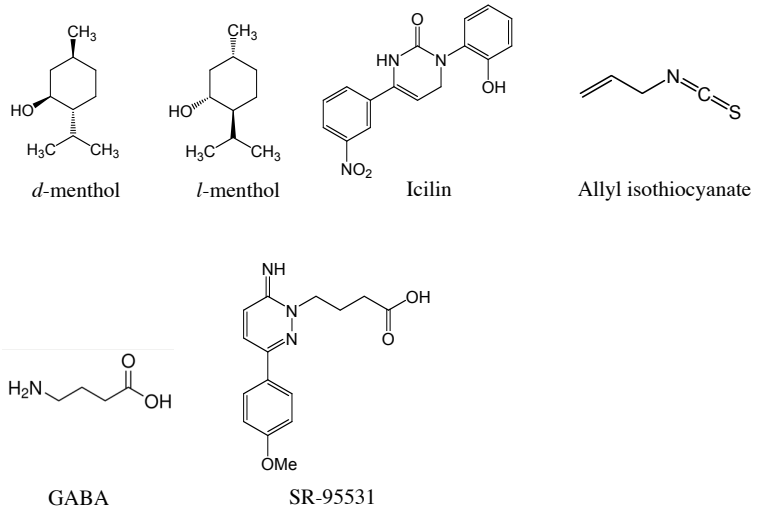

Fig. (1). Chemical structure of *d*-menthol, *l*-menthol, icilin, ally isothiocyanate, GABA and SR-95531 used in the study. Icilin is transient receptor potential melastatin-8 (TRPM8) receptor agonist and produces cold sensation in mammals. Allyl isothiocyanate is transient receptor potential cation channel subfamily A member 1 (TRPA1) receptor agonist and produces cold nociception. SR-95531 is a specific γ-aminobutyric acid type A ($GABA_A$)-receptor antagonist.

Estimation of Surgical Anesthesia

During exposure to a test solution for 6 min in waterborne application, the fishes were determined to exhibit surgical anesthesia as they lost their sense of equilibrium and were unmoved when touched and lifted with a plastic spoon. When the fishes exhibited surgical anesthesia, it was placed in a tank of fresh water. The latency of the response for surgical anesthesia was measured. When it exhibited voluntary swimming activity, the time was recorded as recovery time.

Data Analysis and Animal Ethics

All data are expressed as mean ± standard deviation (SD) and the mean differences between groups with values $P < 0.05$ were considered statistically significant by Student's *t*-test. All experiments in this study were performed in accordance with the Ethics Committee for Experimental Animals of the National Institute for Environmental Studies, Japan.

RESULTS

Surgical Anesthesia Induced by *d*, *l*-, and *dl*-menthol

The tested fishes displayed anesthesia in a four-stage process when exposed to menthol in an aquarium. The anesthetic effects began with sedation (motion reduction) and was followed by narcosis (loss of balance or motion but reaction to touch stimulus), and subsequently, developed into surgical anesthesia (complete loss of motion and no reaction to touch stimulus). Higher concentrations induced an anesthetic response followed by rapid movement. In the present experiment, latency to surgical anesthesia and recovery time were observed by chemical stimulation. A concentration of 0.1 mM of *d*-, *l*- and *dl*-menthol did not produce an anesthethic effect to medaka, goldfish and zebrafish. A concentration of 0.2 mM of *d*-menthol induced surgical anesthesia in only 1 of the 12 fish tested (8% in medaka, 8% in goldfish, and 8% in zebrafish) as shown in (Fig. **2A**, **B**, and **C**). Latencies of response following 0.2 mM *d*-menthol administration were 225, 330, and 278 s, respectively, as shown in (Fig. **2D**, **E**, and **F**), and recovery times were 350, 453, and 451 s, respectively, as shown in (Fig. **2G**, **H**, and **I**). Both *l*-menthol and *dl*-menthol at 0.2 mM failed to induce a response in medaka and goldfish (Fig. **2A** and **B**). The 0.2 mM dose of the *l*-type induced the response in one fish (8%) and of the *dl*-type in two (17%) zebrafish (Fig. **2C**). The average latencies were 175 s and 206 ± 82 s, respectively, and the average recovery times were 640 s and 228 ± 82 s, respectively. There was no difference in percentages of responsive fish among these fishes or among the three types of menthol. With 0.3 mM menthol, percentages of goldfish exhibiting anesthetic response were lower than those in medaka and zebrafish, although there was no difference in their latencies. Higher concentrations of menthol, >0.4 mM, produced a response in most (80–100%) of the fishes in all three species as shown in (Fig. **2A**, **B**, and **C** and Table **1**). The latency of the response was shorter with increasing dose, although no dose dependency of recovery time was observed. EC50 evaluated by logarithmic dose/response curve was 0.27 mM for *d*-type, 0.30 mM for *l*-type, 0.30 mM for *dl*-type menthol in medaka fish, 0.32, 0.25 and 0.33 mM in goldfish and 0.28, 0.28, 0.32 mM in zebrafish. There was no difference among these fishes or among the three types of menthol.

Rapid Movement Induced by *dl*-, *d*- and *l*-menthol

At concentrations <0.5 mM, *dl*-menthol failed to induce rapid movement in medaka (Fig. **3A**, left panel) or goldfish (Fig. **3B**, left panel); however, at the concentration of 3.0 mM, *dl*-menthol induced rapid movement preceding surgical anesthesia in 16 (73%) of 22 tested medakas and in 8 (40%) of 20 tested goldfish (Fig. **3A** and **B**, left panels and Table **1**). Application of *d*-menthol at concentrations of 3.0 mM induced rapid movements preceding surgical anesthesia in 5 (50%) medakas (Fig. **3A**, right panel and Table **1**) and 6 (60%) goldfish (Fig. **3B**, right panel and Table **1**). Application of 3.0 mM *l*-menthol induced rapid movement in 8 (80%) medakas and 5 (50%) goldfish (Fig. **3A** and **B**, right panels and Table **1**). The latency of rapid movement at high concentration was 4.4 ± 1.2 s and the latency of the surgical anesthesia after the rapid movement was 44 ± 20 s.

Effects of Icilin and Allyl Isothiocyanate on the Surgical Anesthesia

Icilin at concentrations of 30–90 μM and allyl isothiocyanate at 0.5–1.0 μM were applied to medaka and goldfish. Both icilin and allyl isothiocyanate at these concentrations failed to induce surgical anesthesia in all the 10 tested fishes, as shown in Table **1**. Application of 1.0 μM allyl isothiocyanate induced rapid movement without surgical anesthesia in 100 % of the 10 tested medakas (Fig. **3C** and Table **1**) and 50% of the 10 tested goldfish (Fig. **3D** and Table **1**).

Effects of GABA and $GABA_A$-Antagonist on Menthol-Induced Anesthesia

Application of 1, 10 and 100 mM of GABA by waterbone route failed to induce surgical anesthesia in all tested medakas (n = 10). Even when the application period was prolonged to 45 min from 6 min, the medakas exhibited no surgical anesthetic effect. A high concentration (1 M) of GABA induced surgical anesthesia within 45 min in 2 of the 12 tested medakas, with an average latency of the response of 2205 s. GABA at 2 M induced surgical anesthesia within 45 min in all 10 tested medakas with an average latency of 1271 s.

In $GABA_A$-receptor antagonist tests on 8 medakas, pretreated with SR-95531 at 70 μM for 30 min significantly ($P < 0.01$) prolonged the latency of the response of 2.0 mM menthol-induced anesthesia from 26 ± 14 s (n = 8) to 66 ± 14 s (n = 4), and pretreatment for 120 min significantly ($P < 0.01$) prolonged the latency of the response from 26 ± 14 s to 99 ± 40 s (n = 4), respectively. In goldfish, pretreatment with SR-95531 at 50 μM for 120 min prolonged the latency of the response of 0.5 mM menthol-induced anesthesia from 228 ± 10 s (n = 4) to 249 ± 17 s (n = 4) in (Fig. **4A**). Pretreatment of the antagonist with 70 μM significantly ($P < 0.05$) prolonged the latency of the response of 0.5 mM menthol-induced anesthesia to 314 ± 50 s (n = 4) in (Fig. **4A**). At a concentration of 100 μM antagonist, 4 of 6 fish showed surgical anesthetic response to menthol, but others did not showed surgical anesthesia within 6 min. Pretreatment of the antagonist with 100 μM significantly ($P < 0.01$) prolonged

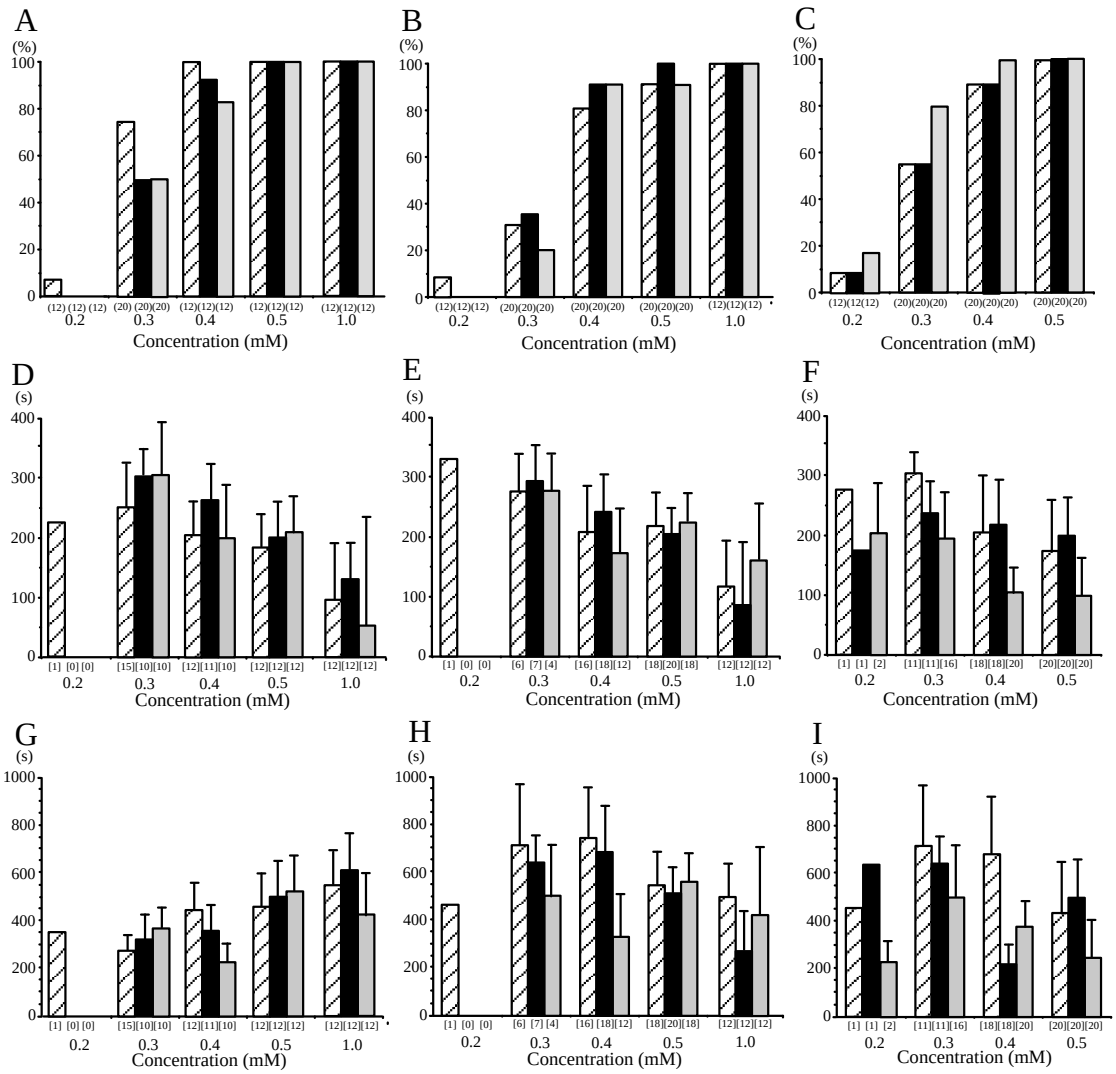

Fig. (2). Surgical anesthesia by *d*-, *l*- and *dl*-menthol on medaka, goldfish, and zebrafish. Each bars shows percentages of medaka (**A**), goldfish (**B**), and zebrafish (**C**) showing surgical anesthesia induced by *d*-menthol (shaded bars), *l*-menthol (black bars), and *dl*-menthol (gray bars) at concentrations of 0.2, 0.3, 0.4, 0.5, and 1.0 mM. D, E and F show average (with SD) latency of the anesthetic response in medaka (**D**), goldfish (**E**), and zebrafish (**F**), respectively. **G**, **H**, and I show recovery times. Numerals in brackets indicate the numbers of tested fish and numerals in parenthesis indicate the numbers of responsive fish.

Table 1. Summary of the effects of tested chemicals effect on fishes.

		% of Fish Showing	
Tested Chemicals	**Specimen**	**Surgical Anesthesia**	**Rapid Movement**
Menthol (0.4 mM *d*-, *l*-, *dl*-type)	Medaka	80%<	0%
	Goldfish	80%<	0%
	Zebrafish	80%<	0%
Menthol (3 mM *d*-, *l*-, *dl*-type)	Medaka	100%	50%<
	Goldfish	100%	40%<
Icilin (90 μM)	Medaka	N	N
	Goldfish	N	N
Allys isothiocyanate (1 μM)	Medaka	N	100%
	Goldfish	N	50%

N; no response in the present experiment.

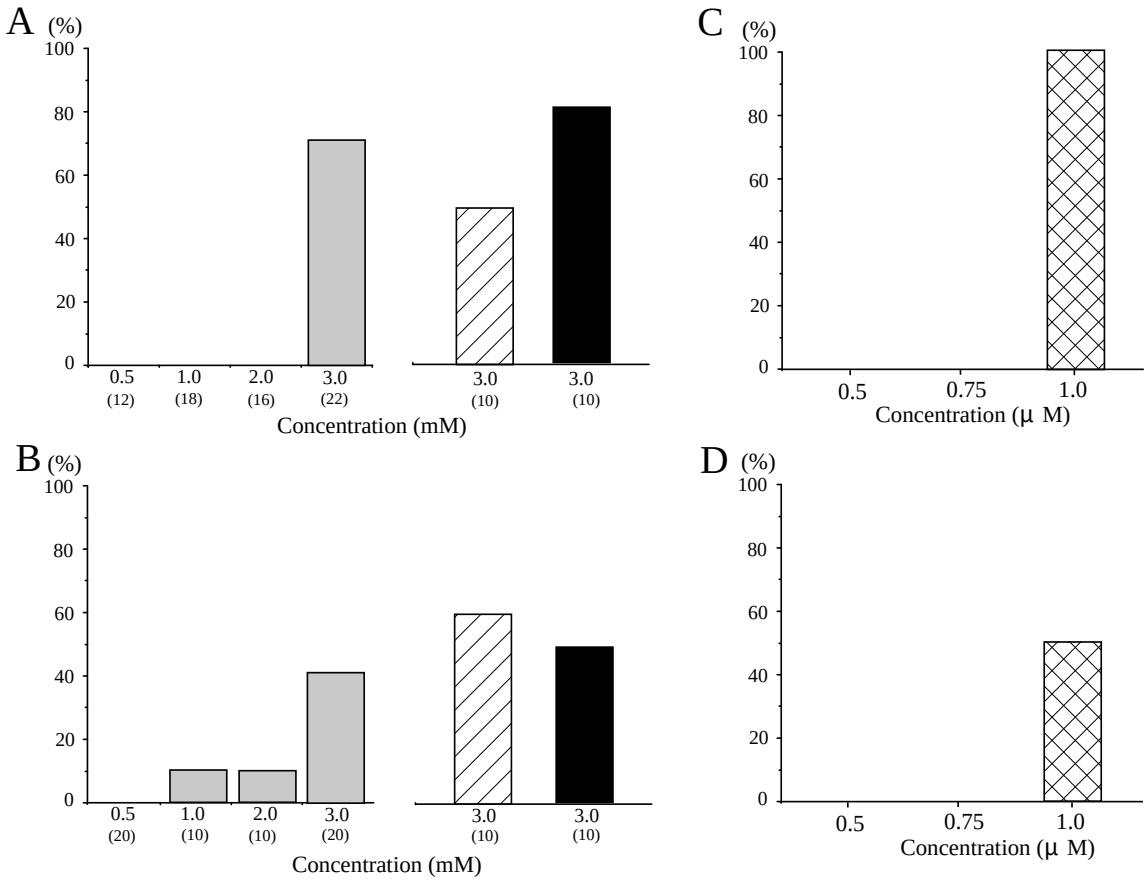

Fig. (3). Rapid movements induced by *dl*-, *d*-, *l*-menthol and allyl isothiocyanate in medaka and goldfish. Percentage of medaka showing rapid movement induced by *dl*-menthol (gray bars) at concentrations of 0.5, 1.0, 2.0, and 3.0 mM in A and of *d*-menthol (shaded bars) at 3.0 mM and *l*-menthol (black bars) at 3.0 mM in the right panel of **A**. **B** shows the percentage in goldfish. Numerals in brackets indicate the numbers of tested fish. **C** and **D** shows percentages of medaka (**C**) and goldfish (**D**) showing rapid movement by allyl isothiocyanate (cross hatched bars) at concentrations of 0.5, 0.75 and 1.0 μM. Numbers of fish tested were 10 in each experiment.

the latency to 351 ± 5 s (n = 4) in (Fig. **4A**). In contrast, pretreatment with SR-95531 at 70 μM for 120 min failed to change the latency of the response of cold (0°C)-induced anesthesia (Fig. **4B**).

Effects of Local Application of Menthol

Menthol (*dl*-type, 10 mM) was locally applied to a gill, oral cavity, body surface, and peritoneal cavity of individual medaka and goldfish. Applications of 50 μl of menthol to the oral cavity (n = 8) and 100 μl to the body surface (n = 12) failed to induce surgical anesthesia in all tested medakas. Application of 100 μl, of menthol to the oral cavity (n = 10) and 300 μl (n = 10) to the body surface also failed to induced surgical anesthesia in all tested goldfish.

Intraperitoneal injection of 10 μl of menthol induced surgical anesthesia in only 1 of the 10 tested medakas and none of 10 tested goldfish, while application of 50 μl to one side of the gill induced surgical anesthesia in 6 of the 10 tested medakas and in 3 of 10 tested goldfish.

DISCUSSION

Concentrations of menthol >0.4 mM produced a response in 80–100% of medaka, goldfish, and zebrafish in the present experiment. After the surgical anesthesia, most fishes recovered within 10 min when returned to fresh water environment. The effective concentration (EC50), with a range of 0.25 – 0.32 mM (0.004–0.005%) is lower than those for oyster (2%) and sea cucumber (0.05%); thus, menthol may be of utility in transportation of fresh water fishes. However, there is no information concerning the effect of menthol on fish health after long exposure during transportation. This problem remains to be addressed by further studies.

Most anesthetics used on fishes and shellfishes are reported to be absorbed into tissues via the gills [22]. In addition, intraperitoneal administration of menthol promoted ambulation in mice [23], suggesting that menthol could enter and influence a specific part of the brain. In the present study, applications of menthol to oral cavity and body surface completely failed to induce a surgical anesthetic response. Intraperitoneal injection of menthol induced surgical anesthesia in only 10% of medakas, whereas application of menthol to the gill induced surgical anesthetic response in over half of the medakas and also induced sedation in goldfish (data not shown). If the action site of menthol was the brain, the latency of the response would be slower because it would need more time to reach from the gill. The average latency of the menthol effect on the surgical anesthesia was slower (200–300 s). With high concentration of menthol (3.0 mM) inducing both rapid movement and surgical anesthesia,

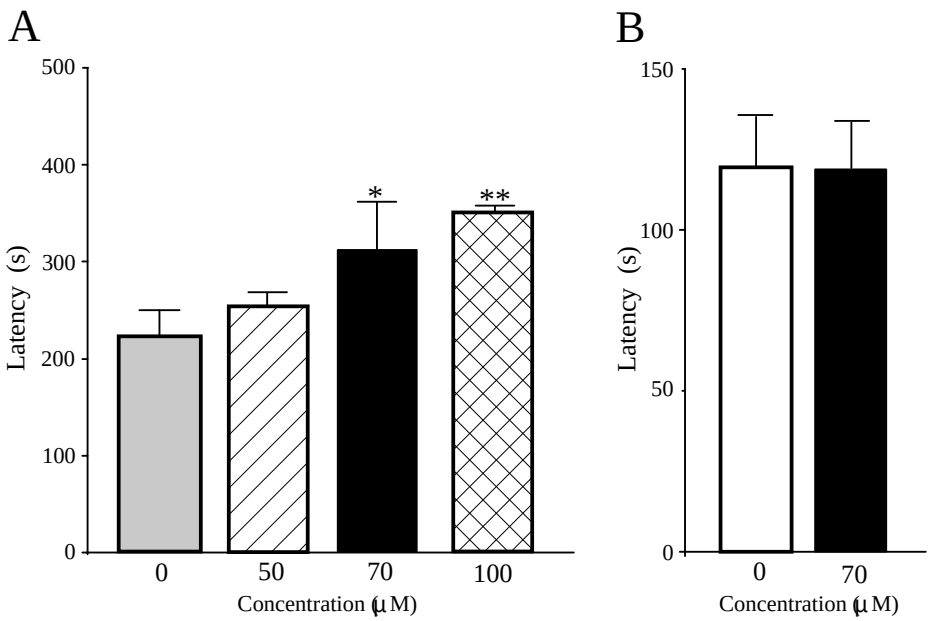

Fig. (4). Effects of a $GABA_A$ receptors antagonist on latency of the anesthetic response to *dl*-menthol and cold stimulation in fish. Average latency (with SD) of the surgical anesthesia by menthol (0.5 mM *dl*-type) in goldfish (A) after pretreatment in the tank with the $GABA_A$ receptors antagonist SR-95531 at 0 μM (in the absence of the antagonist, gray bar), 50 μM (hathched bar), 70 μM (closed bar), 100 μM (black bar) for 120 min. At a concentration of 100 μM antagonist, 4 of 6 fish showed surgical anesthetic response to menthol, but others did not showed surgical anesthesia within 6 min. C shows latencies of the response by cold (0°C) water stimulation in the absence (open bar) and presence (black bar) of the 70 μM antagonist. Asteriskis indicate significant difference from an average at 0 μM. **: $p<0.01$, *: $p<0.05$.

the average latency of surgical anesthesia (40 s) was slower than that of the rapid movement (4 s). These results suggest that menthol may be absorbed from the gill and transported to the brain to produce surgical anesthesia in fishes. The short latency of 4s in the rapid movement suggest that menthol might act through TRPA1 receptors on the gill surface, although it could not exclude a possibility that the rapid movement was caused by an excitation during anesthesia induction.

Menthol is a cyclic terpene alcohol with three asymmetric carbon atoms. Among the optical isomers, *l*-menthol occurs most widely in nature and is the one used in fragrances and flavor compounds such as in toothpaste, other oral hygiene products, and chewing gum [24]. The type of menthol (*d*- or *l*-) effective for anesthetic effect is still unknown, as contradictory results have been reported. The two types, *d*- and *l*-menthol are with respect to local anesthetic activity [25]. *D*-menthol was a moderately potent anesthetic [21], whereas only *l*-menthol induced analgesia in mice [25]. In the present experiment, both types of menthol induced surgical anesthesia with no difference in percentages of responses, latency, or recovery time at different concentrations. These results suggest a possibility that other mechanisms, neither cold receptors and nor cold nociceptors, were involved in the anesthetic response.

GABA receptors are potential candidates for the cellular mechanisms for the anesthesia induction by menthol. GABA is considered one of the major central nervous system (CNS) inhibitory neurotransmitters, which promotes the propagation of neuronal current via the activation of the two main classes of receptors ($GABA_AR$ and $GABA_BR$). $GABA_A$ receptors are well known as the predominant ionotropic receptors for fast inhibitory neurotransmission in the CNS. Electrophysiological and/or immunohistochemical studies demonstrate that $GABA_A$-receptors are distributed in the CNS and the retina [26-29], brainstem [30] and Mauthner cells [31] in goldfish, in the cerebellum of zebrafish [32], and in the optic tectum and the vagal, facial, and glossopharyngeal lobes of the carp [33]. Activation of $GABA_A$ receptors induced a decrease in "free-swimming" of the marine teleost that was reflected mostly in explorative type of behaviors during which the animals bumped against objects in the tank or their tank mates, and in an increase of resting states, although $GABA_A$-receptors agonist muscimol induced an increase in the feeding behavior. This was proved when they craved to swim towards the food sources [34]. On human $GABA_A$ and glycine receptors, menthol isomers act as important targets for modulation by sedative, anxiolytic, and general anesthetic agents [20, 35, 36]. Menthol reduces the excitation of rat hippocampal neurons not only by enhancing the currents induced at low concentrations of GABA but also by directly activating the $GABA_A$ receptor [37]. In addition, menthol is known to act as a potent positive allosteric modulator of $GABA_A$ receptors via sites similar to those of propofol [23], suggesting that $GABA_A$ receptors are involved in the anesthetic response observed in the present experiment. In this study pretreatment with SR-95531, a selective $GABA_A$ receptor antagonist, appreciably prolonged the latency of the anesthetic response to menthol in medaka and goldfish, suggesting that the anesthetic effect of menthol on fishes is mediated at least in part by $GABA_A$ receptors.

It has been demonstrated that application of cooling or cooling compounds such as menthol and icilin activates TRPM8 (CMR1) receptor in mammals [9, 11]. The zebrafish genome contains orthologs of several members of the TRP gene family, but none of these genes are closely related to

TRPM8 [38], suggesting that zebrafish lack a TRPM8 ortholog, and that other genes are used by zebrafish to sense cold temperatures. Thus, the failure of icilin to induce any responses in medaka and goldfish in the present experiment suggests that cold reception through TRPM8 receptors is not present in fishes. Further studies, however, are required to explain this issue.

At relatively high concentrations, topical application of menthol induces cold pain and hyperalgesia, possibly via activation of both cold-sensitive C-type nociceptors and Aδ-fibers [6, 15]. TRPA1 is usually involved in signaling induced by irritant and inflammatory substances [19, 39, 40]. This receptor is expressed in a subset of trigeminal and dorsal root neurons where TRPM8 seems to be absent [16, 41]. A functional role of TRPA1 in nociceptive neurons is known due to the demonstration that pungent chemical ligands, including allyl isothiocyanate such as those found in mustard oil, wasabi, and garlic, as well as other irritant chemicals such as acrolein, are capable of activating this channel [19, 39, 42]. The two zebrafish TRPA1 paralogs (*trpa 1a* and *trpa 1b*) are expressed in sensory neurons of zebrafish larvae. TRPA*1b* is necessary for behavioral responses (increase of locomotor activity) to chemical irritants [43]. In the present experiment, allyl isothiocyanate and high concentrations of menthol induced rapid movement not only in zebrafish but also in medaka and goldfish, suggesting that fishes have cold nociception via TRPA1 receptors. The present behavioral study exhibited the presence of nociceptors that have previously been identified using various neuroanatomical and electrophysiological techniques [44]. The receptor types of trigeminal neuron for rainbow trout were found to correspond with those identified in a teleost fish [45] who defined them as polymodal and mechanothermal nociceptors. However, there is an apparent absence of cold nociceptors in the head region of trout [46]. The present experiment supports the former report. Cold (0°C)-induced rapid movement was observed before anesthesia in medaka and goldfish (data not shown). Rapid movement response was faster than that of the anesthetic response in the present experiment. These results suggest that fishes possess a cold nociception via TRPA1 receptors possibly located on the gills. Further studies are required to explain this issue.

CONCLUSIONS

In the present experiment, three types of menthol (*d*-, *l*-, *dl*-types) at a concentration of 0.4 mM produced surgical anesthesia in medaka, goldfish, and zebrafish. A high (3.0 mM) concentration of any of the three types of menthol induced rapid movement followed by the anesthetic response in medaka and goldfish. Rapid movement was observed with allyl isothiocyanate, a cold nociceptor agonist, but not with icilin, a cold receptor agonist in medaka and goldfish. Both allyl isothiocyanate and icilin failed to induce surgical anesthesia. Pretreatment with a specific $GABA_A$ receptor antagonist prolonged the latency of the anesthetic response to menthol in medaka and goldfish. These results demonstrate that menthol can play a role in the induction of surgical anesthesia in fishes, at least in part by activation of $GABA_A$ receptors, and of rapid movement via cold nociceptors. However, the site of action and mechanisms involved in menthol-induced surgical anesthesia remain unknown and require further studies.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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