

# Effects of isoeugenol on in vitro neuromuscular blockade of rat phrenic nerve-diaphragm preparations

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**Objective**—To investigate in vitro effects of isoeugenol on neuromuscular transmission in tissues obtained from rats.

**Sample Population**—Tissues (phrenic nerve and diaphragm) obtained from 15 male Sprague-Dawley rats.

**Procedure**—Rats were euthanized, and tissues (phrenic nerves and diaphragm) were obtained. Phrenic nerve-diaphragm preparations were examined in vitro. The phrenic nerve was stimulated with weak electrical impulses. Muscle-twitch responses were recorded before and after the addition of drugs (pancuronium, neostigmine, isoeugenol, and benzocaine).

**Results**—Pancuronium and isoeugenol in low concentrations (10 to 206 $\mu$ M) caused a distinct decrease in twitch response, which could be reversed by the addition of neostigmine. The decrease in twitch response caused by benzocaine or high concentrations of isoeugenol could not be reversed by the addition of neostigmine.

**Conclusions and Clinical Relevance**—Isoeugenol caused a competitive blockade of neuromuscular transmission. Neostigmine restored this transmission by inhibiting acetylcholinesterase, which led to increased concentrations of acetylcholine. Because isoeugenol is used as an anesthetic in fish, further investigations are necessary to determine whether fish exposed to isoeugenol are sedated and unconscious or whether they are only paralyzed but have intact perception in afferent sensory nerves. (*Am J Vet Res* 2003;64:690–693)

Numerous anesthetic regimens have been developed within veterinary medicine during the past decade. However, when compared with anesthesia in other animals, anesthesia in fish has received little attention, and the pharmacodynamics and pharmacokinetics of drugs used for general anesthesia in fish are poorly known. Isoeugenol, eugenol, guaiacol, and vanillin are all derivatives of hydroxymethoxybenzene. When used in high concentrations, each of these substances can cause neurologic depression and coma in

rats.<sup>1</sup> Eugenol reportedly has local anesthetic effects as well as general anesthetic effects.<sup>2</sup> Eugenol derivatives have also been used IV to induce general anesthesia in humans,<sup>3</sup> and isoeugenol has general anesthetic properties in mice.<sup>4</sup> There are several reports<sup>5–7</sup> on the use of eugenol (clove oil) as an anesthetic in fish to aid in capture, handling, vaccination, and transport. However, neither clove oil nor any individual active ingredient of clove oil (eugenol [85 to 95%], isoeugenol, or methyleugenol [5 to 15%]) is approved for use as an anesthetic in fish in the United States.<sup>8</sup> Isoeugenol is currently used as an anesthetic in fish in New Zealand and Australia to aid in harvesting and in Norway to aid in vaccination.<sup>9</sup> However, the mechanism of action as well as several of its pharmacologic effects are unknown. Eugenol and isoeugenol have similar properties as free-radical scavengers and inhibitors of lipid peroxidation, but isoeugenol is a more powerful inhibitor of lipid peroxidation than eugenol.<sup>10,11</sup> Eugenol works synergistically with *d*-tubocurarine; thus, it is believed to block nicotine<sub>2</sub>-receptors at the motor end plate in muscles.<sup>12</sup> Blockade of nicotine<sub>2</sub>-receptors causes paralysis without analgesic or hypnotic effects.

The objective of the study reported here was to investigate the in vitro effects of isoeugenol on neuromuscular transmission in rats. We tested 2 hypotheses. The first hypothesis was that isoeugenol used in combination with neostigmine has the same effect on the phrenic nerve-diaphragm preparations as pancuronium or benzocaine used in combination with neostigmine. The second hypothesis was that the effects of isoeugenol on the phrenic nerve-diaphragm preparations were independent of concentration.

## Materials and Methods

**Animals**—Fifteen male Sprague-Dawley rats<sup>a</sup> that each weighed approximately 200 g were used for the study. Rats were housed at 22°C with a cycle of 12-hours light and 12-hours dark; rats had unlimited access to tap water and a standard pellet<sup>b</sup> diet. The study protocol was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

**Collection and preparation of tissue samples**—Rats were exposed to a mixture of carbon dioxide and air. After 45 to 60 seconds, the rats became unconscious. They were then euthanized by exposure to pure carbon dioxide for 2 to 3 minutes. Rats were then exsanguinated by cutting the carotid vessels.

After rats were euthanized and exsanguinated, the left hemidiaphragm and attached left phrenic nerve were removed from each rat and immersed in Tyrode solution (149mM Na<sup>+</sup>, 2.7mM K<sup>+</sup>, 1.8mM Ca<sup>2+</sup>, 0.2mM Mg<sup>2+</sup>, 144mM Cl<sup>-</sup>, 12mM HCO<sub>3</sub><sup>-</sup>, 0.4mM H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, and 5.6mM glucose). Silk sutures were used to secure each tissue preparation between a stationary glass holder and a high-sensitivity isometric

Received October 15, 2002.

Accepted January 23, 2003.

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Supported by the Norwegian Research Council (grant No. 141716/120).

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force transducer<sup>c</sup> in a 100-mL tissue chamber. Signals were recorded on a compact flat-back recorder.<sup>d</sup> The phrenic nerve was connected to an electrode for stimulation.<sup>e</sup> The phrenic nerve was stimulated by use of an electric impulse (0.5 to 0.9 mA), which was 50% greater than the threshold value, at a frequency of 1 Hz.

**Experimental procedure**—In each tissue chamber, the Tyrode solution was aerated with a mixture of 93.5% oxygen and 6.5% carbon dioxide; temperature was maintained at 37°C. After mounting in the tissue chamber, preparations were allowed to equilibrate in the oxygenated solution for approximately 30 minutes before experimental procedures were started. The phrenic nerve was then stimulated, and when the recorded values had stabilized, various substances were added to the tissue bath.

To examine the possible effects of isoeugenol at the nerve-muscle junction, pancuronium,<sup>f</sup> neostigmine,<sup>g</sup> isoeugenol,<sup>h</sup> or benzocaine<sup>i</sup> was added to the tissue bath. Drugs were added in a cumulative manner until sufficient effects were observed. For each phrenic nerve-diaphragm preparation, the effects of isoeugenol and benzocaine, each of which was used in combination with neostigmine, were recorded. Serial concentrations (0.1, 0.32, 1.0, 3.2, 10, 32, 100, 320, and 1,000mM) of isoeugenol and benzocaine were used. For the highest concentrations (ie, 320 and 1,000mM), ethanol was used as a solvent, whereas distilled water was used as a solvent for the lower concentrations. One hundred microliters of each solution was added to the tissue chamber, yielding concentrations of 0.1 to 1,000μM.

**Statistical analysis**—Results were reported as mean ± SD, and number of experiments was indicated. Data were evaluated by use of the Student paired *t* test. Values of *P* < 0.05 were considered significant.

## Results

**Effects of ethanol on twitch responses**—The addition of 100 μL of ethanol to the tissue chamber did not influence recorded twitch responses.

**Effects of pancuronium on twitch responses**—Pancuronium (6.6μM) caused a typical blockade and reversion pattern (Fig 1). When the full effect of pancuronium was observed, addition of neostigmine (9.6μM) also produced a typical blockade and reversion pattern. The same pattern was evident when neostigmine (initially) and then pancuronium were added to the tissue bath.

**Effects of isoeugenol on twitch responses**—Isoeugenol (concentrations of 10 to 1,000μM) caused a distinct decrease in twitch response. When neostigmine was added to the bath before isoeugenol, isoeugenol reversed the decrease in twitch response caused by neostigmine (Fig 1). Adding neostigmine (0.24 to 0.48μM) to the tissue bath after isoeugenol reversed the effects of isoeugenol when concentrations of isoeugenol did not exceed 174μM (Fig 2). For isoeugenol concentrations of 342 to 1,000μM, subsequent addition of neostigmine to the tissue bath did not reverse the effects of isoeugenol.

**Effects of benzocaine on twitch responses**—Benzocaine (10 to 1,000μM) caused a decrease in twitch response. Adding neostigmine (0.24 to 4.78μM) to the tissue bath after benzocaine had no or minimal efficacy in reversing the effects of benzocaine. Adding

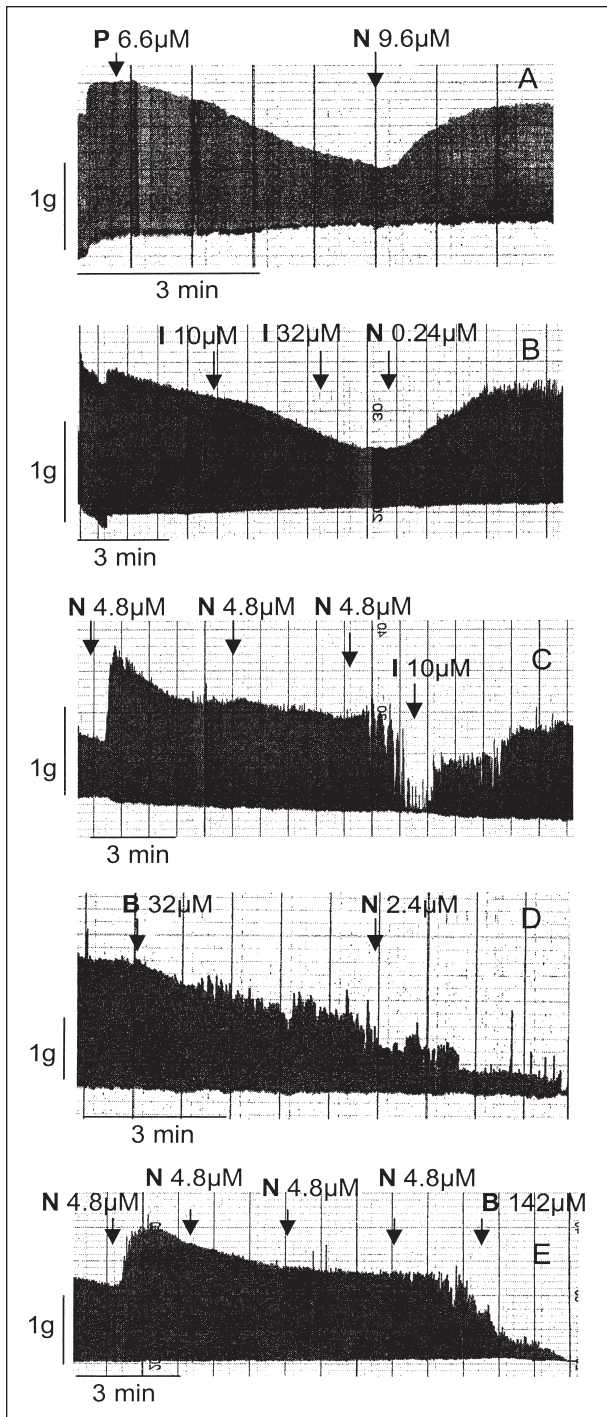


Figure 1—Effects of various drugs on the in vitro twitch response of the diaphragm of rats. The phrenic nerve was electrically stimulated, and contraction of the diaphragm was recorded isometrically. Numbers represent concentrations of each drug in the tissue chamber. A—Addition of pancuronium (P) to the tissue bath caused blockade of the twitch response, which was reversed by addition of neostigmine (N). B—Addition of isoeugenol (I) to the tissue bath caused blockade of the twitch response, which was reversed by addition of neostigmine. C—Addition of neostigmine to the tissue bath caused a depolarization blockade of muscle twitch, which was reversed by addition of isoeugenol. D—Addition of benzocaine (B) to the tissue bath caused blockade of the twitch response, which was not affected by addition of neostigmine. E—Addition of neostigmine to the tissue bath caused a depolarization blockade of muscle twitch, and addition of benzocaine amplified this effect of the twitch response.

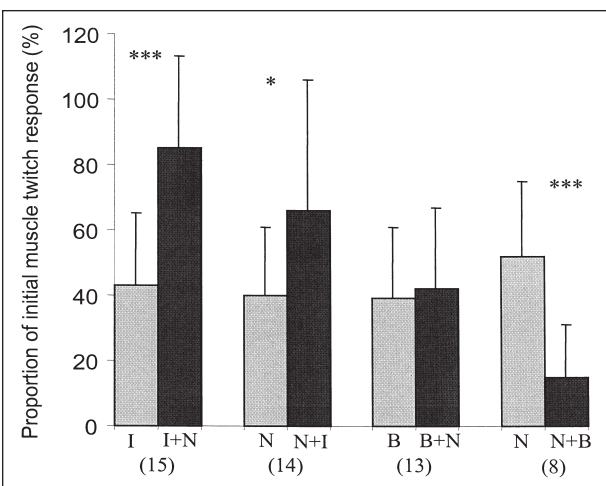


Figure 2—Effects of the addition of isoeugenol (I), benzocaine (B), and neostigmine (N), alone and in combination, to the tissue bath on twitch response of rat diaphragm preparations. Each bar represents mean  $\pm$  SD. Numbers in parentheses are the number of experiments. \*Within each treatment pair, values differ significantly ( $P < 0.05$ ). \*\*\*Within each treatment pair, values differ significantly ( $P < 0.001$ ).

neostigmine to the tissue bath before the addition of benzocaine further decreased the twitch response (Fig 1 and 2).

## Discussion

Similar to all other vertebrates, fish have acetylcholine receptors in the motor end plates of their muscles; these receptors are of a nicotinic nature.<sup>13,14</sup> Thus, the muscles of fish should react to substances affecting the motor end plate in a similar manner to that of muscles of other vertebrates. Therefore, results from the study reported here on the effects of various drugs on the twitch response in rat muscle should also be applicable in fish. The underlying rationale for the use of rats in this study was the suitability of the phrenic nerve-diaphragm preparation for in vitro studies.

To evaluate the possible effects of isoeugenol on the motor end plate, pancuronium and neostigmine were used as reference substances. Pancuronium causes a competitive nondepolarizing blockade of the acetylcholine receptor, and neostigmine restores this transmission by inhibiting acetylcholinesterase, which leads to an increased concentration of acetylcholine (Fig 1). Benzocaine was used because of its local anesthetic properties, and because it blocks the initiation and propagation of action potentials in the nerve membrane.<sup>15</sup> Also, similar to isoeugenol, benzocaine is used as a general anesthetic in fish.<sup>16</sup> Neostigmine should have minimal or no effects on the twitch response caused by benzocaine. However, when neostigmine was used to induce a depolarizing blockade of neuromuscular transmission, benzocaine amplified the effect on the twitch response. This could be explained by the fact that when benzocaine was added, membranes of the motor end plate were already partly depolarized as a result of the high amounts of acetylcholine in the synaptic cleft, and benzocaine blocked the remaining nerve fibers that still were functional.

Effects of isoeugenol and neostigmine on the

twitch response were similar to the effects induced by pancuronium and neostigmine (Fig 1). This strongly indicated that isoeugenol induces a competitive nondepolarizing blockade of the acetylcholine receptor, because neostigmine restores neuromuscular transmission by increasing the amount of acetylcholine. When neostigmine was used to induce a depolarizing blockade of the acetylcholine receptor, isoeugenol restored neuromuscular transmission as a result of competitive inhibition of the receptor, leading to repolarization of the motor end plate. Neostigmine reversed the effects of isoeugenol when the concentrations of isoeugenol ranged from 10 to 206  $\mu$ M. In another study,<sup>12</sup> investigators reported that eugenol at a concentration of 650  $\mu$ M worked synergistically with *d*-tubocurarine to competitively blockade nicotine<sub>2</sub>-receptors. Eugenol, when used alone at that same concentration, did not have an effect on muscle twitches. This indicates that isoeugenol is more potent than its isomer, eugenol. The LD<sub>50</sub> after oral administration to rats is lower for isoeugenol (1,560 mg/kg) than for eugenol (2,680 mg/kg); however, the LD<sub>50</sub> of isoeugenol is approximately the same as the LD<sub>50</sub> for vanillin (1,580 mg/kg).<sup>1</sup>

When high concentrations of isoeugenol (342 to 1,000  $\mu$ M) were added to the tissue chamber, subsequent addition of neostigmine could not reverse the effects. This pattern was similar to that seen for various concentrations of benzocaine (Fig 2). Eugenol has local anesthetic properties.<sup>2</sup> It is probable that isoeugenol also has local anesthetic properties, and these effects were evident when high concentrations of isoeugenol were added to the tissue preparations.

Isoeugenol and eugenol have general anesthetic properties.<sup>3,4</sup> They reportedly are appropriate for use as general anesthetics in fish and can induce effective immobilization at low concentrations.<sup>5-8</sup> In the investigation reported here, we determined that isoeugenol in low concentrations appears to have neuromuscular blocking properties in rats. Although it has not been explicitly documented that this compound causes neuromuscular blockade in fish, there is no reason to assume that the mechanism of neuromuscular transmission should be different in fish species.<sup>13,14</sup> However, an important aspect to consider is possible species differences in the dose-dependent response regarding a more general inhibition of nerve function (anesthetic or analgesic effect) and the specific blockade of nicotinic receptors (paralytic effect) of isoeugenol, as indicated by the results reported here. Further investigations are necessary to determine whether fish exposed to isoeugenol are sedated and unconscious or are only incapable of movement but retain intact afferent sensory pathways.

<sup>1</sup>BK:SD, Baintain & Kingman, Sollentuna, Sweden.

<sup>2</sup>R36, Lactamin AB, Vadstena, Sweden.

<sup>3</sup>High sensitivity isometric transducer, TRN001, Radnoti Glass Technology Inc, Monrovia, Calif.

<sup>4</sup>SE120, Asea Brown Boweri, Vienna, Austria.

<sup>5</sup>Stimulator and electrode were created in the Research Workshop, Uppsala Biomedical Center, Uppsala University, Uppsala, Sweden.

<sup>6</sup>Pavulon solution for injection (2 mg/mL), N. V. Organon, Oss, Netherlands.

<sup>a</sup>Neostigmine solution for injection (2.5 mg/mL), Pharmacia Sverige AB, Stockholm, Sweden.

<sup>b</sup>Aqui-S, New Zealand Ltd, Lower Hutt, New Zealand.

<sup>c</sup>Ethyl *p*-aminobenzoate (benzocaine), Sigma Chemical Co, St Louis, Mo.

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