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The Effect of Levamisole on Sheep Trachea Alone and With Trichlorfon Combination

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Abstract: The aim of this study is to determine the effect of levamisole and levamisole-trichlorfon combination on isolated sheep trachea. Contraction was achieved with levamisole concentrations (10^{-8}-10^{-3} M) on tracheal strips of adult sheep (>1 year of age). Pretreatment with trichlorfon (10^{-7} M)-levamisole (10^{-7} M) decreased the pD_2 and E_{max} of Ach when compared to levamisole pretreatment ($p \leq 0.01$). Pretreatment with levamisole (10^{-7} M), decreased the E_{max} ($p \leq 0.01$) and pD_2 ($p<0.05$) of bethanechol concentrations (10^{-8}-10^{-3} M) significantly. Pretreatment with atropine (10^{-6} M) decreased the E_{max} of levamisole (10^{-4} M) significantly ($p<0.05$). To conclude, levamisole acted mainly on the muscarinic receptors of the sheep trachea and this effect was partly inhibited by atropine. Adverse interaction was present between levamisole and trichlorfon.

Key words: Levamisole, trachea, trichlorfon

INTRODUCTION

Levamisole, a levo isomer of tetramisole, is used as an anthelminthic (Sanchez Bruni *et al*., 2006) and immune modulator (Krastev *et al*., 1999; Sajid *et al*., 2006) in humans and animals. There are theories about the mode of action of levamisole on the host and parasites. The most mentioned is nicotine like activity. Levamisole has cholinergic activity at the ganglionic and neuromuscular junctions (Martin, 1997; Martin *et al*., 2005; Reinemeyer and Courtney, 2001). Eyre (1970) suggested that levamisole had both muscarinic and nicotinic effects by the inhibition of acetylcholinesterase (AchE) and the pharmacodynamic actions of levamisole in the host suggested that the drug exerted both muscarinic and nicotinic effects (Barragry, 1994; Reinemeyer and Courtney, 2001). But Hsu (1981) suggested that levamisole did not inhibit AchE activity. On the other hand, Caposso *et al*. (1982) observed that some effects of levamisole were not related with the autonomic system, but Shah *et al*. (1986) indicated that sympathetic and parasympathetic effects were observed at the same time.

Trichlorfon is an organophosphorus (OP) compound which has both anthelminthic and insecticidal activity. This agent shows cholinergic activity on parasites, inhibits the action of the enzyme AchE resulting in accumulation of acetylcholine (Ach) and overstimulation of nicotinic and muscarinic receptors. Overdoses of this agent result in toxication in the host by the same mode of action (Barragry, 1994; De Silva *et al*., 2006).

Acetylcholinesterase inhibitors (e.g., OP, neostigmin) may potentiate the toxicity of levamisole (Sanchez Bruni *et al*., 2006). It is recommended not to use OP compounds within 14 day period if levamisole treatment is needed (Barragry, 1994; Bishop, 1996). However, Aldabagh and Mohammad (1999) showed that combination of these two compounds did not increase the toxic effect, even the preuse of levamisole protected host from OP toxication.

This study was aimed to determine the interaction between levamisole and trichlorfon on sheep trachea which acts as the sensitive target animal in *in vitro* pharmacodynamical experiments.

MATERIALS AND METHODS

Forty two tracheal strips were obtained from slaughtered sheep, from the licensed municipal slaughterhouses of Kazan and Cubuk provinces of Ankara in Turkey, under the supervision of a veterinary surgeon. The animals were 1-3 years old and 30-50 kg weight. Preliminary studies were performed on 20 tracheal

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strips from 10 sheep. The study was carried out from September to December in 2004. The sheep tracheas obtained from the slaughtered animals at the slaughterhouse were placed in the Krebs solution which contained 95% oxygen and 5% carbon dioxide gases and were transferred to the laboratory of Pharmacology and Toxicology Department, Ankara University, Faculty of Veterinary Medicine (Turkey) in cold chain where the studies were conducted.

Mecamylamine (Sigma) 10^{-2} M stock solutions, levamisole (Sigma), nicotine hydrogene tartrate (Sigma), atropine sulphate (Sigma), trichlorfon (Sigma), acetylcholine chloride (Sigma) and bethanechol (Sigma) 0.1 M stock solutions were used. Krebs-Henseleit solutions were used to dilute the stock solutions. Solutions were prepared daily.

Modified Krebs-Henseleit solution (mM) consisted of NaCl: 118; $NaHCO_3$: 25; Anhydrate glucose: 11.1; KH_2PO_4: 1.2; KCl: 4.8; $Mg_2SO_4.7H_2O$: 1.2; $CaCl_2.2H_2O$: 1.2 (pH of the solution was 7.4).

In vitro studies were performed by using TDA 97 polygraph system (MAY), FDT-10A isometric tension transducer (MAY) and WBC 3446V2 water bath and circulation system (MAY).

Smooth muscle preparations were harvested according to the method described by Thulesius and Mustafa (1994) and Jackawski *et al.* (1993). Ten millimeter in length muscle strips were dissected free from the underlying cartilage and placed under a load of 2 g in the isolated organ baths which contained 15 mL of Krebs solution (37°C). Tissues were treated with 95% oxygen and 5% carbon dioxide gases continuously during the experiments (Mustafa and Orowo, 1999; Thulesius and Mustafa, 1994).

Each preparation was allowed to equilibrate for at least 60 min prior to initiating the experimental procedure. During the stabilization period; bath solution was changed 4 times. After the equilibrium, contraction was achieved with Ach (10^{-3} M) to determine the response of the tissue. Bath fluid was changed in every 15 min.

In preliminary tests, levamisole alone was applied on ovine tracheal strips of different ages (<1 year of age was considered as young and >1 year of age as adult).

Studies on adult ovine isolated tracheal strips (Set of experiments): Each set of experiments were achieved on tracheal strips which were isolated from at least 4 different sheep. n represents the number of tracheal strips.

The effect of levamisole on ovine tracheal strips: Levamisole was applied cumulatively (10^{-8}-10^{-3} M) to the tracheal smooth muscle strips of ovine (n:7).

The effect of levamisole and trichlorfon combination on ovine tracheal strips: Ach (10^{-8}-10^{-3} M) concentrations were applied to obtain the corresponding concentration-response curve. After that the incubation medium was changed three times in 45 min, to restore the base line. Before each procedure, incubation medium was changed to restore the base line. These procedures include separate exposure to levamisole (10^{-7} M), trichlorfon (10^{-7} M), levamisole (10^{-7} M) and trichlorfon (10^{-7} M) combination for 20 min, before the addition of further cumulative concentrations of Ach (10^{-8}-10^{-3} M) (n:8).

The effect of levamisole on bethanecol response on ovine tracheal strips: The tracheal strips were exposed to cumulative concentrations of bethanecol (10^{-8}-10^{-3} M) obtaining the corresponding concentration-response curve. The incubation medium was changed three times in 45 min to restore the baseline. This was followed by exposure to levamisole (10^{-7} M) for 20 min, before the addition of further cumulative concentrations of bethanecol (10^{-8}-10^{-3} M) (n:8).

The effect of levamisole on nicotine response on the tracheal strips of ovine trachea: The tracheal strips were exposed to 10^{-3} M nicotine. The incubation medium was changed three times in 45 min, to restore the baseline. Then 10^{-3} M nicotine was applied to the tracheal strips which were pretreated with levamisole (10^{-7} M) for 20 min (n:7).

The effect of atropine on levamisole response on the tracheal strips of ovine trachea: The tracheal strips were exposed to 10^{-4} M levamisole. The incubation medium was changed three times in 45 min, to restore the baseline. Then 10^{-4} M levamisole was applied to the tracheal strips which were pretreated with atropine (10^{-6} M) for 20 min (n:6).

The effect of mecamylamine on levamisole response on the tracheal strips of ovine trachea: The tracheal strips were exposed to 10^{-4} M levamisole. The incubation medium was changed three times in 45 min, to restore the baseline. Then 10^{-4} M levamisole was applied to the tracheal strips which were pretreated with mecamylamine (10^{-5} M) for 20 min (n:6).

Contraction responses are expressed as percentage. The pD_2 and E_{max} responses of the drugs were compared. pD_2 represents the negative logarithm of the drug molarities that is needed to achieve the half of maximum effect. E_{max} is the expression of the maximum efficacy formed by the drug in percent responses. pD_2 responses were calculated using Pharm PCS-Version 4, Pharmacologic calculation packet software program.

Statistical analyses were done using SPSS 11 for Windows software. The results of the study were expressed as arithmetic means and standard errors. Wilcoxon tests were used to compare the matched two sample groups. If the groups to compare were more than two, Friedman variance analyses were used. To determine significance, Wilcoxon test was used.

RESULTS AND DISCUSSION

In preliminary tests, levamisole showed no response on young sheep tracheal strips (<1 year of age) (data not shown), but caused significant contraction on adult tracheal strips (Table 1). Pretreatment of the strips with levamisole only increased the E_{max} responses of Ach ($p<0.05$), trichlorfon decreased the pD_2 responses of Ach ($p<0.05$) and trichlorfon-levamisole combination decreased the pD_2 and E_{max} responses of Ach ($p\leq0.01$) when compared with the responses of control and levamisole treatment groups (Fig. 1, Table 2). Pretreatment of the strips with levamisole (10^{-7} M), decreased the E_{max} ($p\leq0.01$) and pD_2 ($p<0.05$) responses of bethanechol significantly (Fig. 2, Table 4). The effect of levamisole on nicotine response was statistically insignificant (Table 5) and atropine (10^{-6} M) decreased the E_{max} response of levamisole (10^{-4} M) ($p<0.05$) (Table 6). Mecamylamine (10^{-5} M), decreased the response to levamisole (10^{-4} M) insignificantly (Table 3).

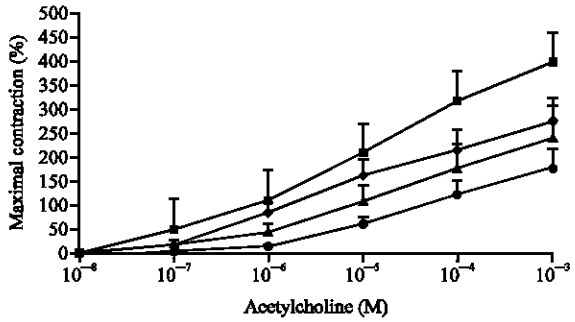

Fig. 1: Effect of levamisole, trichlorfon and levamisole trichlorfon combination on the concentration response curve of acetylcholine in sheep tracheal strips. (♦) represents control (Ach) concentration response curve. (■) represents the concentration response curve in the presence of levamisole. (▲) represents the concentration response curve in the presence of trichlorfon. (●) represents the concentration response curve in the presence of levamisole and trichlorfon combination. Verticle lines indicate the standard errors of the mean (n:8 for each point)

The preliminary tests which were aimed to determine the effect of levamisole on sheep trachea showed that cumulative administration of levamisole (10^{-8}-10^{-3} M) caused no response in the tracheas of young sheep (<1 year). Given the fact that, the responses were related to age, it is quite probable that the tracheal receptor distribution changes with age significantly. In the study of Panitch *et al.* (1989), the responses of the sheep tracheal smooth muscles to Ach had changed significantly by age and to achieve a half maximal response, adult sheep were given much less doses than young sheep. In another word, adult sheep tracheal muscle responded to Ach better than young ones.

Table 1: The effect of levamisole on ovine trachea

(n:7)	pD_2 (mean±SE)	E_{max} (mean±SE)
Levamisole (10^{-8}-10^{-3} M)	4.83±0.22	92.69±20.01

n: The number of tracheal strips

Table 2: The effect of levamisole, trichlorfon and trichlorfon-levamisole combination pretreatment on Ach concentration (10^{-8}-10^{-3} M) response

(n:8)	pD_2 (mean±SE)	E_{max} (mean±SE)
Ach (Control)	5.38 ± 0.03^{a}	275.78 ± 48.72^{a}
Levamisole (10^{-7} M)	5.39 ± 0.03^{ac}	395.13 ± 85.41^{b}
Trichlorfon (10^{-7} M)	5.11 ± 0.11^{bd}	242.48 ± 65.20^{ac}
Levamisole (10^{-7} M)+ Trichlorfon (10^{-7} M)	5.14 ± 0.06^{d}	178.87 ± 40.48^{c}

n: The number of tracheal strips, ^{abcd}: The degree of difference between the pD_2 and E_{max} groups that carry different letters was significant, $^{a-b}$: The degree of significance between the E_{max} groups ($p<0.05$), $^{a-c,\ b-ac,\ b-c}$: The degree of significance between the E_{max} groups ($p\leq0.01$), $^{a-bd}$: The degree of significance between the pD_2 groups ($p<0.05$), $^{a-bd,\ a-d,\ ac-bd,\ ac-d}$: The degree of significance between the pD_2 groups ($p\leq0.01$)

Table 3: The effect of mecamylamine pretreatment on levamisole response

(n:6)	E_{max} (mean±SE)
Levamisole (Control)	69.64±10.28
Mecamylamine (10^{-5} M)	50.98±17.72

n: The number of tracheal strips

Table 4: The effect of levamisole pretreatment on bethanecol concentration (10^{-8}-10^{-3} M) response

(n:8)	pD_2 (mean±SE)	E_{max} (mean±SE)
Bethanecol (Control)	5.15±0.05	538.75±53.28
Levamisole (10^{-7} M)	4.93±0.06*	177.28±35.72**

n: The number of tracheal strips, *: The difference between the pD_2 of the control and levamisole groups are significant ($p<0.05$), **: The difference between the E_{max} of the control and levamisole groups are significant ($p\leq0.01$)

Table 5: The effect of levamisole pretreatment on nicotine (10^{-3} M) response

(n:7)	E_{max} (mean±SE)
Nicotine (Control)	13.41±3.60
Levamisole (10^{-7} M)	16.40±2.58

n: The number of tracheal strips

Table 6: The effect of atropine pretreatment on levamisole (10^{-4} M) response

(n:6)	E_{max} (mean±SE)
Levamisole (Control)	77.97±11.81
Atropine (10^{-6} M)	42.34±10.48*

n: The number of tracheal strips, *: The difference between the E_{max} of the control and levamisole groups are significant ($p<0.05$)

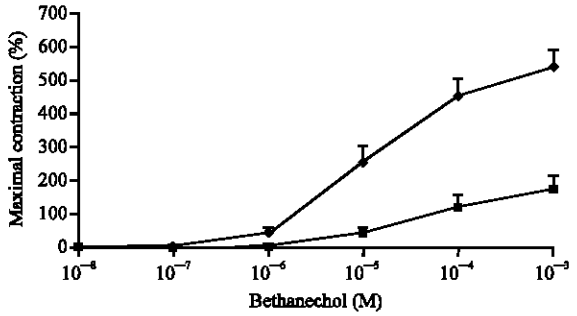

Fig. 2: Effect of levamisole on the concentration response curve of bethanechol in sheep tracheal strips. (♦) represents control (Bethanecol) concentration response curve. (■) represents the concentration response curve in the presence of levamisole. Verticle lines indicate the standard errors of the mean (n:8 for each point)

Although a different drug was in consideration in this study, these studies provide supportive evidence and are compatible with the results of our study that responses in sheep change with age.

Levamisole caused a significant contraction in the tracheal strips of sheep (1-3 years of age) (Table 1). As the tissue interaction time was increased, the effect of levamisole became more dominant. This effect of levamisole on smooth muscles was not observed in other studies. For example in the study of Gulati *et al.* (1985), on guinea pig vas deference, levamisole alone neither caused a relaxation nor contraction, as in the study by Vanhoutte *et al.* (1977) on isolated dog saphenous vein and by Yildirim (2005) on rabbit trachea. Contraction of sheep tracheal smooth muscle with levamisole was an outstanding finding in our study which may be related to species and tissue specific receptor distribution.

Pretreatment of levamisole (10^{-7} M) did not change the pD_2 of Ach concentration-dose response while it significantly increased the E_{max} responses ($p<0.05$). In the study of Gulati *et al.* (1985) which was aimed to determine the interaction of levamisole with other autonomic drugs on guinea pig vas deference, levamisole increased the Ach response. The investigators have suggested that this effect is due to inhibition of cholinesterase by levamisole. Also Aldabagh and Mohammad (1999) suggested that levamisole and tetramisole inhibited AchE activity *in vitro*.

Pretreatment of levamisole (10^{-7} M) decreased the E_{max} ($p\leq0.01$) and pD_2 ($p<0.05$) of bethanecol (10^{-8}-10^{-3}M) which is a muscarinic agent that is resistant to AchE. This showed that levamisole binds to the muscarinic receptor where bethanecol binds. The increase of Ach maximum response and decreased bethanecol response due to the pretreatment of levamisole may be because of the direct effect of levamisole on postsynaptic cholinergic receptor as well as the inhibition of AchE (Table 4). Pretreatment with atropine (10^{-6} M) which is an antimuscarinic agent, decreased ($p<0.05$) the response of levamisole (10^{-4} M) (Table 6). In general, positive responses with atropine treatment in levamisole intoxication, support this evidence (Cook *et al.*, 1985; Hsu, 1980; Kaya and Imren, 1984).

Levamisole (10^{-7} M) increased the nicotinic effects insignificantly ($p>0.05$) (Table 5) and mecamylamine (10^{-5} M) which is a ganglion blocker decreased the levamisole (10^{-4} M) responses slightly ($p>0.05$) (Table 3). Both results indicated that the effect of levamisole on nicotinic receptors of the sheep trachea were poor.

Pretreatment with trichlorfon and levamisole combination decreased the pD_2 and E_{max} of Ach compared to pretreatment with levamisole alone ($p\leq0.01$) and control group (Table 2). In spite of the findings of other referred studies, adverse interaction between trichlorfon and levamisole were observed in the present study. Thus a decrease in the toxic effect could be mentioned when the two compounds were present together. Similarly in the study on rabbits about the interaction between levamisole and dichlorvos, levamisol decreased the toxic effects caused by dichlorvos (Aldabagh and Mohammad, 1999). The decrease in the pD_2 and E_{max} responses of Ach pretreated with levamisole-trichlorfon combination according to levamisole pretreatment is may be due to the down regulation caused by the combination of levamisole and trichlorfon. Trichlorfon inhibited the AchE activity by converting to dichlorvos (Barragry, 1994). It also shows cholinergic activity by binding to cholinergic receptors. A number of studies evaluated the effects of anticholinesterases on muscarinic receptor bindings. Dichlorvos inhibited the binding of the non-selective muscarinic antagonist quinuclidinyl benzilate at low concentrations (Pope *et al.*, 2005). In the present study; trichlorfon decreased the pD_2 ($p<0.05$) and E_{max} ($p>0.05$) responses of Ach concentrations (Table 2). In this manner, besides trichlorfon effects by blocking AchE, it also effects by causing a decrease of cholinergic response of Ach through muscarinic receptor occupation and down regulation upon long term exposure.

To conclude, the results suggested that in sheep; levamisole acts significantly on muscarinic receptors. The effect of levamisole on nicotinic receptors was poor in sheep trachea. Levamisole and trichlorfon combination result in a decrease in the Ach response *in vitro* and this indicates the presence of a counter interaction in between. Measured responses may deviate according to age and species. So atropine, although a strong antagonist, may not be sufficient in the treatment of levamisole toxicity alone.

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