

Prolonged Administration of Pyridostigmine Impairs Neuromuscular Function with and without Down-regulation of Acetylcholine Receptors

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ABSTRACT

Background: The acetylcholinesterase inhibitor, pyridostigmine, is prophylactically administered to mitigate the toxic effects of nerve gas poisoning. The authors tested the hypothesis that prolonged pyridostigmine administration can lead to neuromuscular dysfunction and even down-regulation of acetylcholine receptors.

Methods: Pyridostigmine (5 or 25 mg·kg⁻¹·day⁻¹) or saline was continuously administered *via* osmotic pumps to rats, and infused for either 14 or 28 days until the day of neuromuscular assessment (at day 14 or 28), or discontinued 24 h before neuromuscular assessment. Neurotransmission and muscle function were examined by single-twitch,

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What We Already Know about This Topic

- The acetylcholinesterase inhibitor pyridostigmine is prophylactically administered to mitigate the toxic effects of nerve gas poisoning, as used in the Gulf War.

What This Article Tells Us That Is New

- Prolonged administration of pyridostigmine (25 mg·kg⁻¹·day⁻¹) leads to neuromuscular impairment, even if discontinued for 24 h. This impairment appears to be associated with but is also independent of acetylcholine receptor down-regulation.

train-of-four stimulation and 100-Hz tetanic stimulation. Sensitivity to atracurium and acetylcholine receptor number (quantitated by ¹²⁵I- α -bungarotoxin) provided additional measures of neuromuscular integrity.

Results: Specific tetanic tensions (Newton [N]/muscle weight [g]) were significantly ($P < 0.05$) decreased at 14 (10.3 N/g) and 28 (11.1 N/g) days of 25 mg·kg⁻¹·day⁻¹ pyridostigmine compared with controls (13.1–13.6 N/g). Decreased effective dose (0.81–1.05 *vs.* 0.16–0.45 mg/kg; $P < 0.05$) and decreased plasma concentration (3.02–3.27 *vs.* 0.45–1.37 μ g/ml; $P < 0.05$) of atracurium for 50% paralysis (controls *vs.* 25 mg·kg⁻¹·day⁻¹ pyridostigmine, respectively), irrespective of discontinuation of pyridostigmine, confirmed the pyridostigmine-induced altered neurotransmission. Pyridostigmine (25 mg·kg⁻¹·day⁻¹) down-regulated acetylcholine receptors at 28 days.

Conclusions: Prolonged administration of pyridostigmine (25 mg·kg⁻¹·day⁻¹) leads to neuromuscular impairment, which can persist even when pyridostigmine is discontinued 24 h before assessment of neuromuscular function. Pyridostigmine has the potential to down-regulate acetylcholine receptors, but induces neuromuscular dysfunction even in the absence of receptor changes.

ACETYLCHOLINESTERASE inhibitors, including pyridostigmine, are used in clinical practice to treat the symptoms associated with myasthenia gravis, Alzheimer disease, and multiple sclerosis.^{1–3} Pyridostigmine also has been approved by the Federal Drug Administration for pretreatment of humans against nerve gas poisoning,^{4,5} and was used in over 41,000 soldiers

prophylactically during the Gulf War.^{6–8} Soldiers who were deployed to the Gulf War received pyridostigmine orally three times a day from 1–21 days prior to departure to the war zone, and continued to take it throughout their stay in the desert area.

Fatigue, mood-cognitive disorders, and skeletal muscle symptoms (*a.k.a.*, Gulf War syndrome) were more common in the veterans deployed to the Gulf War, than in those deployed elsewhere or not deployed.^{9–11} Chronic inhibition of the acetylcholinesterase enzyme by pyridostigmine has been implicated in the Gulf War syndrome.^{12–15} The fatigue related to Gulf War syndrome may have a central and/or peripheral component, and these symptoms can persist even after termination of pyridostigmine. In myasthenia gravis, treatment with acetylcholinesterase inhibitors, even for prolonged periods, improves and maintains muscle function.³ Acute or prolonged exposure to reversible acetylcholinesterase inhibitors in healthy subjects, however, can have positive or negative effects on neuromuscular transmission and muscle-ultrastructure.^{16–24} Anderson *et al.*¹⁸ reported a dose-dependent, frequency-dependent, and time-dependent decrement in muscle function, which remained depressed during 20 days of pyridostigmine administration. Histological changes associated with prolonged administration of acetylcholinesterase inhibitors include disruption of organelles in the axon terminal, regional or total withdrawal of the nerve terminal from postsynaptic junctional folds, invasion of Schwann cell fingers into the synaptic cleft, degeneration of postsynaptic folds in end-plates, the dissolution of Z-discs, dilation of mitochondria, and destruction of the sarcoplasmic reticulum.^{19–22}

The classical theory of receptor control suggests that chronic agonist stimulation of any receptor can lead to receptor desensitization and even down-regulation in a concentration- and time-dependent manner. Plasticity and altered function due to persistent agonist stimulation has been documented for many receptors, including the adrenoceptors, glutamate, opiate, and the muscarinic acetylcholine receptors.^{25–28} This study examined the subacute neuromuscular (peripheral) effects of pyridostigmine administration for up to 28 days. We tested the hypothesis that chronic agonist stimulation of the acetylcholine receptors by long-term administration of acetylcholinesterase inhibitors, specifically pyridostigmine, induces muscle weakness, and has the potential to even down-regulate acetylcholine receptors on the muscle membrane in a time- or concentration-dependent manner, as a result of excessive agonist stimulation by acetylcholine and/or the direct effects of pyridostigmine. In addition to conventional nerve-muscle contraction studies, pharmacological sensitivity to an antagonist of acetylcholine receptors (atracurium) and biochemical (acetylcholine receptor) changes in muscle provided additional characterizations of the neuromuscular integrity associated with prolonged pyridostigmine administration.

Materials and Methods

Animal Model and Study Design

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY), weighing 220–260 g, each were included in this study after approval by the Subcommittee on Animal Care Research at Massachusetts General Hospital, Boston, Massachusetts. The animals were housed under a 12-h light and dark cycle with free access to rat chow and water and were permitted to acclimate to our animal care facility for at least one week before the study.

Acute Pyridostigmine Administration

To distinguish the acute from chronic effects of pyridostigmine, a group of rats under anesthesia (described in detail *infra*) received incremental doses of pyridostigmine acutely every 10 min ($n = 6$). To prevent the muscarinic side effects of pyridostigmine (bradycardia, lacrimation, salivation, and bronchorrhoea), 350- $\mu\text{g}/\text{kg}$ atropine was injected subcutaneously. For the functional studies, the animals were anesthetized and monitored as described below (see Neuromuscular Function Studies). Pyridostigmine was administered by intravenous injection in doses of 0, 0.03, 0.07, 0.14, 0.27, 0.55, and 1.1 mg/kg (cumulative total dose 2.16 mg/kg), and corresponding evoked muscle tension of the tibialis muscle after train-of-four stimulation was recorded. At the end of the experiment, the animals were euthanized with an intravenous overdose of pentobarbital.

Chronic Pyridostigmine Administration

On the basis of previous studies,¹⁸ low- (5 mg·kg⁻¹·day⁻¹) and high-dose (25 mg·kg⁻¹·day⁻¹) pyridostigmine infusion rates were established. Rats were randomly assigned to one of six groups ($n = 18$ per group; fig. 1). The four experimental groups received low- or high-dose pyridostigmine by osmotic pump for 14 or 28 days. All groups received 350- $\mu\text{g}/\text{kg}$ of atropine subcutaneously before the osmotic pump was inserted. The two control groups received saline *via* osmotic pump. Atropine was repeated as necessary for the first 48–72 h, based on side effects enumerated above. All groups were further divided into two subgroups. In one subgroup ($n = 8–9$ per dose per time period), the pyridostigmine or saline infusion was discontinued by explanting the pump 24 h in advance of neuromuscular assessment, whereas in the other subgroup, the infusion was continued until the day of the functional studies, which were performed at 14 or 28 days after start of the infusion (fig. 1). The reason for discontinuing or continuing pyridostigmine before neuromuscular assessment was to differentiate the allosteric interaction of pyridostigmine and that of increased acetylcholine levels with acetylcholine receptors,¹⁷ from the truly chronic effects of pyridostigmine.

Pyridostigmine bromide (ICN Biomedicals Inc., Aurora, OH) or saline was continuously infused *via* Alzet osmotic pumps (Alzet model 2ML4; DURECT Corporation, Cupertino, CA) placed subcutaneously on the rodent's back. Before

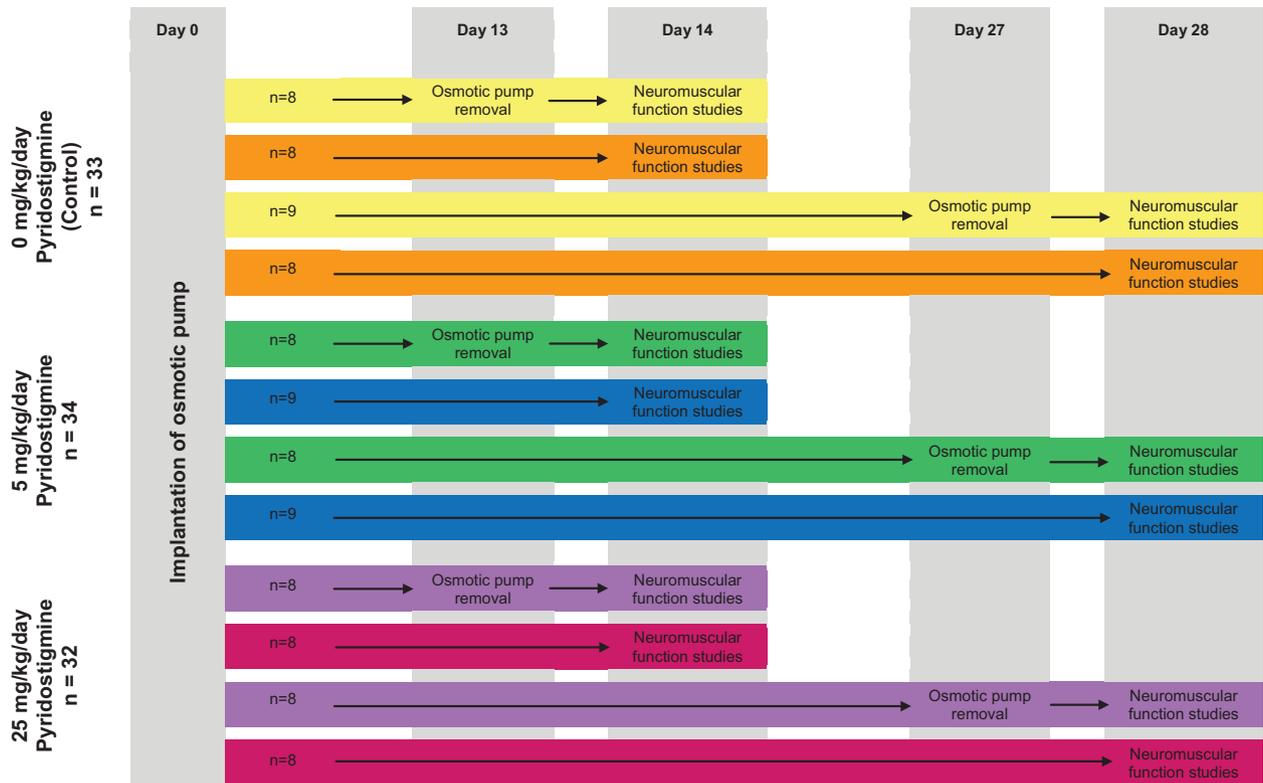


Fig. 1. Study design and final number of animals for chronic pyridostigmine administration. The protocol consists of three groups: pyridostigmine 5 or 25 mg · kg⁻¹ · day⁻¹, and saline infusion group. The infusion was continued for 13, 14, 27, or 28 days. Functional and pharmacological tests were performed at 14 or 28 days only. The number of animals for each group is indicated in the figure.

implantation, the rats were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally, with additional doses (5–10 mg/kg) as required. Adequacy of the depth of anesthesia was confirmed by the absence of withdrawal response to toe clamping. The rat's back was shaved, the skin was disinfected with iodine solution, and an incision (~2 cm) was made at the lower back. A subcutaneous pocket cranial to the incision was constructed by blunt dissection. The osmotic pump, filled with either pyridostigmine or saline, was placed into the pocket. The osmotic pumps delivered a constant infusion of $2.54 \pm 0.06 \mu\text{l/h}$. To achieve the two different infusion rates (5 or 25 mg · kg⁻¹ · day⁻¹), either 22 mg pyridostigmine, or 110 mg pyridostigmine was dissolved in 1 ml saline solution and spiked into the pump. The incision was closed with (4-0) silk, and antibiotic ointment applied to the wound. After emerging from anesthesia, the rats were returned to the animal care area. The osmotic pumps were explanted, using the same anesthetic and surgical techniques used for implantation.

Anesthesia and Vital Parameters

For the acute and day 14 or 28 neuromuscular function tests, the animals were anesthetized with pentobarbital, as described previously. Tracheostomy was performed, and the animals were ventilated with room air to maintain Paco_2 between 35 and 45 mmHg, using a Harvard rodent ventilator. The right jugular vein was catheterized for drug administration. The

right carotid artery was cannulated for hemodynamic monitoring and blood gas analysis throughout the experiment. Blood gas analyses were performed intermittently and ventilation adjusted to pH values between 7.36 and 7.46. Body temperature was monitored with a rectal probe and maintained between 37° and 38°C with a warming pad and heat lamp. Anesthesia was maintained with intermittent intravenous doses of pentobarbital. The administration of repeat doses of pentobarbital was based on cardiovascular signs of inadequate anesthesia. If a rat was hemodynamically unstable (mean arterial blood pressure <80 mmHg), or had blood gas values beyond the predefined physiologic ranges (PaO_2 <70 mmHg, Paco_2 <35 or >45 mmHg, arterial blood pH <7.36 or >7.46), the animal was excluded from the experiment.

Neuromuscular Function Studies

Neuromuscular transmission and muscle function were monitored by evoked mechanomyography, using a peripheral nerve stimulator (NS252; Fisher & Paykel Health Care, Irvine, CA) along with a Grass FT03 force transducer and software (Grass Instruments, Quincy, MA) as described previously.²⁹ Both sciatic nerves were exposed and stimulated at the thigh with supramaximal stimuli (0.2 ms duration, 1 Hz). The resulting contractions of both tibialis muscles were recorded. To yield maximal evoked twitch tensions, a baseline

tension of approximately 50 g was applied to the tendons of the tibialis muscles. A 50-Hz tetanic stimulation (5 s) was applied to recruit all muscle fibers. After stable single-twitch tensions were elicited for at least 15 min, the stimulation was changed to the train-of-four pattern (2 Hz for 2 s every 12 s). To assess muscle function during increased repetitive workload, a 100-Hz tetanic stimulation (5 s) was applied, and maximal tension was recorded.

When assessing efficacy and integrity of neurotransmission, in addition to examining mechanical muscle responses to nerve stimulation, pharmacological tests (*e.g.*, curare sensitivity) and biochemical assays (*e.g.*, acetylcholine receptor number) have been used to increase sensitivity and improve the assessment of neuromuscular integrity and/or changes.^{30–32} Therefore, in this study, the assessment of neuromuscular sensitivity to atracurium and changes in acetylcholine receptors at the tibialis muscle membrane provided additional measures of neuromuscular function. After the tetanic muscle contraction tests, the muscle was permitted to recover for 30 min from the effects of the preceding tetanus. Subsequently, neuromuscular sensitivity to atracurium, a nondepolarizing muscle relaxant, was examined with the cumulative dose–response method, as described previously.^{29,33} Incremental doses of atracurium (0.2–0.4 mg/kg) were given intravenously until the first twitch height (T1) of the train-of-four was below approximately 5% of the baseline twitch tension (>95% twitch depression). After the last dose of atracurium, the twitch tension (paralysis) was permitted to recover and an infusion of atracurium was started to maintain muscle paralysis at 50 ± 5% twitch depression. After 10 min of stable 50 ± 5% twitch depression, a pseudo steady-state was assumed to be present between plasma and the neuromuscular junction; a blood sample was drawn to assess the plasma atracurium level. The blood was immediately transferred to Eppendorf tubes containing 20 µl 1 M H₂SO₄ and centrifuged (3,500 rpm, 10 min, 4°C). The supernatant was collected and 0.2 ml portions were aliquoted into Eppendorf tubes containing 0.8 ml 15 mM H₂SO₄.

Acetylcholine Receptor and Atracurium Assays

After the muscle function and pharmacological studies, the animals were euthanized with an intravenous overdose of pentobarbital, and the tibialis muscles of both hindlimbs were harvested and weighed. The muscles were immediately frozen in liquid nitrogen and stored at –80°C for later analysis of acetylcholine receptor concentrations. Total acetylcholine receptor concentration in the tibialis muscle was determined using the ¹²⁵I- α -bungarotoxin binding assay, as described previously.³⁴ The protein concentration of the muscle extract was assayed using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), and the content of acetylcholine receptors was calculated and expressed in femtomole acetylcholine receptors per milligram protein. Plasma concentrations of atracurium were determined

by high-performance liquid chromatography as described previously.³⁵

Statistical Analyses

All values are expressed as mean ± SD. The effective doses to achieve 50% neuromuscular paralysis (ED₅₀) were calculated from the cumulative dose–response curve for each rat by interpolation from linear regression of the degree of block in logit scale and the respective cumulative dose of atracurium in log scale. Data were analyzed with general linear models. Results of the acute pyridostigmine administration experiment were analyzed with repeated measures of ANOVA, with the dependent within-groups variable being the cumulative pyridostigmine dose. *Post hoc* values of consecutive doses as well as T1 and T4 values at a respective dose were compared with two-tailed paired Student *t* tests and adjusted according to Bonferroni. Results of the chronic pyridostigmine administration experiments were compared by factorial analyses of variance, using pyridostigmine dose (0, 5, *vs.* 25 mg · kg⁻¹ · day⁻¹), duration of administration (14 *vs.* 28 days), and osmotic pump removal *versus* nonremoval as independent between-groups factors. In case of a significant family-effect of pyridostigmine dose, all pairs of the three doses were compared according to Bonferroni *post hoc* strategy. For the further *post hoc* testing a hierarchical approach was chosen to address the multiple comparisons. Two-tailed paired or unpaired Student's *t* tests were calculated between those subgroups for which the respective group effect proved to be significant. The level of statistical significance was chosen as a *P* value less than 0.05. Statistical analyses were performed using SPSS 20 for Macintosh (IBM, Armonk, NY).

Results

Acute Pyridostigmine Administration

As the tibialis muscle mass (590 ± 60 mg) did not differ significantly between the six rats, all muscle forces developed during acute administration of pyridostigmine are given as absolute tensions. Hemodynamic and metabolic variables were stable and within the predefined physiologic ranges in the six animals during these experiments. Cumulative intravenous bolus doses of pyridostigmine administered acutely resulted in a dose-dependent increase in the evoked-muscle force during train-of-four stimulation at and above doses of 0.24 mg/kg (fig. 2). The muscle force of the fourth twitch (T4), during train-of-four stimulation, increased to a lesser degree when compared with the T1 responses, resulting in decrease of the train-of-four ratio to a mean of 0.87 ± 0.05 at and above cumulative doses of 0.24 mg/kg.

Chronic Pyridostigmine Administration

Stability of the Model. Four animals died within the first 2 days of high-dose (25 mg · kg⁻¹ · day⁻¹) pyridostigmine administration. All other animals survived until the day of the functional studies (14 or 28 days, respectively), but the animals

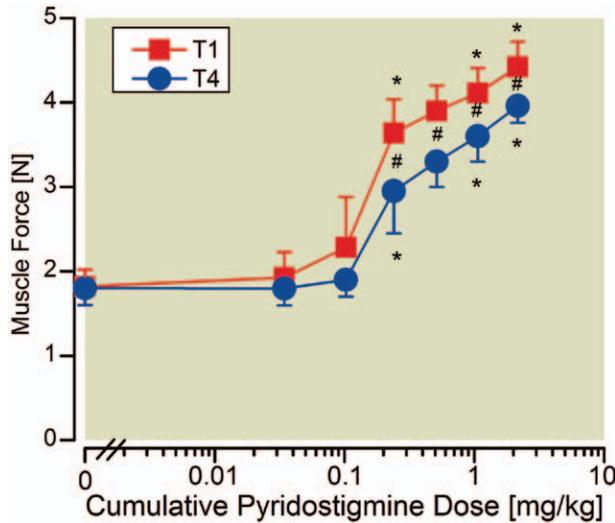


Fig. 2. Evoked-muscle tension increases with acute administration of pyridostigmine. During train-of-four stimulation, tension in the tibialis muscle was recorded. There was a dose-dependant increase of both the first (T1) and fourth (T4) twitches. The increase in T4 was less than that of T1, resulting in fade of the train-of-four responses. The mean train-of-four ratio was 0.87 ± 0.05 at a cumulative pyridostigmine dose of 0.24 mg/kg and above. * $P < 0.05$ when T1 or T4 is compared with the respective previous T1 or T4 value. # $P < 0.05$ when T4 is compared with the respective T1 value.

exhibited mild cholinergic side effects, including increased oral and ocular secretions as well as muscle fasciculations on the first 2 to 3 days of pyridostigmine administration. The saline group showed no such changes. The animals in the $25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ pyridostigmine group lost approximately 10% of their initial body weight during the first 3 days of pyridostigmine administration. From day 4 onward, they

started gaining weight ($2\text{--}5 \text{ g/day}$). Five animals had to be excluded from the neuromuscular function studies because of hemodynamic or metabolic instability at the beginning of or during the functional studies. This resulted in final numbers of animals in each of the subgroups as follows (fig. 1): 16 in the 14 days saline group (not explanted: $n = 8$, explanted: $n = 8$); 17 in the 28 days saline group (not explanted: $n = 8$, explanted: $n = 9$); 17 in the 14 days low-dose pyridostigmine group (not explanted: $n = 9$, explanted: $n = 8$); 17 in the 28 days low-dose pyridostigmine group (not explanted: $n = 9$, explanted: $n = 8$); 16 in the 14 days high-dose pyridostigmine group (not explanted: $n = 8$, explanted: $n = 8$); and 16 in the 28 days high-dose pyridostigmine group (not explanted: $n = 8$, explanted: $n = 8$).

Muscle Mass and Nerve-stimulated Contractile Responses after Chronic Pyridostigmine. The tibialis muscle mass and the nerve-stimulation evoked changes in contraction are presented in table 1. As there were significant differences in the weights of the tibialis muscle between groups at different study periods, the muscle force is reported relative to muscle mass, referred to as specific muscle force (twitch tension [Newton, N]/muscle weight [g]). The first twitch of the train-of-four (T1) during evoked-muscle stimulation increased significantly in all pyridostigmine groups when the administration of pyridostigmine was not discontinued (osmotic pump not explanted) 24 h before the functional studies. This increase was dose-dependent, developing the highest tension at 28 days with $25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ pyridostigmine administration. The specific single-twitch tensions were not different between groups when the osmotic pump was removed 24 h before functional studies. The fourth twitch of the train-of-four (T4) increased as well in all pyridostigmine groups, when the administration of pyridostigmine was not discontinued, but to a lesser extent than the first twitch. This

Table 1. Muscle Mass and Nerve-stimulation Evoked Muscle Function Studies

		Pyridostigmine Dose					
		$0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$		$5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$		$25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$	
		14 d	28 d	14 d	28 d	14 d	28 d
Specific twitch tension, T1 [N/g]	Not explanted	3.8 ± 0.6	3.6 ± 0.3	$5.3 \pm 1.1^{* \#}$	$4.9 \pm 1.0^{* \#}$	$6.0 \pm 0.9^{* \#}$	$6.2 \pm 0.7^{* \# \phi}$
	Explanted	3.2 ± 0.3	3.4 ± 0.3	3.6 ± 0.7	3.3 ± 0.2	3.0 ± 0.2	3.2 ± 0.3
Train-of-four ratio	Not explanted	0.98 ± 0.01	0.98 ± 0.01	$0.87 \pm 0.06^{* \#}$	$0.82 \pm 0.09^{* \#}$	$0.89 \pm 0.02^{* \#}$	$0.89 \pm 0.04^{* \#}$
	Explanted	0.99 ± 0.01	0.98 ± 0.01	$0.98 \pm 0.01^{*}$	0.98 ± 0.01	$0.96 \pm 0.02^{*}$	0.98 ± 0.01
Specific tetanic tension [N/g]	Not explanted	13.1 ± 0.6	13.6 ± 1.0	12.7 ± 1.3	13.4 ± 0.8	$10.3 \pm 1.0^{* \phi}$	$11.1 \pm 1.7^{* \phi}$
	Explanted	13.2 ± 0.9	13.0 ± 0.9	12.8 ± 0.4	12.8 ± 0.7	$10.8 \pm 1.5^{* \phi}$	11.9 ± 0.9
Muscle mass [mg] (M. tibialis)	Not explanted	603 ± 29	659 ± 70	634 ± 47	$718 \pm 60_{\S}$	571 ± 56	$689 \pm 73_{\S}$
	Explanted	585 ± 55	$724 \pm 65_{\S}$	628 ± 68	711 ± 69	$549 \pm 33_{\phi}$	$705 \pm 64_{\S}$

* $P < 0.05$ when compared with $0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ pyridostigmine. $\phi P < 0.05$ when compared with $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ pyridostigmine for the same duration of infusion. # $P < 0.05$ when compared with the group with the same dose and length of infusion but removal of the osmotic pump 24 h before. $\S P < 0.05$ when compared with the same dose for 14 d of treatment.

T1 = first twitch of a train-of-four.

resulted in a statistically significant decrease of the train-of-four ratios in the chronic pyridostigmine groups at day 14 and 28, when the osmotic pump was not removed (table 1). After removing the osmotic pumps on day 13 and day 27, none of the groups had a decreased train-of-four ratio of less than 0.9. The specific tetanic tension (100 Hz for 5 s) was statistically significantly decreased in the pyridostigmine 25 mg·kg⁻¹·day⁻¹ groups compared with control and pyridostigmine 5 mg·kg⁻¹·day⁻¹ groups, when pyridostigmine was administered until the day of functional studies at 14 and 28 days. The tetanic tension in the 25 mg·kg⁻¹·day⁻¹ groups was not decreased consistently relative to controls, when pyridostigmine was removed 24 h before the functional studies.

Pharmacological and Biochemical (Acetylcholine Receptor) Measures of Neuromuscular Function. The administration of high-dose pyridostigmine for 14 and 28 days shifted dose-response curves of atracurium to the left (fig. 3), resulting in a statistically significant decrease in effective dose for 50% twitch depression (ED₅₀), whether or not the osmotic pump was removed 24 h before neuromuscular evaluation (table 2). When the pump was not removed, however, the ED₅₀ was lower than that of the group where the pump was removed a day earlier, suggesting a more marked impairment of neuromuscular integrity in the former. The effective plasma concentration for 50% paralysis (EC₅₀) followed a similar pattern, where pyridostigmine at a dose of 25 mg·kg⁻¹·day⁻¹ decreased the atracurium plasma concentration required for 50% paralysis at 14 and 28 days, irrespective of pump removal. The continuation of the pyridostigmine infusion until the day of functional studies had a more significant effect, however, leading to a lower EC₅₀ compared with removal of the pump the day before (table 2). The slopes of the dose-response curves for atracurium were also less steep in the 25 mg·kg⁻¹·day⁻¹ pyridostigmine groups at both 14 and 28 days. The slopes reversed toward controls after removal of the pumps, but were still statistically significantly less steep than controls. A consistent change in slope was not observed in the pyridostigmine 5 mg·kg⁻¹·day⁻¹ groups. The membrane acetylcholine receptor expression in the tibialis muscle was statistically significantly reduced after 28 days of high-dose pyridostigmine treatment compared with the low-dose and control groups, irrespective of the discontinuation of pyridostigmine. High-dose pyridostigmine did not change acetylcholine receptor expression at 14 days of pyridostigmine administration (table 2). Low-dose pyridostigmine, also, did not change acetylcholine receptor number at both 14 or 28 days.

Discussion

Increasing evidence indicates prolonged exposure to pyridostigmine as the etiological factor for Gulf War syndrome, which includes skeletal muscle symptoms.⁷⁻¹³ This study in rats was performed to investigate the acute and subacute neuromuscular effects of pyridostigmine. The data for our study

demonstrate that prolonged exposure to high-dose pyridostigmine (25 mg·kg⁻¹·day⁻¹) leads to neuromuscular impairment at 14 and 28 days, even when the pyridostigmine infusion is stopped 24 h before neuromuscular assessment. The impairment was evidenced as decreased specific tetanic tension, as well as increased sensitivity to the neuromuscular blocking drug, atracurium, with and without reduced acetylcholine receptor expression. The study confirmed the hypothesis that acetylcholinesterase inhibitor-induced agonist stimulation can down-regulate acetylcholine receptors in a concentration- and time-dependent manner. These neuromuscular changes were not consistently seen in the 5 mg·kg⁻¹·day⁻¹ pyridostigmine group, even when infused for 28 days.

Acute experiments with pyridostigmine were performed to examine the immediate effects of the drug on train-of-four stimulation. Consistent with many other studies on acetylcholinesterase inhibitors,^{16,23,24,36} our studies show that acute pyridostigmine administration produces fade and/or neuromuscular changes during acute administration. Similar to the acute neuromuscular responses to pyridostigmine, prolonged pyridostigmine administration until the time of the functional studies resulted in a dose-dependent increase of the first and the fourth twitch of a train-of-four. The fourth twitch increased to a lesser extent than the first, resulting in a small but statistically significant fade. The train-of-four ratio in controls was 0.98 or more, whereas, in the pyridostigmine groups it was 0.90 or less, a value previously noted to be associated with muscular weakness.³⁷⁻³⁹ In view of increases in both T1 and T4 responses, the clinical significance of fade (≤0.90), during pyridostigmine administration in our study, is not entirely clear. The train-of-four fade was absent when pyridostigmine was discontinued 24 h before the muscle contraction studies. The response to acute incremental doses of pyridostigmine resulted in similar increase of both T1 and T4 responses (fig. 2).

The decrease of specific tetanic tension during high-dose (25 mg·kg⁻¹·day⁻¹) pyridostigmine is noteworthy. Tetanic tension reflects the ability to do high-intensity repetitive work. The decrease in specific tetanic tension was observed at both 14 and 28 days of high-dose pyridostigmine administration. The findings of increased single-twitch tension and the decreased tetanic tensions at the same time period in the high-dose groups are not contradictory. Single-twitch tension is not a clear indicator of muscle function, particularly when assessing the ability to do repetitive work. The most likely reason for the increased single-twitch tension during high-dose pyridostigmine infusion is as follows: single twitch results in the release of acetylcholine into the synaptic cleft. Due to the inhibition of its breakdown by high-dose pyridostigmine, the acetylcholine induces repetitive channel opening, resulting in enhanced single-twitch tension. In contrast, with tetanic stimulation during high-dose pyridostigmine, the repetitive release and maintenance of continued high levels of acetylcholine because of inhibition of its breakdown, leads to desensitization of the acetylcholine

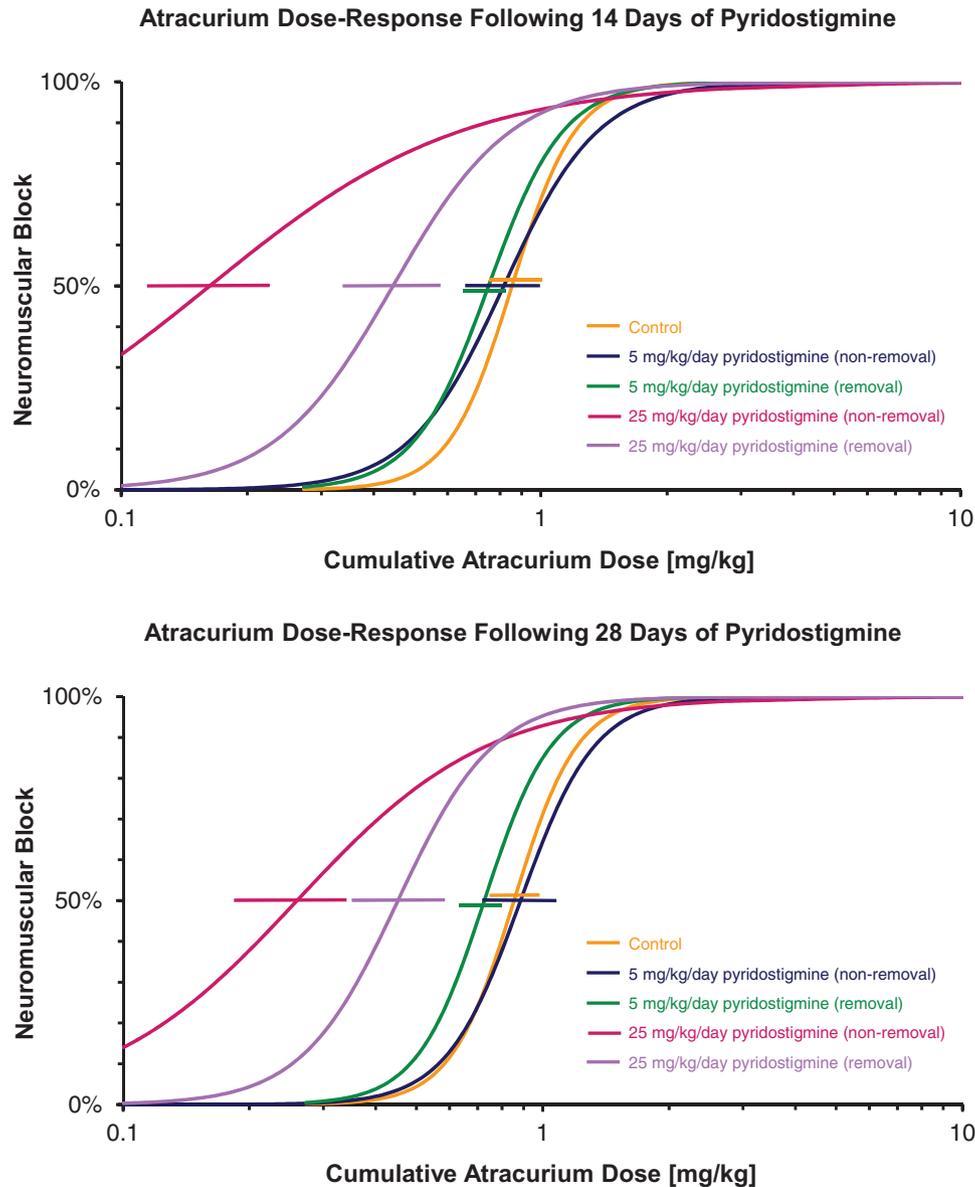


Fig. 3. Increased sensitivity to atracurium in the high-dose pyridostigmine groups at 14 and 28 days, with and without removal of pyridostigmine 24 h before neuromuscular assessment suggests decreased margin of safety. Incremental doses of atracurium were administered and dose–response curves at both 14 and 28 days of pyridostigmine administration were calculated for each group. The 95% CIs of the doses to produce a 50% neuromuscular paralysis are presented as horizontal bars. The doses differ significantly if the bars do not overlap. The animals receiving high-dose pyridostigmine showed a significant leftward shift in the dose–response curves at both 14 and 28 days of pyridostigmine infusion, indicating that the neuromuscular transmission was not as robust compared with controls, irrespective of whether or not the osmotic pump infusing pyridostigmine was removed 24 h before pharmacological testing of neuromuscular function. The $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ pyridostigmine dose did not impair neuro-transmission.

receptors, resulting in decreased specific tetanic tensions at 14 and 28 days.

When measurable change in the mechanical response to nerve stimulation is absent, pharmacological tests (*e.g.*, regional curare sensitivity test) have proved useful for diagnosing aberrations of neurotransmission. In the early diagnosis of myasthenia gravis, for example, pharmacological tests, such as sensitivity to nondepolarizing relaxant, together with changes in acetylcholine

receptor number, were used to verify neuromuscular impairment.^{30–32} The pharmacological and biochemical tests proved more sensitive when classical nerve-muscle contractions did not reveal any overt defect. To further characterize the nature of the observed neuromuscular impairment, we measured neuromuscular sensitivity to atracurium and the changes in the acetylcholine receptor number at the tibialis muscle membrane. In both high-dose pyridostigmine groups, that is, at 14 and 28

Table 2. Pharmacological and Biochemical (Acetylcholine Receptors) Measures of Neuromuscular Integrity

	Osmotic Pump	Pyridostigmine Dose					
		0 mg · kg ⁻¹ · day ⁻¹		5 mg · kg ⁻¹ · day ⁻¹		25 mg · kg ⁻¹ · day ⁻¹	
		14 d	28 d	14 d	28 d	14 d	28 d
Receptor expression [fmol/mg protein]	Not explanted	23.8 ± 4.5	21.1 ± 4.2	23.7 ± 6.6	18.8 ± 4.3	18.0 ± 5.6	13.2 ± 3.1* ϕ
	Explanted	27.8 ± 4.5	22.4 ± 5.0	22.2 ± 3.9	19.2 ± 5.1	20.8 ± 5.7	12.6 ± 3.3* ϕ \S
Atracurium ED ₅₀ [mg/kg]	Not explanted	0.81 ± 0.15	0.82 ± 0.08	0.81 ± 0.28	0.93 ± 0.17	0.16 ± 0.03* ϕ $\#$	0.21 ± 0.08* ϕ $\#$
	Explanted	1.05 ± 0.30	0.87 ± 0.15	0.77 ± 0.15	0.82 ± 0.20	0.45 ± 0.14* ϕ	0.45 ± 0.07* ϕ
Atracurium Plasma EC ₅₀ [μ g/ml]	Not explanted	3.27 ± 0.44	3.02 ± 0.52	3.37 ± 0.44 $\#$	3.14 ± 0.68	0.51 ± 0.16* ϕ $\#$	0.45 ± 0.23* ϕ $\#$
	Explanted	3.06 ± 0.66	3.21 ± 0.53	2.41 ± 0.22	3.00 ± 0.98	1.34 ± 0.39* ϕ	1.37 ± 0.37* ϕ
Slope of dose–response curve	Not explanted	5.98 ± 0.90	5.97 ± 0.92	3.85 ± 1.27*	4.89 ± 0.71	1.45 ± 0.28* ϕ $\#$	1.90 ± 0.40* ϕ $\#$
	Explanted	6.07 ± 0.71	5.11 ± 0.53	4.86 ± 1.33	5.41 ± 1.11	3.07 ± 0.54* ϕ	3.78 ± 0.84* ϕ

* $P < 0.05$ when compared with 0 mg · kg⁻¹ · day⁻¹ pyridostigmine. $\phi P < 0.05$ when compared with 5 mg · kg⁻¹ · day⁻¹ pyridostigmine for the same duration of infusion. $\#P < 0.05$ when compared with the group with the same dose and length of infusion but removal of the osmotic pump 24 h before. $\S P < 0.05$ when compared with the same dose for 14 d of treatment.

EC₅₀ = effective plasma concentration of atracurium for 50% paralysis; ED₅₀ = effective dose of atracurium for 50% paralysis.

days of infusion, the ED₅₀ and EC₅₀ of atracurium were decreased, whether or not pyridostigmine was stopped 24 h before neuromuscular assessment. Consistent with observations in myasthenic patients, our pharmacologic tests were more sensitive in detecting neuromuscular impairment than contractile (tetanus and fade) responses. The flatter slope of the dose–response curves suggests that during the continued presence of pyridostigmine, the affinity of the receptors to atracurium may also be decreased. The slopes improved and the dose–response curves shifted to the right, indicating increased ED₅₀s, when the pyridostigmine infusion pumps were removed (table 2). This suggests that when pyridostigmine infusion is discontinued, the high level of acetylcholine- or pyridostigmine-induced desensitization of acetylcholine receptors is mitigated, resulting in improved neuromuscular function. Hence, the rightward shift in the dose–response curve to atracurium after explantation of the pyridostigmine pumps. Agonist-induced desensitization is a feature well documented for adrenoceptor and opiate receptors.^{25,26} The right-shift or improvement of the slopes, and the increase of ED₅₀ and EC₅₀ with discontinuation of pyridostigmine 24 h before functional studies may also be due to the lack of direct allosteric interaction of pyridostigmine with acetylcholine receptors after its removal.¹⁷ It is noteworthy that high-dose pyridostigmine treatment for 28 days is also capable of down-regulating acetylcholine receptor number, mimicking the state of myasthenia gravis, although through a different mechanism, namely agonist stimulation. In myasthenia gravis, the down-regulation is due to antibodies against acetylcholine receptors.^{30,31}

The increased sensitivity to the nondepolarizing muscle relaxant, atracurium, in the presence of 25 mg · kg⁻¹ · day⁻¹ pyridostigmine seems counter-intuitive because pyridostigmine is known to reverse the effects of muscle relaxants.^{37–41} Our studies, combined with the observations of others, confirm that acute and chronic effects of a drug may be dramatically different. In the presence of acute exposure to acetylcholinesterase inhibitors, because of the larger than normal levels of acetylcholine present at the neuromuscular junction, the dose–response curve to nondepolarizers may be expected to shift to the right.⁴² However, soldiers pretreated with pyridostigmine for prolonged periods did not require larger than normal doses of nondepolarizers to maintain paralysis.^{43,44} Consistently, patients exposed to organophosphate poisoning are more sensitive than resistant to pancuronium.⁴⁵ The increased sensitivity to atracurium in our studies reflects the decreased margin of safety of neurotransmission induced by pyridostigmine, either due to its direct allosteric interaction with the acetylcholine receptors, or due to desensitization of acetylcholine receptors by acetylcholine.¹⁷ Our observation that the dose–response curves of atracurium can indeed shift to the left is in agreement with the clinical scenario, where chronic cholinesterase inhibition induced by organophosphate poisoning made patients more sensitive to nondepolarizers.⁴⁵

Although the current study was not performed in humans, the findings may have implications relative to neuromuscular function during prophylactic administration of pyridostigmine to soldiers at war. The known consequences of prolonged pyridostigmine therapy on immunological, behavioral, and cognitive function,^{46–51} even after its

termination of administration, is well documented. Our studies suggest that the continued presence of high-dose pyridostigmine impairs neurotransmission in subtle ways, not easily detectable by conventional means, such as fade and single-twitch tension development. The more sensitive pharmacological tests used by us confirm the presence of altered neurotransmission, even when pyridostigmine was stopped 24 h in advance of testing. Furthermore, the findings demonstrate that pyridostigmine has the potential to down-regulate acetylcholine receptors. Down-regulation of acetylcholine receptors in and of itself can decrease the margin of safety, as shown for myasthenia gravis.^{30,31} Thus, despite the advantages of pyridostigmine pretreatment for the prevention of toxicity from nerve gas poisoning,^{6–8} the subacute neuromuscular changes call for careful further examination of the use of pyridostigmine as prophylaxis against nerve gas poisoning in human studies.

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