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Effect of chronic pyridostigmine bromide treatment on cardiovascular and behavioral parameters in mice

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Abstract

Experiments were performed to determine the effect of chronic low-dose pyridostigmine bromide (PB) treatment on blood acetylcholinesterase (AChE), cardiovascular (CV) function, and behavior in C57BL/6J male mice. Chronic carotid arterial catheters were used for long-term CV measurements and for collection of blood samples. Separate groups of mice were used for behavioral open field tests. PB was administered subcutaneously using osmotic minipumps at 1 and 3 mg/kg/day for 7 days. Blood pressure and heart rate (HR) were measured continuously for 24 h before treatment and on Days 3 and 7 after minipump insertion. Blood samples were collected on the same days. Mean arterial pressure (MAP) of the control group was 108 ± 2 and 104 ± 2 mm Hg during the dark and light periods, respectively. HR was 510 ± 18 and 493 ± 19 beats/min during the dark and light periods, respectively. PB treatment had no effect on MAP or HR in either dark or light period. Basal AChE activity was 0.42 ± 0.1 μmol/min/ml, with no changes observed with PB at 1 mg/kg/day. The higher PB dose (3 mg/kg/day) decreased blood AChE activity by 85% on Day 7. Despite the reduction in blood AChE activity, there were no alterations in open field behaviors (locomotor activity, rearing, distance traveled, rest time, number of entries, and pokes). In conclusion, chronic low-dose PB exposure decreased blood AChE activity but had no effect on CV function or behavior in mice.

Keywords: Blood pressure; Heart rate; Open field; Acetylcholinesterase; Gulf War Syndrome

1. Introduction

Pyridostigmine bromide (PB) is a quaternary ammonium compound that inhibits the hydrolysis of acetylcholine (ACh) by reversibly binding to acetylcholinesterase (AChE). PB is used clinically in the treatment of myasthenia gravis (Breyer-Pfaff et al., 1985). It has also been used as a prophylactic agent against nerve gas exposure, particularly during the Persian Gulf War (Sapolsky, 1998). The scientific rationale for this treatment is that PB competitively blocks the binding of irreversible organophosphate AChE inhibitors, such as soman (Blick et al., 1994). Additionally, its lipophobicity and charge on the quaternary ammonium group inhibit its passage across the blood–brain barrier, thus limiting the central nervous system effects. There is little evidence of adverse effects of PB treatment in humans (Izraeli et al., 1990; Cook et al., 1992; Wenger et al., 1993).

Despite its relative safety, exposure to PB along with other chemicals and/or chronic stress has been implicated in the development of "Gulf War Syndrome" (Haley and Kurt, 1997; Haley et al., 1997). Chronic fatigue, muscle and joint pain, headache, sensorimotor difficulties, and problems with concentration are just a few of the complaints of veterans of the Persian Gulf War (Institute of Medicine, 1995, 2000; Knoke et al., 2000). Because of the possible involvement of PB in this cadre of symptoms, there has been much interest in studying its effects in various models. Behavioral studies showed that PB had marked behavioral effects in rats. PB at doses less than or equal to 0.10 LD_{50} interfered with avoidance learning, open-field behavior, and complex coordinated movements (Wolthuis and van Wersch, 1984; Wolthuis et al., 1995). There are also reports of decreased

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locomotor activity, exaggerated acoustic startle response, or impeded response acquisition with immediate or delayed reinforcement after PB treatment (Hoy et al., 2000, 1999; van Haaren et al., 2001; Servatius et al., 2000). Cardiovascular (CV) studies in rats showed that acute PB administration increased blood pressure (Chaney et al., 2002) or caused no change (Bataillard et al., 1990). In humans, there was a decrease in HR with no changes in reflex activity (Castro et al., 2000; Nobrega et al., 2001). These PB effects may be mediated by cholinergic interactions with cardiac function and peripheral cholinergic vasodilatory mechanisms. Central stimulation of muscarinic receptors triggers a BP increase mediated by an increase of sympathetic tone (Buccafusco, 1996; Buccafusco and Brezenoff, 1979) and release of vasopressin (Rascol et al., 1990).

We have chosen to use a model of chronic PB treatment in mice, using osmotic minipumps. The rationale for this model is that the military personnel of the Gulf War were given uncontrolled access to PB tablets. Thus, they were exposed chronically to varying dosage levels of PB. This oral self-treatment may result in underdosing as well as overdosing, both with possible lethal consequences. Thus, in the present study, we evaluated the behavioral and CV effects of chronic subcutaneous PB treatment using osmotic pumps that provide a method for continuous and constant drug delivery in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (Harlan Sprague–Dawley, Indianapolis, IN), 10–12 weeks of age, with body weight of 24–26 g, were used in the present study. The mice were housed at 22 °C with a 12:12-h dark/light cycle (0500–1700 h lights on). Animals were housed individually in plastic cages with wooden shavings. They were maintained on a standard pellet diet (0.5% sodium by weight; Harlan Teklad) with tap water ad libitum. After 10 days of acclimatization, the mice were randomly assigned for individual experiments. The Laboratory Animal Care and Use Committee of the Wright State University approved all experiments.

2.2. PB treatment

PB (Sigma, St. Louis, MO, USA) was infused subcutaneously at 1 mg/kg/day (PB 1) or 3 mg/kg/day (PB 3) by means of Alzet minipumps (model 1007D, volume of 0.5 μl/h over 7 days; DURECT, Cupertino, CA, USA). This treatment results in blood levels of PB approximately 8 and 12 ng/ml, respectively, in a separate experiment. Osmotic pumps were implanted subcutaneously on the back of the mice under anesthesia using a ketamine–xylazine mixture (6:1 mg/kg im). In the control group, minipumps were filled with isotonic saline.

2.3. Blood cholinesterase (ChE) activity

Total blood ChE, AChE, and butyrylcholinesterase (BChE) activities were determined before treatment (basal values) and on Days 3 and 7 of treatment. Total ChE activity was determined by a modified version of the colorimetric method of Ellman et al. (1961) using a Packard Fusion Microplate analyzer at 25 °C. The ChE measurements were made in whole blood collected from carotid arterial catheters. The blood samples were stored at 4 °C and enzyme activities were determined within 4 h of collection. For assay, the blood was diluted 1:100 with 0.1 M $NaPO_4$ buffer (pH 7.4). Blood AChE activity was determined by inhibiting BChE activity with 25 μM tetraisopropylpyrophosphoramide (iso-OMPA; Sigma) and then by calculating BChE activity by subtracting AChE activity from total ChE activity.

2.4. CV measurements

Mice were prepared with chronic carotid arterial catheters (Li et al., 1999; Bernatova et al., 2002). This method allows for continuous, long-term measurement of BP and HR in conscious animals. After surgery, a heparinized saline solution (100 U/ml) was continuously infused into the catheter at 25 μl/h using a syringe pump (Model 220; KD Scientific, Boston, MA, USA). The infusion is required in order to maintain catheter patency over the time course of the experiment. The catheter was covered with a metal spring that was attached to a fluid swivel at the top of the cage. The animals were allowed to recover from surgery for at least 4–5 days. Blood pressure (BP) and heart rate (HR) were recorded continuously (24 h) before minipump implantation (basal values) and on Days 3 and 7 of the treatment. Systolic and diastolic BP were recorded directly using a sampling rate of 85 samples per second using the Biopac System MP100 (BIOPAC Systems, Santa Barbara, CA, USA). HR is derived from the BP data. The data were converted from digital to numeric form using acquisition software. Data were processed by calculation of 10-min means of the respective variable. These 10-min means were averaged for the calculation of the dark and light period means.

2.5. Behavioral tests

Separate groups of mice ($n = 9–12$) were tested using an automated open field system combined with hole board (infrared photobeam technology, Motor Monitor, Version 3.11, 2000; Hamilton Kinder, Poway, CA, USA). The open field (40 × 40 cm) contained nine holes (diameter 4 cm, depth 7.5 cm) and was divided into central (20 × 20 cm), intermediate, and peripheral (both 5 cm wide) zones. Illumination (300 lx) as well as background noise levels were identical in the animal housing room and the testing room. The mouse was placed in the center of the open field and the following variables of motor activity were recorded: loco-

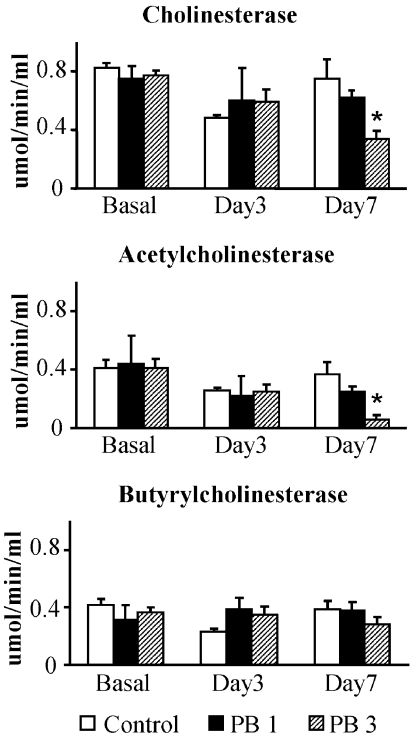

Fig. 1. Effect of PB (1 and 3 mg/kg/day) on ChE, AChE, and BChE activity. There was a significant main effect of PB treatment on ChE [$F(1,41)=12.6$, $P<.001$] and AChE [$F(1,41)=14.8$, $P<.001$] activity. * $P<.03$ vs. basal value. Values are mean ± S.E.M.

motor activity, fine movements (grooming), rearing, and head dipping. Moreover, distance traveled, total time, rest time, number of entries, head pokes, and head dips into the holes in individual zones were recorded. Mice were assigned to the experimental groups, according to the baseline values of locomotor activity. Animals were tested after 7

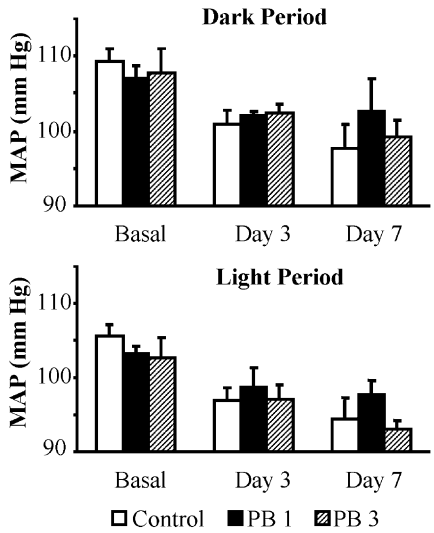

Fig. 2. Effect of PB (1 and 3 mg/kg/day) on MAP during dark and light periods. MAP in the dark period was significantly higher than in the light period [$F(1,82)=13.76$, $P<.0001$ main effect of the circadian factor]. Values are mean ± S.E.M.

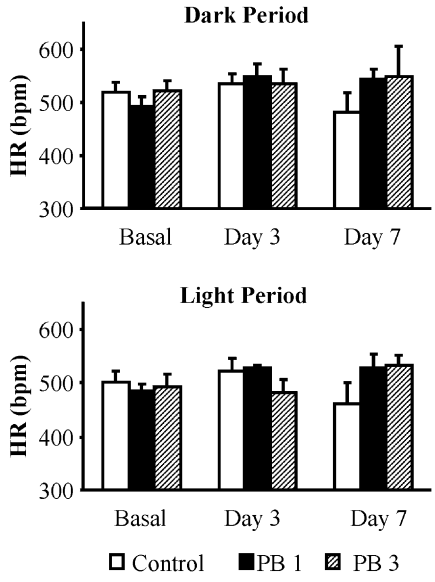

Fig. 3. Effect of PB (1 and 3 mg/kg/day) on HR during dark and light periods. Values are mean ± S.E.M.

days of continuous PB treatment. Mice were exposed to the open field in 15-min sessions once daily in the morning between 0900 and 1300 h using the same time schedule. After the testing session, the number of fecal boli (defecation rate) was noted for assessment of emotional reactivity. The open field chamber was cleaned with 70% alcohol solution between animals.

2.6. Statistical analysis

Differences in MAP and HR were evaluated by three-way ANOVA (Group × Day of Treatment × Day Period)

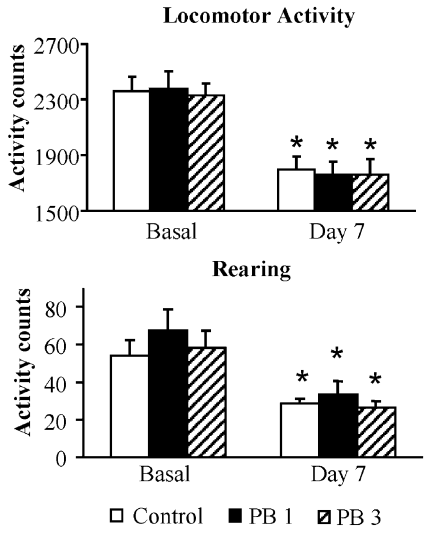

Fig. 4. Effect of PB (1 and 3 mg/kg/day) on locomotor activity and rearing. There was a significant main effect of time in the intensity of locomotor activity [$F(1,59)=42.57$, $P<.0001$] and rearing [$F(1,59)=17.86$, $P<.0001$], which reflects a habituation to the testing. * $P<.01$ vs. respective basal value. Values are mean ± S.E.M.

Table 1
Effect of PB treatment at doses of 1 and 3 mg/kg/day on central zone activities using open field testing

Central activities	Controls ($n=11$)		PB, 1 mg/kg/day ($n=12$)		PB, 3 mg/kg/day ($n=9$)	
	Basal	Day 7	Basal	Day 7	Basal	Day 7
Distance (cm)[a]	652±238	430±161	716±276	451±225	587±167	421±155
Time in zone (s)	62±27	63±55	66±22	45±19	61±25	48±16
Rest time (s)	5.9±4.7	19.9±54	5.9±5.8	4±4.3	3.9±4.5	5.2±4.3
Number of entries[a]	32±9	21±7	35±13	23±12	28±8	20±7
Number of pokes[a]	29±10	19±9	25±8	19±9	25±9	20±7

Values are mean±S.E.M.
[a] Main effect of time was significant.

followed by Duncan's test. ChE, AChE, and BChE were analyzed using two-way ANOVA (Group × Day of Experiment) and Tukey HSD test. Behavioral data were analyzed by means of two-way ANOVA (Group × Day of Treatment) followed by Duncan's post-hoc test. Values were considered to differ significantly if the P value was $<.05$. Statistical analyses were performed using Statistica, 1999 Edition (StatSoft, Tulsa, OK, USA). The results are presented as a mean±S.E.M.

3. Results

3.1. Blood ChE activity

Average basal values of ChE, AChE, and BChE were 0.78±0.05, 0.42±0.10, and 0.36±0.06 μmol/min/ml, respectively (Fig. 1). There was a significant main effect of PB treatment on ChE [$F(1,41)=12.6$, $P<.001$] and AChE [$F(1,41)=14.8$, $P<.001$] activity. PB treatment at the dose of 3 mg/kg/day significantly decreased ChE and AChE activity on Day 7 by 56% ($P<.03$ vs. basal value) and 85% ($P<.02$ vs. basal value), respectively. There were no significant differences in ChE, AChE, and BChE in mice treated with PB at the dose of 1 mg/kg/day.

3.2. Blood pressure and HR

Mean arterial pressure (MAP) and HR were analyzed using a program that compiles all of the data over the 12-h dark/light periods (3.7×10^6 samples per 12-h period). There was a significant main effect of the circadian rhythm in MAP [$F(1,82)=13.76$, $P<.0001$]. Average basal MAP in all groups was 108±2 and 104±2 mm Hg during dark and light periods (12-h averages), respectively (Fig. 2). There were no significant changes in MAP between controls and PB-treated animals. Average basal HR of mice was 510±18 and 493±19 beats/min during dark and light periods (12-h averages), respectively (Fig. 3). There were no significant differences in HR between control and PB-treated animals.

3.3. Open field test

PB treatment for 7 days did not affect the behavior of the mice in any of the parameters investigated. The two-way ANOVA revealed only a significant main effect of time on some behavioral variables. There was a significant main effect of time in the intensity of locomotor activity [$F(1,59)=42.57$, $P<.0001$] and rearing [$F(1,59)=17.86$, $P<.0001$]. Post-hoc test demonstrated significant habituation—a decrease in these behavioral activities on Day 7 compared to basal testing in all experimental groups ($P<.01$ for both activities) (Fig. 4).

Zone analysis revealed a significant main effect of time on distance traveled in the central zone [$F(1,59)=16.69$, $P<.0001$], number of entries to the central zone [$F(1,59)=16.34$, $P<.0001$], and number of head pokes in the central zone [$F(1,59)=9.72$, $P<.003$; Table 1]. In the peripheral zone, time significantly affected the distance traveled [$F(1,59)=19.45$, $P<.0001$], rest time [$F(1,59)=4.72$, $P<.04$], and number of entries [$F(1,59)=18.23$, $P<.0001$; Table 2]. Emotional reactivity, in the number of fecal boli, did not decrease after repeated exposure of mice to the open field (data not shown).

Table 2
Effect of PB treatment at doses of 1 and 3 mg/kg/day on peripheral zone activities using open field testing

Peripheral activities	Controls ($n=11$)		PB, 1 mg/kg/day ($n=12$)		PB, 3 mg/kg/day ($n=9$)	
	Basal	Day 7	Basal	Day 7	Basal	Day 7
Distance (cm)[a]	2565±836	1958±599	2490±429	1788±609	2372±539	1626±644
Time in zone (s)	421±118	452±116	412±59	415±156	416±105	373±108
Rest time (s)[a]	71±34	100±49	65±40	96±66	64±47	88±67
Number of entries[a]	87±18	69±16	94±20	72±17	91±19	75±16
Number of pokes	57±26	47±17	54±18	50±26	52±16	62±24

Values are mean±S.E.M.
[a] Main effect of time was significant.

Two-way ANOVA did not reveal any significant effects of interaction of time and PB treatment in the behavioral parameters investigated.

4. Discussion

The present study examined the effect of chronic subcutaneous PB treatment on CV and behavioral parameters in mice. The results show that while PB inhibited blood ChE and AChE activity, it had no effect on MAP, HR, or open field behavior.

CV effects are observed with some AChE inhibitors. For example, the cholinergic stimulation produced by sarin or soman evoked hypertensive responses in rats and humans (McGee and Brezenoff, 1987; Letienne et al., 1999). On the other hand, Anzueto et al. (1990) found a decline in blood pressure and bradyarrhythmias in baboons after inhalation of these organophosphates. These effects were supposedly linked with central cholinergic stimulation (Letienne et al., 1999; Smith et al., 2001) even though peripheral effects may also be involved (Buccafusco and Brezenoff, 1979). PB, due to its lipophobic structure, is unlikely to cross the blood–brain barrier under normal conditions; thus, any effect should be related to peripheral rather than central actions.

The majority of studies that have looked for CV effects of PB have used acute treatments. A decrease in HR was observed after a single dose of PB in anesthesized dogs (Caldwell et al., 1989), while no alterations were observed in marmosets (Wolthuis et al., 1995) and rats (Bataillard et al., 1990). Blood pressure was either increased or unchanged after acute PB treatment (Caldwell et al., 1989; Bataillard et al., 1990; Chaney et al., 2002). On the other hand, PB pretreatment before central stimulation with L-glutamate blunted the pressor response (Grabe-Guimaraes et al., 1999).

In humans, a single oral dose of PB (30 mg) was well tolerated and caused no alterations in BP, but produced a drop in HR (de Pontes et al., 1999; Nobrega et al., 2001) and an increase in HR variability (Nobrega et al., 2001). A higher dose of PB (45 mg) caused no alterations in systolic or diastolic pressure (Nobrega et al., 1999), but reduced HR at rest (Serra et al., 2001). When the same dose of PB was administered before mental stress, the stress-induced increase of BP and tachycardia were blunted as compared to placebo (Nobrega et al., 1999).

There is little information on the CV effects of prolonged PB exposure. Wenger et al. (1993) and Cook et al. (1992) investigated the effect of a 7-day PB treatment (3×30 mg PB/day) on red blood cell ChE activity, BP, and HR in soldiers. After 4 days of exposure, ChE activity was reduced by approximately 28%. There were time-related effects of PB on HR during exercise. By the fourth day of treatment, exercise tachycardia was reduced in PB-treated soldiers as compared to the placebo group (Wenger et al., 1993). Resting diastolic pressure was also slightly reduced (-4 mm Hg) after PB treatment (Cook et al., 1992). In contrast, chronic PB administration to nonhuman primates caused no change in CV parameters (Avlonitou and Elizondo, 1988).

In our experiment, the high PB dose (3 mg/kg/day), administered via a slow infusion, produced no change in blood BChE, but caused a decrease in AChE activity after 7 days of treatment. No changes in AChE and BChE activity were observed in mice treated with PB at 1 mg/kg/day. The data are in agreement with the study of Somani et al. (2000), which showed no differences in AChE and BChE activity after 2 weeks of oral PB treatment in mice (1.2 mg/kg/day). It is of interest that blood BChE in mice was not affected by even higher dose of PB since plasma BChE activity is often used as a marker for AChE activity. Thus, the data raise a question as to whether BChE activity is always a reliable marker of AChE.

The decreases in blood ChE and AChE activity were not accompanied by alterations in MAP or HR. This is consistent with studies that show little relationship between peripheral ChE activity and CV parameters (Avlonitou and Elizondo, 1988; Caldwell et al., 1989). The data suggested that alterations in the peripheral cholinergic signaling mechanisms could be successfully compensated by other regulatory mechanisms in the face of ChE inhibition. Certainly, the method of drug delivery may be a factor in explaining the lack of changes. The continuous slow infusion of PB (0.125 mg/kg/h) by osmotic minipump may produce the gradual development of cholinergic tolerance by reducing sensitivity to cholinergic stimulation.

While there is much information on the behavioral effects of PB in the rat, there is little information on mice. Wolthuis and van Wersch (1984) determined that PB decreased two-way shuttle box avoidance efficiency, decreased open field locomotion, and produced a decrease in stepping activity. PB was also found to decrease locomotor activity in a dose-dependent manner with obvious gender differences (Hoy et al., 2000). Other studies showed that acute and repeated PB administration negatively affected learning paradigms, such as fixed-ratio, fixed-interval, and progressive-ratio performances (van Haaren et al., 1999). Despite the generally accepted notion that PB does not cross the blood–brain barrier, the behavioral alterations indicate that there may be interactions with central cholinergic systems. However, the exact mechanism by which this compound exerts behavioral effects in rats remains to be determined.

In contrast to the results in rats, chronic PB treatment did not have any effect on open field behaviors in mice. In both control and PB groups, there was a significant decline in the behavioral variables recorded when mice were repeatedly tested in the open field. This behavioral decline represents a normal physiological habituation to repeated testing. The lack of a PB behavioral effect may be related to the accommodation to the drug treatment (slow and accumulative infusion) as seen with the CV parameters. However, it may also be related to species and strain differences. For

example, the C57BL/6J mouse strain used in our study was found to be nonemotional and less fearful as compared to other strains (Van Gaalen and Steckler, 2000). Furthermore, other features of the laboratory environment—such as housing in isolation or in a group, and standard vs. enriched housing or handling procedure—may also affect the experimental results (Wahlsten, 2001).

Even though our results suggest that PB treatment does not affect CV or behavioral parameters in the laboratory environment, it is not possible to directly translate these results to the human condition. During the military deployment, the soldiers were treated with PB, but they were also exposed to other environmental conditions, such as, heat, physical and psychological stress, smoke, and chemical exposure. Investigators have postulated that it was the interaction of these various influences which led to the Gulf War Syndrome (Haley and Kurt, 1997; Haley et al., 1997). Recently, it has been shown that stress disrupted the blood–brain barrier and allowed PB penetration across the blood–brain barrier (Friedman et al., 1996) even though these results were not replicated by others investigators (Servatius et al., 2000; Kant et al., 2001). In rats, short-term treatment with PB (1.85 mg/kg twice daily for 4 days) induced a prolonged apoptotic response, which was evident in rat cortex up to 30 days after the last dose. These observations indicate that PB can initiate a prolonged neurodegeneration (Li et al., 2000). PB treatment in mice was also found to inhibit hypothalamic AChE activity and to decrease the peak intensity for hypothalamic peptide/protein profiles in mice (Ropp et al., 2002). Moreover, atypical genetic predisposition of some individuals resulting in the lack of BChE, a plasma scavenger of PB, may allow free PB to influence central cholinergic transmission (Loewenstein-Lichtenstein et al., 1995). Thus, the role of PB in Gulf War Syndrome is not satisfactorily understood and more experiments are needed to elucidate the cause of this illness.

Taken together, we believe that the finding of no CV or behavioral effects after chronic subcutaneous PB administration may have important clinical implications. Perhaps, chronic subcutaneous dosing may be a useful way of PB administration for the prophylactic treatment of military personnel. Development of cholinergic tolerance may provide protection against nerve gas poison. Moreover, it would eliminate the problems of underdosing as well as overdosing of soldiers. The methods of long-acting subcutaneous treatment in human have been developed and refined over the last 15 years, mainly as a part of conception control (Olsson et al., 1990; Pollanen et al., 2001). Similar methods could be developed for PB administration.

In conclusion, we tested the effect of chronic PB treatment on behavioral and CV parameters in mice. A comprehensive investigation using long-term blood pressure and HR monitoring, and behavioral evaluation revealed no changes in any parameters. This occurred concurrent with significant inhibition of blood ChE activity. The results suggest that prolonged low-dose PB treatment itself does not represent a significant risk factor.

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