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PERSISTENT/DELAYED TOXIC EFFECTS OF LOW-DOSE SARIN AND PYRIDOSTIGMINE UNDER PHYSICAL STRESS (EXERCISE) IN MICE

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Abstract : Pyridostigmine bromide, a reversible anticholinesterase drug, was used by military personnel during the Gulf War. They were under physical stress and might have been exposed to low-dose nerve gas, sarin. This study examined the interactions of low-dose sarin and pyridostigmine in exercised mice. Male NIH Swiss mice were treated as follows : 1) Control; 2) Sarin (0.01 mg/kg, sc); 3) exercise; 4) sarin plus exercise; 5) pyridostigmine; 6) pyridostigmine plus exercise; 7) pyridostigmine plus sarin; 8) pyridostigmine plus sarin plus exercise. Exercise was given daily for 10 weeks on treadmill and pyridostigmine and sarin were administered daily during the 5th and 6th weeks only. Respiratory exchange ratio decreased significantly during the dosing period of 5th and 6th weeks in groups 4, 6, and 8. Animals were sacrificed 24 hours after the ten-week exercise, tissues isolated and analyzed. Sarin significantly decreased butyrylcholine esterase (BChE) activity in plasma; AChE activity in platelet, triceps muscle, and striatum; neurotoxic esterase (NTE) activity in platelets, spinal cord, cortex and striatum and malondialdehyde (MDA) levels in sciatic nerve and cord. Sarin plus exercise significantly reduced BChE activity in plasma; acetylcholinesterase (AChE) activity in platelets, muscle, nerve and striatum; NTE activity in platelets, cord, cortex and striatum; and increased creatinine phosphokinase (CK) activity in plasma and MDA levels in cord. Pyridostigmine plus exercise significantly decrease BChE activity in plasma; AChE activity in muscle and enhanced malondialdehyde (MDA) levels in muscle. Pyridostigmine plus sarin significantly decreased NTE activity in platelets, cord, cortex and striatum. Pyridostigmine plus sarin plus exercise significantly altered AChE activity and MDA levels in muscle; and NTE activity in platelets, nerve, cord and cortex. Exercise significantly augmented the changes in plasma CK activity, muscle and nerve AChE activity, platelet NTE activity and cord MDA levels induced by sarin. It is concluded that physical stress (exercise) enhanced the persistent/delayed toxic effects of low-dose sarin and pyridostigmine in specific tissues of mice.

Key words : sarin, pyridostigmine, lipid peroxidation, cholinesterase, neurotoxic esterase, physical stress

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INTRODUCTION

Pyridostigmine is a reversible anticholinesterase drug most frequently used in the treatment of patients with myasthenia gravis and as a pretreatment drug against nerve gas exposure. Most of the U.S. and British troops took pyridostigmine for atleast two weeks as a pretreatment drug against possible exposure to nerve gases during the Gulf War (1, 2). Sarin (methylisopropyl phosphonofluoridate) is an organophosphorus nerve gas with a potent anticholinesterase property in humans (3). Sarin has been used as a chemical warfare agent since World War II and also during the Iran-Iraq conflict and the Gulf War (4, 5, 6). It has been reported that personnel exposed to low-dose sarin during its production in sarin manufacturing plants suffered neurological and psychiatric abnormalities even 5 to 10 years after their last exposures (7, 8, 9, 10). Neurological dysfunctions have also been reported 6–8 months after sarin exposure to humans in Japan (11). Exposure to nerve gases may produce acute cholinergic toxicity, which is related to inhibition of cholinesterase at neuromuscular junctions and the neuronal membrane and the accumulation of acetylcholine (12, 13). The persistent/ delayed neurotoxic effect induced by nerve agents is referred to as organophosphate-induced delayed neurotoxicity (OPIDN), which is due to inhibition and subsequent aging of enzyme neurotoxic esterase or neuropathy target esterase (NTE) in the neuronal membrane of the nervous system (14, 15). A minimum of 70% NTE inhibition after single exposure and 45% after multiple exposures to neuropathic organophosphorus compounds is required to initiate OPIDN (15, 16, 17, 18). Recent data show that NTE is comprised of two functional domains : an N-terminal regulatory domain and C-terminal effector domain (19). It is proposed that the N-terminal domain may bind a cyclic nucleotide and thereby modulate the activity of the C-terminal effector domain. The non-esterase function of NTE may be important for axonal maintenance and neuron-glia cells signalling. Platelet NTE has been demonstrated as a peripheral biochemical marker for exposure to neuropathic organophosphorus compounds (18, 20, 21). Physical stress has been known to influence the disposition and pharmacokinetics of drugs/chemicals and (22, 23) and also alter the cholinergic system (24). During exercise, blood flow is redistributed so that the active muscle tissue receives the greater cardiac output. Vasodilatation of arterioles occurs during exercise, thus there may be an increased uptake of the drug/agent by the muscle and it may get trapped and persist for a longer time. Recently, our laboratory has shown that physical stress enhanced the oxidative injury specifically to skeletal muscle of mice exposed to pyridostigmine (25). Although Gulf War veterans were exposed to pyridostigmine, or might have been exposed to low doses of sarin for weeks, during combat, the symptoms continued for months and years. It is hypothesized that under stressful conditions, sarin/pyridostigmine will cause delayed injury to specific tissues of mice. Therefore, this study was undertaken to elucidates the mechanism of interaction of low-dose sarin and pyridostigmine (for two weeks) with physical stress (ten weeks) on esterases, creatine phosphokinase, and lipid peroxidation in central and peripheral tissues of mice.

METHODS

Chemicals

Sarin was provided by US Army Institute of Chemical Defense, Aberdeen Proving Ground, MD. Pyridostigmine bromide, creatin phosphokinase kit, acetylthiocholine iodide, butyrylthiocholine iodide, dithiobisnitrobenzoic acid (DTNB), 1,1,1,1-tetraethoxypropane and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co., St. Louis, MO.

Animals

Adult male Swiss mice (Harlan Industries, Indianapolis, IN), (25–30 gm) were used in this study. They were kept in quarantine for one week and were fed *ad libitum* with Rodent Laboratory Chow (Ralston Purina Company, Indianpolis, IN) and tap water. Feed consisted of protein (23.4%), fat (4.5%) and was balanced with carbohydrates, fibers, vitamins and minerals. The animals were maintained on a 12 : 12 hr light : dark photo period. They were randomly divided into different groups and were treated as follows :

Experimental groups

Group I (Sedentary Control): For an equivalent handling, control mice were put on the treadmill at a low belt speed of 5 m/min for 5 min daily for 10 weeks. Mice received saline orally in the 5th and 6th weeks (n=15).

Group II (Sarin): Mice were put on the treadmill at a belt speed of 5 m/min for 5 min daily for a total of 10 weeks. They were administered sarin (0.01 mg/kg, sc [1/20 of LD_{50}] daily during the 5^{th} and 6^{th} weeks (n=15).

Group III (Exercise): Mice were exercised on the treadmill daily for 10 weeks as per the protocol shown in Table I. They were administered saline orally during the 5^{th} and 6^{th} weeks (n=15).

Group IV (Sarin plus Exercise): Mice exercised on the treadmill daily for 10 weeks as per exercise protocol shown in Table I. They were administered sarin (0.01 mg/kg, sc) daily during the 5^{th} and 6^{th} weeks (n=15).

Table I: Exercise training protocol for mice on treadmill.

Week	*Belt speed (m/min)*	*Inclination (degree)*	*Total time (min)*
1	12	0°	30
2	24	0°	30
3	30	8°	45
4	30	8°	45
5	30	8°	60
6	30	8°	60
7	30	8°	60
8	30	8°	60
9	30	8°	60
10	30	8°	60

Group V (Pyridostigmine): Mice were put on treadmill daily for 10 weeks. They were administered pyridostigmine (1.2 mg/kg, po) during the 5^{th} and 6^{th} weeks (n=15).

Group VI Pyridostigmine plus exercise): Mice were exercised on the treadmill daily for 10 weeks. They were administered pyridostigmine (1.2 mg/kg, po) during the 5^{th} and 6^{th} weeks (n=15).

Group VII (Pyridostigmine plus Sarin): Mice were put on treadmill daily for 10 weeks. They were administered pyridostigmine (1.2 mg/kg, po) followed by sarin (0.01 mg/kg, sc) daily during the 5^{th} and 6^{th} weeks (n=15).

Group VIII (Pyridostigmine plus Sarin plus Exercise): Mice were exercised on treadmill daily for 10 weeks. They were administered pyridostigmine (1.2 mg/kg, po) followed by sarin (0.01 mg/kg, sc) daily during the 5^{th} and 6^{th} weeks (n=15).

Exercise training

The Oxyscan System and Omni-Pacer Treadmill (Omnitech Inc., Columbus, OH) were used for exercising the mice as per protocol shown in Table I. The Oxyscan System determined the rate of oxygen consumption (VO_2, ml/kg/min), exchange ratio (VCO_2/VO_2), (RER) and heat production ΔH every 2.5 minutes. This ΔH is related to caloric expenditure (Kcal/kg/hr).

The five animals from each group were sacrificed 24 hr after the 10-week treatments for biochemical analysis. The remaining ten mice from each group were used for physiological and histological work for a separate study.

Sacrifice and tissue isolation

The animals were sacrificed by decapitation using decapicones (Braintree, MA) and blood was collected in heparinized vials. Plasma and RBC were separated by centrifugation at 2000 rpm for 10 min. The animals were dissected and the brain regions (cerebral cortex and corpus striatum), spinal cord, sciatic nerve and right leg triceps muscle were quickly isolated, rinsed in cold saline, immersed in liquid nitrogen and stored in a –80°C biofreezer.

Preparation of tissue extract

Cerebral cortex, corpus striatum, spinal cord, sciatic nerve and triceps muscle were homogenized in 0.5 M phosphate buffer pH 7.0 using motor driven glass homogenizer (Eberbach Corporation, Ann Arbor, MI). The homogenates (5%, w/v) were used to determine protein and enzyme activity.

Isolation of platelets

Blood was collected in tubes containing 0.1 M citrate buffer for pH 7.4 and was centrifuged at 600 rpm for 45 min. Platelet-rich plasma was transferred to another tube and was centrifuged at 2000 rpm for 30 min. The plasma was decanted and sedimented platelets were carefully resuspended in cold saline to wash out adhering blood proteins. The tube was centrifuged at 2500 rpm for 20 min and the saline was discarded. Platelets were lysed with saponin and diluted with 50 mM Tris-HCI/0.02 mM EDTA buffer of pH 8 (20).

Determination of protein in tissues

Protein concentrations in plasma, platelets and tissue homogenates were determined by the Coomassie reagent using serum bovine albumin as a standard (26).

Enzyme assays

Acetylcholinesterase

Enzyme activity was determined in tissues according to the modified method of Ellman et al (27). In a cuvette, 100 µl of tissue extract was added to 780 µl of 0.1 M phosphate buffer pH 8. One hundred µl of 0.01 M dithiobisnitrobenzoic acid (in 0.1 M phosphate buffer pH 7.0) was added to the cuvette. The reaction was started after the addition of 20 µl of acetylthiocholine (0.075 M) as a substrate, and optical density was recorded at 412 nm every 30 seconds, up to 4 minutes, using a Hitachi U-2000 spectrophotometer. The change in absorbance per minute and molar extinction coefficient for Thiocholine (1.36×10^4) and enzyme activity was calculated by a computerized enzyme kinetic program. The enzyme activity was expressed as µmoles of acetylthiocholine hydrolyzed/min/mg protein.

Butyrylcholinesterase

This enzyme activity in plasma was determined by the modified method of Ellman et al (27). In a cuvette containing 920 µl of 0.1 M phosphate buffer (pH 8), 10 µl of plasma and 50 µl of 0.01 M dithiobisnitrobenzoic acid (in 0.1 M phosphate buffer pH 7) was added. The reaction was started with the addition of 20 µl of 0.075 M butyrylthiocholine as substrate as substrate and optical density was recorded at 412 nm every 30 sec, up to 4 minutes The enzyme activity was calculated using the change in absorbance per minute and molar extinction coefficient of thiocholine (1.36×10^4) by a computerized enzyme kinetic program. The specific activity was expressed as µmoles of butyrylthiocholine hydrolyzed min/mg protein.

Neurotoxic esterase (NTE)

Enzyme activity was determined by the method of Johnson (28). In a paired tube (A and B) containing 940 µl and 920 µl of 50 mM Tris-EDTA buffer (pH 8), respectively, was added 10 µl of 4 mM paraoxon (in acetone and 10 µl of 4 mM paraoxon plus 10 µl of mM mipafox (in 5 mM Tris-citrate buffer, pH 6.0), respectively. In both tubes, 50 µl of tissue homogenate or platelet extract was added, vortexed and was incubated at 37°C for 20 min. The substrate 1 ml phenyl valerate prepared in dimethyl formamide (15 mg/ml) and 0.03% Triton X–100 (1:30 v/v) was added, mixed and incubated for 15 min. at 37°C. After incubation, the reaction was stopped by adding 500 µl of 0.5% potassium ferricyanide was added and the absorbance was measured spectrophotometrically at 510 nm. The activity was calculated by subtracting the absorbance of tube B from tube A. The enzyme activity was expressed as nmoles of phenyl valerate hydrolyzed/ min/mg protein.

Creatine phospho kinase (CK)

The enzyme activity in the plasma was determined by the spectrophotometric method using a commercial kit from Sigma (520–C). A tube containing 600 µl of 0.1 M Tris–buffer (pH 7.4), 200 µl phosphocreatine and 20 µl of ADP-glutathione reagent, received 10 µl of plasma and the tube was incubated for 30 min at 37°C. After

incubation, the reaction was stopped by adding 500 µl of 4N NaOH. Then 100 µl of 0.05 M p-hydroxy mercuribenzoate, followed by 100 µl of diacetyl and 100 µl of α-nephthol, was added to all tubes at room temperature. The tube was vortexed and color (pink) intensity was measured at 540 nm. The enzyme activity was expressed as µmoles of creatine formed/min/ml of plasma.

Lipid peroxidation

This assay was used to determine malondialdehyde (MDA) levels as described by Ohkawa et al (29). Two hundred µl of tissue homogenate was added to 50 µl of 8.1% sodium dodecyl sulfate (SDS), vortexed, and was incubated for 10 minutes at room temperature. Three hundred seventy five µl of 20% acetic acid (pH 3.5) and 375 µl of thiobarbituric acid (0.6%) were added and placed in a boiling water bath for 60 min. The samples were allowed to cool at room temperature, then 1.25 ml of butanol: pyridine (15:1) was added, vortexed and centrifuged at 1000 rpm for 5 minutes. Five hundred µl of the colored layer was measured at 532 nm using 1,1,3,3-teteraethoxy propane as a standard.

Statistical analysis

Values for RER were averaged per day and then per week for the ten-week period (n = 15) per group. Data analyses for RER were performed using a two-factor split-plot analysis of variance. The biochemical data were expressed as mean ± SEM (n = 5) and statistical analyses were performed using two-way analysis of variance (ANOVA) with Duncan's follow-up procedure using the SAS statistical software package (SAS Institute, Cary, NC). Statistical significance was set at the 5 percent level.

RESULTS

Respiratory exchange ratio (RER)

RER value significantly ($P<0.05$) decreased after sarin, pyridostigmine or pyridostigmine plus sarin dosing compared to the initial week in exercising mice (Fig. 1). Interestingly, the recovery in RER values to towards normal level were different in sarin plus exercise and pyridostigmine plus sarin plus exercise

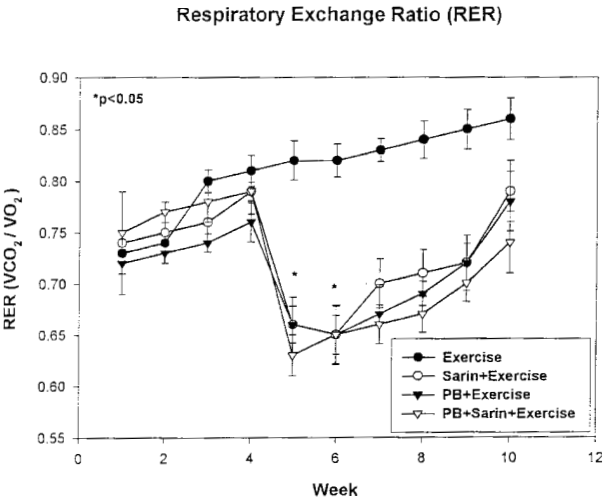

Fig. 1 : Changes in respiratory exchange ratio (RER) at different week in mice treated with sarin (0.01 mg/kg, sc [1/20 of LD_{50}]) plus exercise for ten weeks (-O-), pyridostigmine (1.2 mg/kg, po) plus exercise (-t---), and pyridostigmine plus sarin plus exercise for ten weeks (-∇-) and exercise for ten weeks (-•-). Sarin or pyridostigmine were administered during 5^{th} and 6^{th} weeks only. RER significantly decreased after sarin, pyridostigmine or pyridostigmine plus sarin dosing compared to initial week (*$P<0.05$) in exercising mice.

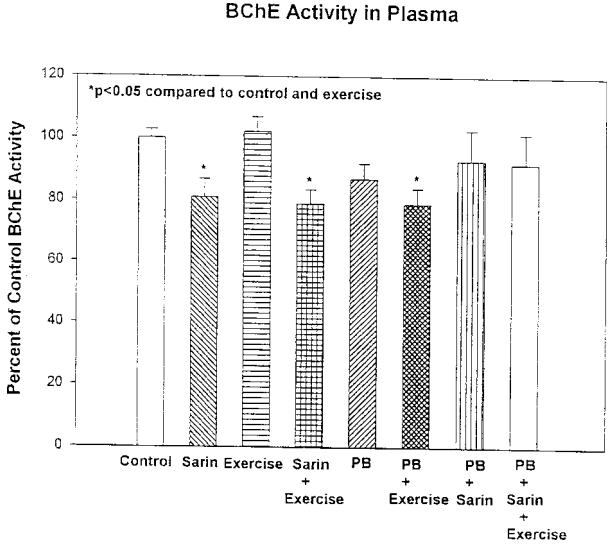

Fig. 2 : Effects of sarin (0.01 mg/kg, sc [1/20 of LD_{50}]), exercise for ten weeks, sarin plus exercise for ten weeks, pyridostigmine (1.2 mg/kg, po) pyridostigmine (PB) plus exercise, PB plus sarin and PB plus sarin plus exercise for ten weeks on plasma butyrylcholinesterase (BChE) activity in mice. BChE activity significantly decreased (*P<0.05) compared to control and exercise groups. Control plasma BChE activity was 537.05 ± 28.80 u moles of butyrylthiocholine hydrolyzed/min/mg protein.

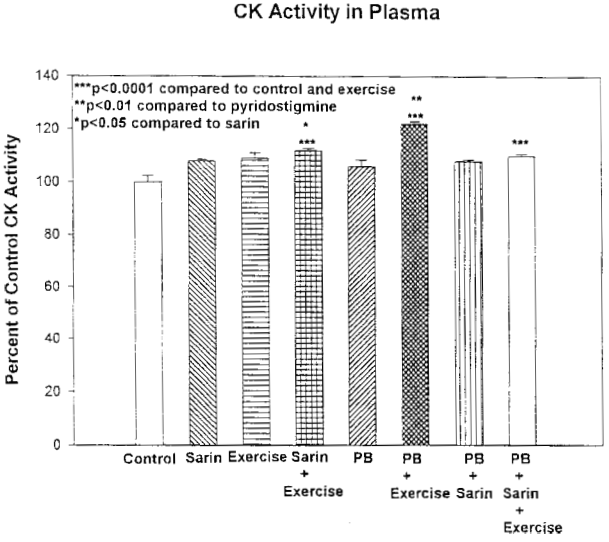

Fig. 3 : Effects of sarin (0.01 mg/kg, sc [1/20 of LD_{50}]), exercise for ten weeks, sarin plus exercise for ten weeks, PB (1.2 mg/kg, po), PB plus exercise, PB plus sarin, and pyridostigmine plus sarin plus exercise for ten weeks on plasma creatine phosphokinase (CK) activity in mice. CK activity significantly increased (***P<0.0001) compared to control and exercise groups, (**P<0.01) compared to control and exercise groups and (P<0.05) compared to sarin group. Control plasma CK activity was 22.92 ± 3.42 u moles of creatine formed/min/ml.

groups. The recovery in RER value was slower in pyridostigmine plus sarin plus exercise compared to sarin plus exercise group (Fig. 1).

Plasma butyrylcholinesterase (BChE) activity

BChE activity decreased (81, 79, 87, 79, 93 and 92% of control) in sarin, sarin plus exercise, pyridostigmine, pyridostigmine plus exercise, sarin plus pyridostigmine and sarin plus pyridostigmine plus exercise group, respectively (Fig. 2). The significant (P<0.05) depression of BChE activity was noticed in sarin plus exercise and pyridostigmine plus exercise groups as compared to control (Fig. 2).

Plasma creatine phosphokinase (CK) activity

CK activity in plasma increased (108, 112, 108, 122 and 110% of control) in sarin, sarin plus exercise, pyridostigmine plus exercise, sarin plus pyridostigmine and sarin plus pyridostigmine plus exercise group, respectively (Feg. 3). The significant (P<0.0001) elevation of plasma CK was noticed in sarin plus exercise and sarin plus pyridostigmine plus exercise groups compared to control. A significant (P<0.05) increase of plasma CK activity in sarin plus exercise and in pyridostigmine plus exercise group (P<0.01) was noted when compared to sarin and pyridostigmine alone groups (Fig. 3).

TABLE II : Effects of sarin (0.01 mg/kg, sc [1/20 of LD_{50}]), exercise, sarin plus exercise, pyridostigmine (1.2 mg/kg, po), pyridostigmine plus exercise, pyridostigmine (1.2 mg/kg, po) plus sarin, and pyridostigmine plus sarin plus exercise for ten weeks on acetylcholinesterase (AChE) activity in peripheral and central tissues of mice. Sarin and pyridostigmine were administered daily during 5th and 6th week. Each value represents mean ± SEM (n=5).

Group	*Acetylcholinesterase activity**						
	RBC	*Platelets*	*Triceps muscle*	*Sciatic nerve*	*Spinal cord*	*Cerebral cortex*	*Corpus striatum*
Control	4.94±0.44	10.98±0.87	12.47±0.88	16.30±1.60	67.06±3.06	82.15±7.05	169.92±7.00
Sarin	4.31±0.20	7.77±0.89b	8.51±1.34c	14.42±1.71	59.69±5.72	61.84±9.45	107.35±5.06d
Exercise	5.10±0.51	10.02±2.01	13.20±1.25	15.80±2.02	73.70±8.70	85.22±8.50	170.40±7.52
Sarin+exercise	4.00±0.46	6.33±1.12b	6.94±1.33c,e	12.37±1.33a,e	50.64±8.96	60.94±7.61	106.94±9.59b
Pyridostigmine	4.58±0.26	9.74±1.85	11.88±1.44	15.33±2.12	70.77±2.11	81.85±4.31	169.72±7.50
Pyridostigmine+exercise	4.87±0.65	9.47±1.65	9.73±0.40a	15.11±2.13	66.83±6.50	78.63±8.24	165.15±8.24
Pyridostigmine+sarin	4.94±0.47	10.62±1.01	11.08±1.23	18.98±1.44	64.60±4.87	77.67±3.54	154.30±7.93
Pyridostigmine+ sarin+exercise	4.39±0.18	9.03±0.37	8.95±0.40c	16.34±1.73	56.24±9.07	75.31±6.38	111.22±5.49d

*Activity is expressed as umoles of acetylthiocholine hydrolyzed/min/mg tissue protein.
a=P<0.05 compared to Groups 1, 3, 7 and 8; b=P<0.008 compared to Groups 1 and 3; c=P<0.004 compared to Groups 1 and 3; d=P<0.0001 compared to Groups 1 and 3; e=P<0.05 compared to Group 2.

Acetylcholinestease (AChE) activity

A significant (P<0.05) inhibition of AChE activity was observed in the sarin plus exercise group compared to sarin alone group, indicating the interaction of sarin and physical stress (Table II). The significant (P<0.004) inhibition of muscle AChE activity was observed in sarin, sarin plus exercise and sarin plus pyridostigmine plus exercise groups and in pyridostigmine plus exercise group (P<0.01) compared to control. The significant (P<0.05) decrease in sciatic nerve AChE activity was observed in sarin plus exercise group compared to sarin alone group. The significant (P<0.05) decrease in sciatic nerve AChE activity was observed in sarin plus exercise group compared to sarin alone group. The significant (P<0.001) decrease in AChE activity observed in corpus striatum of

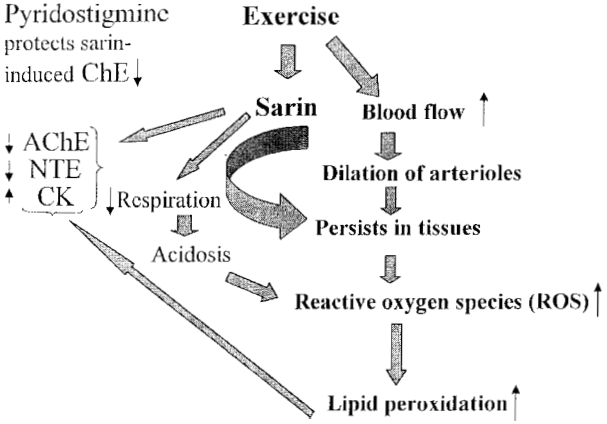

Fig. 4 : Influence of exercise on possible pathways of sarin and pyridostigmine-induced delayed/ persistent toxic effects in tissues of mice. Exercise enhances the blood flow, dilates arterioles leading to persistence of sarin and pyridostigmine in tissues, specifically in muscle. Exercise also enhances sarin-induced hypoxia, acidosis leading to excess ROS generation. ROS-induced membrane lipid peroxidation leads to decline in AChE and NTE activity and enhances in CK activity. Pyridostigmine protected the decline in cholinesterase activity caused by sarin.

TABLE III: Effects of low-dose sarin (0.01 mg/kg, sc [1/20 of LD_{50}]), exercise, sarin plus exercise, pyridostigmine (1.2 mg/kg, po), pyridostigmine plus exercise, pyridostigmine (1.2 mg/kg, po) plus sarin, and pyridostigmine plus sarin plus exercise for ten weeks on neurotoxic esterase (NTE) activity in tissues of mice. Sarin and pyridostigmine were administered daily during 5th and 6th week. Each value represents mean ± SEM (n=5).

Group	*Neurotoxic esterase (NTE) activity**				
	Platelets	*Sciatic nerve*	*Spinal cord*	*Cortex*	*Striatum*
Control	1.22±0.22	0.80±0.09	2.07±0.18	4.45±0.41	2.85±0.23
Sarin	0.66±0.03c	0.66±0.04	1.55±0.13b	2.82±0.15c	2.15±0.21a
Exercise	1.25±0.22	0.88±0.11	2.14±0.23	4.65±0.42	2.83±0.27
Sarin+exercise	0.51±0.02c,d	0.63±0.04	1.43±0.05b	2.60±0.14c	2.06±0.13a
Pyridostigmine	1.35±0.15	0.83±0.10	2.17±0.21	4.35±0.31	2.92±0.21
Pyridostigmine+exercise	1.21±0.12	0.79±0.09	2.06±0.12	4.25±0.42	2.84±0.25
Pyridostigmine+sarin	0.63±0.10c	0.69±0.07	1.49±0.09b	3.10±0.25c	2.22±0.14a
Pyridostigmine+sarin+exercise	0.59±0.07c	0.62±0.07	1.41±0.21b	3.10±0.22c	2.23±0.14a

*Enzyme activity was expressed as n moles of phenyl valerate hydrolyzed/min/mg tissue protein.
a=P<0.05 compared to Groups 1 and 3; b=P<0.003 compared to Groups 1 and 3; c=P<0.001 compared to Groups 1 and 3; d=P<0.001 compared to Group 2.

mice in sarin, sarin plus exercise and sarin plus pyridostigmine plus exercise groups (Table II).

Neurotoxic esterase (NTE)

Platelets NTE activity significantly (P<0.0001) decreased (55, 42, 52 and 49% of control) in sarin, sarin plus exercise, sarin plus phridostigmine, and sarin plus pyridostigmine plus exercise group, respectively (Table III). Exercise significantly (P<0.05) enhanced the changes in platelet NTE activity induced by sarin. NTE activity significantly decreased (82, 79, 86 and 77% of control) in sciatic nerve; (75, 69, 72 and 68% of control in spinal cord (P<0.003); (63, 58, 70 and 70% of control) in cerebral cortex (P<0.0001) and (75, 72, 78 and 78% of control) in corpus striatum (P<0.05) of mice in sarin, sarin plus exercise, sarin plus pyridostigmine and sarin plus pyridostigmine plus exercise group, respectively (Table III).

Lipid peroxidation end product (MDA) concentrations

MDA concentration significantly enhanced (110, 126 (P<0.05), 121 (P<0.05), 106 and 125% (P<0.05) of control) in triceps muscle and (122 (P<0.05), 114 (P<0.05), 103, 116 and 109% of control) in sciatic nerve of mice in sarin, sarin plus exercise, pyridostigmine plus exercise, sarin plus pyridostigmine and sarin plus pyridostigmine plus exercise group, respectively (Table IV). MDA concentration significantly increased (126 (P<0.008), 138 (P<0.008), 103 and 112% of control) in spinal cord of mice in sarin, sarin plus exercise, sarin plius pyridostigmine and sarin plus

TABLE IV : Effects of low-dose sarin (0.01 mg/kg, sc [1/20 of LD_{50}]), exercise, sarin plus exercise, pyridostigmine (1.2 mg/kg, po), pyridostigmine plus exercise, pyridostigmine plus sarin and pyridostigmine plus sarin plus exercise for ten weeks on lipid peroxidation end product, malonidialdehyde (MDA) concentration in peripheral and central tissues of mice. Sarin and pyridostigmine were administered daily during 5th and 6th week. Each value represents mean ± SEM (n=5).

Group	*MDA concentration (n moles/mg protein)*				
	Triceps muscle	*Sciatic nerve*	*Spinal cord*	*Cerebral Cortex*	*Corpus Striatum*
Control	7.75±0.66	6.72±0.43	17.67±1.27	18.35±0.81	19.55±1.03
Sarin	8.49±0.37	8.21±0.22a	22.35±0.60b	21.63±1.93	22.07±2.07
Exercise	7.85±0.61	6.88±0.23	17.69±1.14	18.05±0.92	20.21±1.05
Sarin+exercise	9.73±0.91a	7.64±0.48a	24.26±0.83b,c	22.24±1.10	22.79±1.77
Pyridostigmine	7.92±0.48	6.78±0.32	17.33±1.23	19.20±1.12	20.15±0.83
Pyridostigmine+exercise	9.61±0.39a	6.91±0.25	17.83±1.23	19.35±1.20	20.28±1.22
Pyridostigmine+sarin	8.22±0.5	7.79±0.45	18.22±1.17	19.65±1.25	20.46±1.16
Pyridostigmine+sarin+exercise	9.72±0.38a	7.32±0.46	19.76±1.50	20.83±1.82	21.67±1.54

a=significant $P<0.05$ compared to Groups 1 and 3; b=significant $P<0.008$ compared to Groups 1 and 3; c=$P<0.05$ compared to Group 2.

pyridostigmine plus exercise group, respectively. A significant elevation ($P<0.05$) of MDA in spinal cord of animals in sarin plus exercise group, respectively. A significant elevation ($P<0.05$) of MDA in spinal cord of animals in sarin plus exercise group compared to sarin alone group (Table IV).

DISCUSSION

We consider that this is the first report that has systematically analyzed the effects of anticholinesterase and exercise on oxygen inhalation and carbon dioxide exhalation in a mice model. Previous studies from our laboratory have shown a significant increase in oxygen consumption, respiratory exchange ratio and heat production after 8–10 weeks exercise in rats (30, 31, 32, 33). Exercising mice had significant lower RER values after pyridostigmine bromide or sarin doses compared to exercised controls during the 5^{th} and 6^{th} weeks. This indicated that the mice had greater respiratory problems due to pyridostigmine and sarin alongwith exercise. It is possible that the differences in RER values between exercise and sarin plus exercise and pyridostigmine plus sarin plus exercise could reflect a lower elimination of carbon dioxide via respiration due to the effect of sarin during treadmill exercise. Lower carbon dioxide loss is normally reflected as the RER values decrease. However, it has been reported that performance of soldiers did not decrease due to Pyridostigmine intake (1). The decreased RER due to sarin plus exercise, pyridostigmine plus exercise and pyridostigmine plus exercise group may be explained due to alterations in lung mechanics. A previous study in rats has shown that sarin exposure depresses the respiratory rate, airflow and minute volume and enhances the transthoracic pressure and tidal volume (34). Interestingly, the recovery of RER in sarin plus exercise and pyridostigmine plus sarin plus exercise

group is different indicating the interaction of pyridostigmine and sarin on the pulmonary mechanics of mice.

Low-dose sarin (1/20 of LD 50) significantly inhibited BCDhE activity in plasma as well as AChE activity in platelet 4 weeks after the last dose suggests the persistent/delayed effects of sarin on blood cholinesterase activity significantly 4 days after the last dose of sarin in mice (35). Data of the present study show that pretreatment with pyridostigmine protected the cholinesterase in the blood. However, physical stress enhanced sarin and pyridostigmine-induced depression of cholinesterase activity in mice. An increase in plasma volume due to exercise and a decrease in hepatic blood flow may be responsible for decline in enzyme activity. Data on muscle and nerve AChE activity demonstrate that physical stress amplifies the persistent/delayed effects of sarin and pyridostigmine exposure on the neuromuscular system of the mice. A previous study has shown that exercise increases the severity of myonecrosis caused by irreversible cholinesterase inhibitors (36). Muscle fatigability is also caused by AChE inhibition in rats (37). The data of the present study also demonstrate an enhanced activity of plasma CK in mice treated with sarin plus exercise, pyridostigmine plus exercise and pyridostigmine plus sarin plus exercise. Thus, physical stress seems to be one of the factors that may be responsible for enhancing the effects of sarin and pyridostigmine on the skeletal muscle. The data further demonstrate the ability of sarin (a lipid soluble organophosphorus compound) to penetrate into deeper area of the brain (striatum) and interact with AChE. This area of the brain contains highest AChE activity and controls motor functions (38). Even a very small change in AChE activity can be detected in this brain region (39). The present data suggest that exercise does not influence sarin-induced changes in striatal AChE activity.

This is the first report to demonstrate the interaction of exercise and cholinesterase inhibitors on tissue NTE activity in mice. The data show that sarin significantly inhibited NTE activity in platelets, spinal cord, cerebral cortex and corpus striatum. But the NTE inhibition was close to the threshold levels in platelets and was below the threshold levels in CNS. A minimum of 70% NTE inhibition after a single exposure and 45% after multiple exposures to neuropathic organophosphorus compounds, and subsequent aging of NTE, is the prerequisite for the development of OPIDN (16, 17, 40, 41). The data further indicate that NTE inhibition was enhanced in sarin plus exercise group compared to sarin alone in platelets. It is suggested that exercise seems to increase the susceptibility of mice to the inhibition of NTE thereby enhancing the delayed neurotoxicity of sarin. Previous studies have shown that sarin inhibits NTE activity in platelets and induces delayed neurotoxicity in hens (41) as well as in mice (35, 40) at low-dose repeated subcutaneous and inhalation exposures, respectively. However, studies with a single high dose of sarin in protected hens also showed significant NTE inhibition in the brain but OPIDN symptoms were not observed (42, 43). Crowell et al (43) have reported that sarin inhibited NTE activity (60% of control) in lymphocytes but less inhibition was observed in the brain of hen

24 hr after sarin dosing. Pyridostigmine pretreatment did not protect NTE in platelets as observed in pyridostigmine plus sarin and pyridostigmine plus sarin plus exercise groups. Previous studies have suggested the lymphocyte NTE inhibition as a marker for risk assessment for OPIDN in experimental animals (44, 45, 46, 47), but many problems have been encountered (20, 48, 49, 50, 51, 52, 53). Later platelet NTE as a peripheral biochemical marker has been proposed and established (20, 21, 41, 54, 55). Platelet NTE is a food peripheral models for central neurons and can be reliably measured in humans as well as in experimental animals for monitoring exposure risk to neuropathic organophosphorus compounds.

During exercise, the arterioles dilate, and this might enhance the entry of pyridostigmine, a quaternary ammonium compounds, into the tissue compartment causing alteration in enzyme activity (23). Exercise is likely to enhance the uptake of sarin in the tissues. Exercise increase the oxygen species in tissues and exerts oxidative stress response (32, 33, 56). Reports from our laboratory have demonstrated that exercise and ethanol interaction resulted in a decrease in AChE activity that was correlated with enhanced lipid peroxidation in tissues of rats (31, 57). Data show that physical stress further increased membrane lipid peroxidation (MDA) levels in the spinal cord and triceps muscle of mice exposed to sarin and pyridostigmine indicating oxidative tissue injury. Exposures to cholinesterase inhibitors have been known to alter the respiration, oxygen consumption and catabolic effects in central and peripheral tissues (60, 61, 62, 63) leading to oxidative damage.

Based on these findings, Fig. 4 illustrates the possible mechanism of persistent/delayed effects due to interaction of low dose sarin and pyridostigmine in exercised mice. The changes in tissue respiration and oxygen consumption following exercise and/or following exposure to anticholinesterase agents may enhance the influx of reactive oxygen species. These reactive oxygen species can initiate lipid peroxidation leading to membrane damage. Thus, the interaction of sarin/ pyridostigmine and exercise result in peroxidative tissue injury in mice. Exercise enhances the blood flow, dilates arterioles leading to persistence of sarin and pyridostigmine in tissues, specifically in the muscle. Exercise also increases sarin-induced hypoxia and acidosis leading to increased ROS generation. ROS-induced membrane lipid peroxidation leads to a decrease in AChE and NTE activity and increase in CK activity resulting in enhanced toxic effects. It is concluded that exercise augmented the persistent/ delayed toxic effects of low-dose sarin and pyridostigmine in specific tissues of mice.

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REFERENCES

1. Keeler JR, Hurst CG, Dunn MA. Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* 1991; 266: 693–695.
2. Dunn MA, Sidell FR. Progress in medical defense against nerve agents. *JAMA* 1989; 262: 649–652.
3. Grob D, Harvey JC. Effect in man of the anticholinesterase compound sarin. *J Clin Invest* 1958; 73: 350–357.
4. Ivarsson U, Nilsson H, H Santesson J (Eds). *Chemical Warfare Agents Today,* Vol. 16 : Chemical weapons-Threat, effects and protection. Ljungforetagen Press, Orebro, Sweden, 1992.
5. Defense Science Board : Report of the Defense Science Board Task Force on Persian Gulf War health effects. Washington, DC : Office of the Under-Secretary of Defense for Acquisition and Technology, 1994.
6. Committee on Banking, Housing and Urban Affairs, US Senate (1994). US Chemical and Biological Warfare-Related Dual Use Exports to Iraq and Their Possible Impact on the Health Consequences of the Persian Gulf War. Washington, DC : US Senate.
7. Spiegelberg U. Psychopathologisch-neurologisch Shaden nach Einwirkung synthetischer Gifte. In : *Wehrdienst and Gesundheit*, Vol. III. Darmastd, Germany : Wehrund Wissen Verlagsgesellschaft mbH, 1961.
8. Stockholm International Peace Research Institute. Delayed toxic effects of chemical warfare agents : A Stockholm International Peace Research Institute Monograph, Almquist and Wiskell International, New York, 1975.
9. Sidell FR, Soman and Sarin. Clinical manifestations and treatment of accidental poisoning by organophosphates. *Clin Toxicol* 1974; 7: 1–17.
10. Duffy FH, Burchfield JL, Bartels PH, Gaon M, Sim VM. Long term effects of an organophosphate upon the human electrocephalogram. *Toxicol Appl Pharmacol* 1979; 47: 161–176.
11. Yokoyama K, Araki S, Murata K, Nishikitani M, Okumura T, Ishimatsu S, Takasu N. A preliminary study on delayed vestibulocerebellar effects of Tokyo subway sarin poisoning in relation to gender difference : Frequency analysis of postural sway. *J Occupational Environ Med* 1998; 40: 17–21.
12. Holmstedt B. Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacol Rev* 1959; 11: 567–688.
13. Marrs TC, Maynard RL, Sidell FR. Organophosphorus nerve agents. In : *Chemical Warfare Agents–Toxicology and Treatment.* Wiley and Sons, New York, 1996; pp.83–100.
14. Johnson MK. The delayed neuropathy caused by some organophosphorus esters : mechanism and challenge. *Crit Rev Toxicol* 1975; 3: 289–316.
15. Abou-Donia MB, Lapadula DM. Mechanisms of organophosphorus ester-induced delayed neurotoxicity : type I and type II. *Ann Rev Pharmacol Toxicol* 1990; 30: 405–440.
16. Lotti M, Johnson MK. Repeated small doses of a neurotoxic organophosphate : Monitoring of neurotoxic esterase in brain and spinal cord. *Arch Toxicol* 1980; 45: 263–271.
17. Johnson MK. The target for the initiation of delayed neurotoxicity by organophosphorus esters : biochemical studies and neurotoxicological applications. *Rev Biochem Toxicol* 1982; 4: 141–212.
18. Husain K. Neurotoxicesterase. *Asia Pac J Pharmacol* 1994a; 9: 119–128.
19. Glynn P. Neuropathy target esterase. *Biochem J* 1999; 3: 625–631.
20. Husain K. Peripheral biochemical marker for organophosphate induced delayed neurotoxicity. *Biochem In* 1991; 24: 1051–1056.
21. Husain K. Phenyl valerate and cholinester hydrolases in the platelets of human, hen, rat and mouse. *Hum Exp Toxicol* 1994b; 13: 157–159.
22. Somani SM, Kamemori GH. Exercise and absorption, distribution, metabolism, excretion and pharmacokinetics of drugs and chemicals. In : *Pharmacology in Exercise and Sport* Ed.: SM Somani. CRC Press, Inc., Boca Raton, FL, 1996; pp.1–38.
23. Somani SM, Husain K, Jagannathan R. Pharmacokinetics and pharmacodynamics of carbamate under stressful conditions. *In Chemical Warfare Agents : Toxicity at Low Level.* Eds.: SM Somani and J Romano. CRC Press, Florida, 2001; pp.145–189.
24. Somani SM, Husain K. Exercise, drugs and cholinergic system. In : *Pharmacology in Exercise*

and Sports. Ed.: SM Somani. CRC Press, Florida, 1996a; pp.263–288.

25. Jagannathan R, Husain K, Somani M. Interaction of pyridostigmine and physical stress on antioxidant defense system in skeletal muscle of mice. *J Appl Toxicol* 2001; 21: 341–348.

26. Read SM, Northcole DH. Minimization of variation in the response to different protein of the Coomassie blue G dye-binding assay for protein. *Anal Biochem* 1981; 116: 53–64.

27. Ellman GL, Courtney KD, Andre VJ, Featherstone RM. A new and colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; 7: 88–95.

28. Johnson MK. Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch Toxicol* 1977; 37: 113–115.

29. Ohkawa H, Ohisi N, Yagi K. Assay for lipid peroxides in animals and tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351–358.

30. Somani SM, Husain K. Interaction of exercise training and chronic ethanol ingestion on antioxidant system of rat brain regions. *J Appl Toxicol* 1997; 17: 329–336.

31. Husain K, Somani SM. Influence of exercise and ethanol on cholinesterase activity and lipid peroxidation in blood and brain region of rat. *Prog Neuro-Psychopharmacol Biol Psychiat* 1997a; 21: 659–670.

32. Husain K, Somani SM. Interaction of exercise training and chronic ethanol ingestion on hepatic and plasma antioxidant system in rat. *J Appl Toxicol* 1997b; 17: 189–194.

33. Somani SM, Husain K. Exercise training alters kinetics of antioxidant enzyme in rat tissues. *Biochem Mol Biol Int* 1996b; 38: 587–595.

34. Husain K, Kumar P, Vijayaraghavan R, Singh R, Das Gupta S. Influence of pretreatment of carbamates on dynamic pulmonary mechanism in rats exposed to sarin aerosols. *Indian J Physiol Pharmacol* 1993; 37: 49–51.

35. Husain K, Vijayaraghavan R, Pant SC, Raza SK, Pandey KS. Delayed neurotoxic effect of sarin in mice after repeated inhalation exposure. *J Appl Toxicol* 1993b; 13: 143–145.

36. Fenichel GM, Kibler WB, Dettborn WD. The effect of immobilization and exercise on acetylcholine-mediated myopathies. *Neurology* 1974; 24: 1086–1090.

37. Panenic R, Gisiger V, Gardiner PF. Fatigability of rat hind limb muscle after acute irreversible acetylcholinesterase inhibition. *J Appl Physiol* 1999; 87: 1455–1462.

38. Matin MA, Husain K. Striatal neurochemical changes and motor dysfunction in mipafox treated animals. *Meth Find Exptl Clin Pharmacol* 1985; 7: 79–81.

39. Husain K, Vijayaraghavan R. DFP induced changes in acetylcholinesterase activity and glucogen level in certain brain regions of mice. *Indian J Physiol Pharmacol* 1989; 33: 250–252.

40. Husain K, Pant SC, Vijayaraghavan R, Singh R. Assessing delayed neurotoxicity in rodents after nerve gas exposure. *Def Sci J* 1994; 44: 161–164.

41. Husain K, Pant SC, Raza SK, Singh R, Das Gupta S. A comparative study of delayed neurotoxicity in hens following repeated administration of organophosphorus compounds. *Indian J Physiol Pharmacol* 1995; 39: 47–50.

42. Gordon JJ, Inns RH, Johnson MK, Leadbeater L, Maidment MP, Upshall DG, Cooper GH, Rickard RI. The delayed neuropathic effects of nerve agents and some other organophosphorus compounds. *Arch Toxicol* 1983; 52: 71–82.

43. Crowell JA, Parker RM, Bucci RJ, Dacre JC. Neuropathy target esterase in hen after soman and sarin. *J Biochem Toxicol* 1989; 4: 14–20.

44. Dudek BR, Barth M, Gephart L, Huggins J, Richardson RJ. Correlation of brain and lymphocyte neurotoxic esterase inhibition in the adult hen following dosing with neurotoxic compounds. *Toxicol Appl Pharmacol* 1979; 48: A198.

45. Dudek BR, Richardson RJ. Evidence for the existence of neurotoxic esterase in neural and lymphatic tissues of adult hen. *Biochem Pharmacol* 1982; 31: 1117–1121.

46. Lotti M, Becher CE, Aminoff MJ, Woodrow JE, Siber JM, Talcott R, Richardson RJ. Occupational exposure to cotton defoliants DE and merphos. A rational approach to monitoring OP-induced delayed neurotoxicity. *J Occup Med* 1983; 25: 517–520.

47. Bertocin D, Russolo A, Caroldi S, Lotti M. Neuropathy target esterase in human lymphocytes. *Arch Environ Health* 1985; 40: 139–144.

48. Richardson RJ, Dudek BR. Neurotoxic esterase : Characterization and potential for a predictive screen for exposure to neuropathic organophosphates. In : *Pest Chem Hum Welfare and Environment* Eds.:

J Miyamoto and PC Kearney. Pergamon Press, Oxford, 1983; pp.491–496.

49. Lotti M. Biological monitoring for organophosphate-induced delayed neuropathy. *Toxicol Letts* 1986; 33: 167–170.

50. Schwab BW, Richardson RJ. Lymphocyte and brain neurotoxic esterase : Dose and time dependence of inhibition in the hen examined with three organophosphorus esters. *Toxical Appl Pharmacol* 1986; 83: 1–9.

51. Lotti M. Organophosphate-induced delayed polyeuropathy on humans : Perspectives for biomonitoring. *Trends Pharmacol Sci* 1987; 8: 175–180.

52. Farage-Elawar M, Magnus-Francis B. The effect of three organophosphorus esters on brain and blood neurotoxic esterase and acetylcholinesterase. *Pest Biochem Physiol* 1988; 31: 175–1181.

53. Lotti M, Moretto A, Zopellari R, Dainese R, Rizzuto N, Barusco GA. Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced delayed polyneuropathy. *Arch Toxicol* 1990; 59: 176–182.

54. Bleecker ML, Maroni M. Neurotoxic esterase activity in human platelets, lymphocytes and monocytes. *Toxicol Letts* 1983; 18: 23–24.

55. Maroni M, Bleecker ML. Neuropathy target esterase in human lymphocytes and platelets. *J Appl Toxicol* 1986; 6: 1–7.

56. Davies JJA, Qintanilla AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* 1982; 107: 1998–1205.

57. Husain K, Somani SM. Effect of exercise training and chronic ethanol ingestion on cholinesterase activity and lipid peroxidation in blood and brain regions of rat. *Prog Neuro-Psychopharmacol Biol Psychiat* 1998; 22: 411–423.

58. Ott P. Membrane acetylcholinesterases : Purification, molecular properties and interactions with amphiphilic environments. *Biochem Biophys Acta* 1985; 822: 375–392.

59. Babu SR, Somani SM, Dube SN. Effect of physostigmine and exercise on choline acetyltransferase activities in fast and slow muscles of rat. *Pharmacol Biochem Behav* 1993; 45: 713–717.

60. Adams GK, Yamamura HI, O'Leary JF. Recovery of central respiratory functions following anticholinesterase intoxication. *Eur J Pharmacol* 1976; 38: 101–112.

61. Rickett DL, Glenn JF, Beers ET. Central respiratory effects vs. neuromuscular actions of nerve agents. *Neurotoxicol* 1986; 7: 225–236.

62. Matin MA, Husain K. The effects of certain drugs on glycogen and acetylcholine levels in cerebral and peripheral tissues of rats with malathion-induced hyperglycaemia. *Arch Ind Hyg Toxicol* 1984; 35: 325–332.

63. Gupta RC, Patterson GT, Dettbarn WD. Acute tabun toxicity : biochemical and histochemical consequences in brain and skeletal muscle of rats. *Toxicol* 1987; 46: 329–341.