Journal of Life Sciences Research
ISSN : 2408-9184
Vol. 1, No. 1, 10-15, 2014
http://asianonlinejournals.com/index.php/Lifsc/index

Effect of Cold Stress on Pyridostigmine Pretreated Rats Exposed to an Organophosphorous Compound

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Abstract

Context: Pyridostigmine bromide (PB) is a quaternary ammonium compound and has been approved as a pretreatment drug against toxic organophosphorous (OP) compounds. The stressful demands of modern military activity include a broad range of activities at extreme cold temperatures along with various physical activities.
Objective: The effect of "sign free" dose of PB (0.075 mg/kg body weight) against a toxic OP compound diisopropyl fluorophosphate (DFP) was reassessed in rats. Electrocardiographic (ECG) studies in hypothermic and pretreatment conditions were undertaken to assess the cardioprotective role of PB. Total Antioxidant Status (TAS) was quantified to assess the degree of oxidative stress imposed under such conditions. Possible protective role of pyridostigmine in rat lymphocytes was also determined.
Materials& Methods: TAS was estimated spectrophotometrically and the expression of interferon-γ (IFNγ) was measured by Fluorescence Activated Cell Sorting. ECG was monitored by standard protocol.
Results: ECG recording showed that the PR and QT interval progressively increased along with widening of QRS complex. There was a progressive fall in heart rate as the body temperature decreased. TAS significantly decreased ($p \leq 0.001$) in hypothermic conditions and when pretreated with sign free dose of PB before cold induction ($p \leq 0.001$). Following immunostaining of lymphocytes by FITC conjugated mouse anti-rat IFNγ monoclonal antibody, 9.1% of lipopolysaccharide elicited parent cells showed positive IFNγ expression. Hypothermic stress inhibited IFNγ expression (3.6% of parent cells) which was recovered to 6.8% upon pre-treatment with sign-free dose of pyridostigmine.
Conclusion: This study is indicative of a possible protective role of PB against hypothermic stress.

Keywords: Pyridostigmine, DFP, hypothermia, IFN-γ.

1. Introduction

Organophosphorus (OP) compounds are used as pesticides, petroleum additives and chemical warfare agents [1]. OP pesticide poisoning is an important clinical problem in rural areas. Treatment against OP intoxication involves administration of a cholinolytic, atropine and an enzyme reactivator oxime [2]. Carbamates are also used as pesticides. Pyridostigmine Bromide (PB) (Mestinon) is a quarternary ammonium compound and a reversible anticholinesterase drug used for treatment in patients with myasthenia gravis. Koelle [3] first proposed the protection of this compound against irreversible inactivation by diisopropylfluorophosphate (DFP) in vitro. Dasgupta, et al. [4] reported the beneficial effect of carbamates against DFP poisoning in rats using atropine as cholinolytic and diazepam as an adjunct. Later study reported different drug combination consisting of a cholinolytic and a cholinesterase reactivator which provided greater therapeutic efficacy in acute OP poisoning in mice [5]. PB has now been registered as a pretreatment drug for giving protection to the soldiers in the event of nerve gas exposure [6].

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The OP compounds are highly toxic irreversible inhibitors of acetylcholinesterase enzyme and cause a variety of physiological effects. Cardiotoxic effects and dose dependent transient decrease of blood pressure and heart rate after subcutaneous administration of DFP in rats have been reported [7]. Earlier, Husain, et al. [8] reported that DFP significantly increased the level of blood glucose and depleted glycogen level in the brain, liver and diaphragm.

Stress is an important factor for military personnel when he is posted at high altitude and experience cold stress as the average ambient temperature is -25°C. This cold-induced stress may enhance the symptoms of OP toxicity as reported by many authors [9, 10].

Hypothermia is a common sign in animals which are exposed to chemical warfare agents (CWA), presumably due to cholinergic activation of the hypothalamic thermoregulatory centre. Honkakoski, et al. [11] observed differences in the toxicity of DFP in inhibition of tissue acetylcholinesterase in experimental animals subjected to a cold environment. It was widely used during the Persian Gulf War but many war veterans complained of various side-effects although the dose given was symptom free. These symptoms are likely to be potentiated by other stress factors like physical exercise, working in extreme hot and cold climates [12].

The rationale for the present investigation was to study the interaction of hypothermia induced stress influencing the physicochemical dynamics of rats against the toxic OP compound – DFP when pretreated with PB. Electrocardiographic (ECG) studies in such conditions were undertaken to assess the cardioprotective role of PB and also to monitor onset of ventricular fibrillation during surface cooling. Total Antioxidant Status (TAS) was estimated to analyze the degree of oxidative stress that is imposed in such multiple stress situations. Possible protective role of pyridostigmine in rat lymphocytes was also determined.

Leon [13] reported that cytokines like interleukins and interferon-gamma (IFN-γ) have been shown to induce or modulate hypothermia but the role of endogenous IFN-γ in hypothermia has not yet been documented. Keeping this in view we investigated the possible role of PB pretreatment in hypothermic rats and IFN-γ expression in rat splenocytes.

The results obtained from this study will help to formulate a prophylactic regimen in counteracting CWA.

2. Materials and Methods

2.1. Animals

Male albino Wistar rats (120 ± 10g) were acclimatized for a week in laboratory conditions. The rats had access to pellet diet and water *ad libitum* where environmental conditions were optimum. Animal care was in accordance with the guidelines set up by the Animal Ethical Committee of West Bengal State University (Registration No. 1394/ac/10/CPCSEA in November 2010).

2.2. Chemicals

Atropine sulfate, PB and DFP were purchased from Sigma Aldrich, USA. All the drugs were dissolved in sterile water. A 10% stock solution of DFP was prepared in propylene glycol, refrigerated and used within five days. Total antioxidant status (TAS) was determined by Callbiochem TAS assay kit catalogue no 615700 [14].

2.3. LD_{50} Determination

PB and DFP were supplied by Sigma Aldrich Co., USA. The LD_{50} was determined by Dixon's Up and Down for small samples [15] using 6-8 animals each. The maximum sign-free or symptom-free dose of pyridostigmine was defined it as the dose which caused no sign of anticholinesterase poisoning such as tremors, muscle fasciculation, salivation, urination, incoordination, etc [16]. Atropine Sulphate was used in the experiments in which DFP was used. A dose of 10 mg/kg body weight was injected intraperitoneally (i.p.) immediately after administration of 0.5 LD_{50} DFP.

2.4. Treatment

All doses were calculated in terms of the salt of the individual drugs. Rats were grouped as given:

Group 1: Control normothermic rats where the rectal temperature and ECG were recorded.

Group 2: Hypothermic rats where rectal temperature and ECG were recorded.

Group 3: Rats were pretreated with sign-free dose of pyridostigmine intramuscularly for 15 min and then induced to hypothermia.

Group 4: Rats were pretreated with a sign-free dose of pyridostigmine, then induced to hypothermia and then injected 0.5 LD_{50} of DFP (subcutaneously) + atropine (10 mg/kg; intraperitoneally)

Group 5: Rats were induced to hypothermia and then injected only 0.5 LD_{50} of DFP + atropine.

2.5. Induction of Hypothermia

Rats were anesthetized with sodium thiopentone (40mg/kg body weight) given intraperitoneally and placed on supine position on a wooden platform. The rectal temperature was recorded by a telethermometer (LCD Portable Digital Multistem Thermometer, Model ST – 9269). The thermistor probe was inserted 4 cm past the anal sphincter. Hypothermia was induced by packing with ice bags around the trunk of the animal. The standard limb leads were used to record the ECG by needle electrodes inserted subcutaneously in the limbs. An indifferent electrode was placed on the chest and recording taken using BPL Cardiart Model 108T- DIGI electrocardiograph machine with a paper speed of 50 mm/ sec. The rats were cooled until the T-wave became flat or widening of QRS complex was observed. Animals were removed from the board after completing the administration of various test drugs. Blood was collected from orbital plexus after light ether anaesthesia for determining the total antioxidant status.

2.6. Determination of Total Antioxidant Status

In this experiment rats were divided into three groups. Group 1 rats were set as the control group. Group 2 rats were exposed to hypothermia till the rectal temperature reached 28°C, while Group 3 animals were pretreated with a

sign-free dose of PB for 15 minutes before induction to hypothermia. Total Antioxidant Status was determined by Calbiochem TAS Assay Kit. Catalogue No 615700 [14].

2.7. Screening IFNγ by FACS

In a separate study we investigated the possible role of PB by screening the expression of IFNγ in the lymphocytes of experimental rats by Fluorescence Activated Cell Sorting (Aria III. BD. Bioscience. USA). Isolated spleen cells of rat were maintained in cell culture and subsequently stimulated by Lipopolysaccharide (LPS). Stimulated splenocytes from control (normothermic) rat were expected to express IFNγ at elevated level. Stimulated cells were subsequently immunostained with monoclonal IFNγ antibody directly conjugated with FITC and screened by FACS.

2.8. Statistics

All the data were expressed as Mean ± S.D. Students t-test or one way ANOVA was applied wherever it was applicable using Sigma Plot 11 Software. The statistical data analysis was carried out using t-test. (Data Analysis Package, Microsoft Excel, 2010). Data was considered significant at $p \leq 0.05$.

3. Results

3.1. Determination of LD_{50}

1The LD_{50} of PB was 1.88mg/Kg body weight and that of DFP was 0.075 mg/Kg body weight after intramuscular injection.

3.2. Determination of Blood Glucose Level and Acetylcholinesterase Activity

The blood glucose level decreased markedly on pretreatment with PB and subsequent exposure to hypothermic condition.

The level of AChE was inhibited significantly ($p \leq 0.05$) in animals given hypothermic stress as compared to the animals exposed to hypothermia and then exposed to DFP. It was also observed that there was a significant fall ($p \leq 0.009$) in AChE level as compared to the control group.

3.3. Electrocardiography in Hypothermia

In the present study the "sign free dose" of PB was used to study some physiological and biochemical parameters against a toxic dose of DFP. There was a progressive fall in heart rate as the body temperature decreased due to induced hypothermia. The electrocardiogram (ECG) of rats exposed to hypothermia revealed the presence of 'P' wave throughout the experiment and it was always positive following a normal sinus mechanism. The PR interval, PQ and QT intervals increased with the fall of rectal temperature (Figure 1).

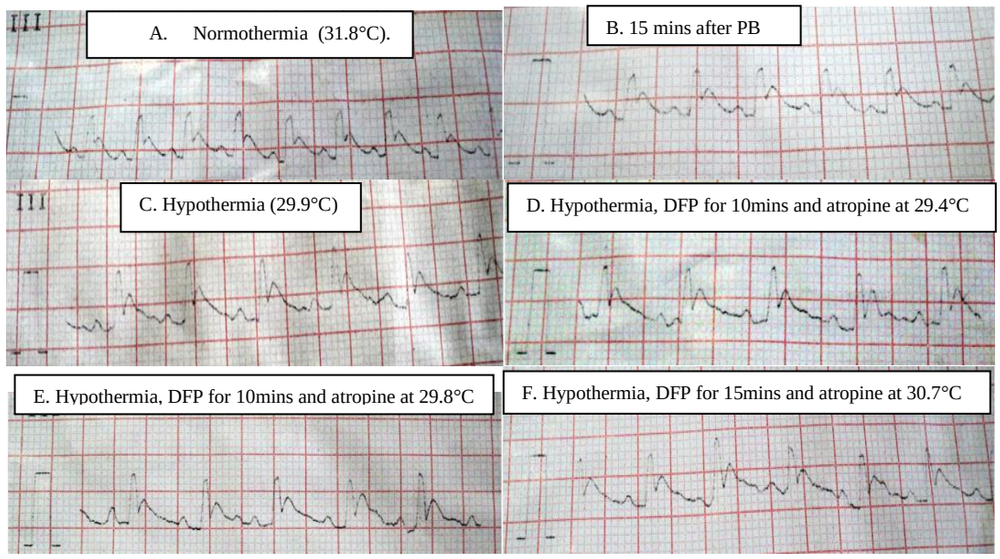

Fig-1. ECG of rats exposed to (A) only normothermia (31.8°C) (B) after 15 mins pretreatment with Pyridostigmine Bromide (PB) in normothermic condition (C) only hypothermia (29.9°C) (D) hypothermia, DFP for 5mins and atropine at 29.4°C (E) hypothermia, DFP for 10mins and atropine at 29.8°C (F) exposed to hypothermia, DFP for 15mins and atropine at 30.7°C.

The duration of QRS complex increased and a widening of QRS complex observed. There was also a decrease in heart rate in rats pretreated with PB and then exposed to DFP. These are commonly observed in hypothermic conditions.

3.4. Protective Role of Pyridostigmine in Hypothermia

3.4.1. Total Antioxidant Status

Environmental stress has been demonstrated to cause an imbalance in the antioxidant status. From the results it has been observed that there was a significant decrease in TAS in animals exposed to hypothermia as compared with the control group ($p \leq 0.001$). TAS was also observed to decrease significantly in animals pretreated for 15 minutes with sign free dose of PB before induced to hypothermic stress ($p \leq 0.001$).

3.4.2. Interferon-γ

FACS analysis revealed that 9.1% of the parent cells were positive for IFNγ expression. Splenocytes of rat exposed to hypothermic stress by surface cooling up to 20.5°C exhibited inhibition of IFNγ expression as revealed by 3.6% of positive parent cell population.

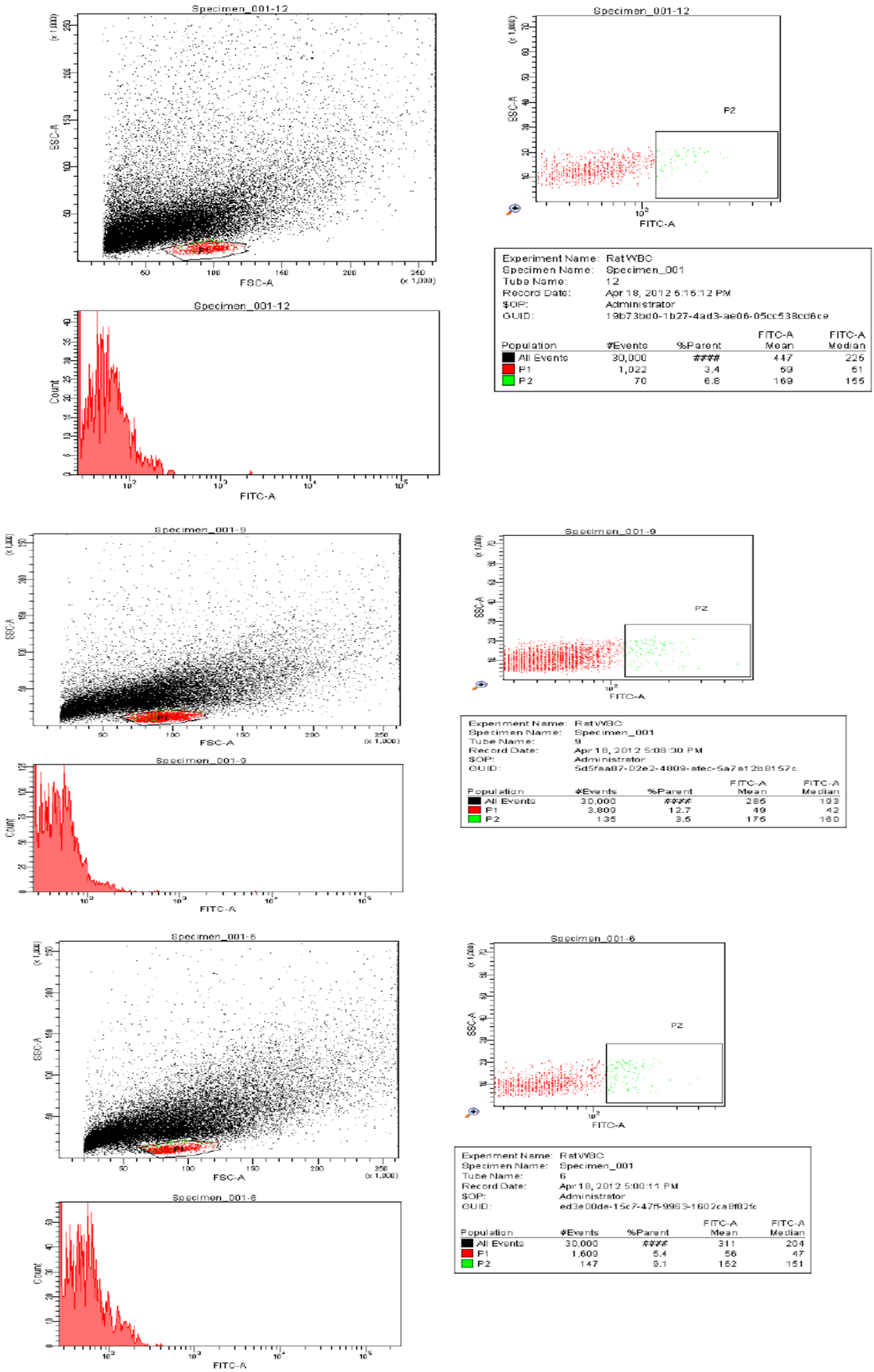

Fig-2. FACS analysis for IFN-γ expression in rat splenocytes in hypothermic and PB pretreated condition of hypothermia.

In a second set of experiments, rats pre-exposed to hypothermia (Fig. 3) were pre-treated with sign free dose of PB and were subjected to FACS analysis for IFN γ expression. The splenocytes of rats exposed to hypothermia and pretreated with PB exhibited an elevation of IFN γ expression from 3.6 % positive parent to 6.8 % of positive parent cells.

4. Discussion

The observed significant delay of the electrocardiographic intervals indicates delay in cardiac conduction in hypothermic and drug superimposed conditions. The decrease in heart rate in such conditions is possibly due to decrease in baroreceptor control that is possibly mediated by central and peripheral components of reflex arc [17]. Mathew, et al. [18] examined the ECG waveform, heart rate and mean blood pressure as potential signatures of hypothermia and rewarming in rats, where both heart rate and blood pressure were observed to decline. It is well documented that in OP poisoning there is onset of arrthymias leading to heart block [19]. However, in the present study, neither bradycardia nor cardiac arrthymia was observed in hypothermic ECG. This might be attributed to the cardioprotective effect of PB pretreatment [20, 21].

The TAS was observed to decrease significantly in hypothermic conditions with and without PB pretreatment. This is in agreement with the earlier findings of Cetinkale et al where it was reported that thermal injury (burn) caused a remarkable decrease (45%) in superoxide dismutase when animals were subjected to 30% surface area burn after their blood was collected at 24 hour post burn. They also reported that TAS was decreased to 14% as compared to the control ($p \leq 0.01$). The plasma malondialdehyde levels were significantly elevated ($p \leq 0.02$) indicating acceleration of lipid peroxidation level due to production of free radicals. Alva, et al. [22] observed deep hypothermia impact on acid base parameters and liver antioxidant status in rat model. Hypothermia decreases endogenous antioxidant consumption and lipid peroxidation after experimental cerebral injury [23].

Immune regulation via the autonomic nervous system or by a non-neuronal cholinergic system suggests that the immune system may be susceptible to disturbance by various components affecting cholinergic function. In our experiment, we established the interacting relationship of hypothermia and IFN-γ expression in rat splenocytes. Furthermore protective role of PB for restoration of IFN-γ under the backdrop of hypothermic stress is also indicated. Griffiths, et al. [24] tested sign free dose of PB on T-cell dependent humoral response to antigen in vivo in the mouse. No remarkable effects of PB were seen during the period of AChE inhibition on the humoral immune response.

5. Conclusion

It is concluded that PB in sign free dose is beneficial as a prophylactic agent against DFP although PB acts mainly on the peripheral nervous system while action of DFP is centrally mediated. The interaction of PB with the peripheral nicotine acetylcholine receptor-ionic channel may be involved in the protection as reported earlier by Akaike, et al. [25]. This observation opens up a new avenue for carrying out further work on this aspect.

6. Acknowledgements

The authors thank the Director, Defence Research and Development Establishment, Gwalior, for providing financial assistance to conduct this study. Authors also acknowledge CRNN, Calcutta University for FACS analysis.
Declaration of Interest: The authors state that there is no conflict of interest.

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