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Cardiorespiratory & Neuromuscular Effects of Freshwater Cyanophyte *Anabena flosaquae* in Rats

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ABSTRACT

Anatoxin-a, recognised as a potent toxin warfare agent, is considered as a weapon of mass destruction due to its lethal anticholinesterase activity. The intravenous administration of cell-free extract of *Anabena flosaquae* UTEX-2383 (anatoxin-a) produced a transient vasodepressor response followed by a sustained rise in blood pressure. The vasodepressor effect was potentiated by physostigmine and antagonised by atropine and bilateral vagotomy, suggesting the involvement of cholinergic system. On the contrary, the vasopressor response was antagonised by hexamethonium, prazosin and hemicholinium-3, indicating that the toxin stimulates the sympathetic system through the release of catecholamines from nerve endings. Prolonged apnoea with attendant bronchoconstriction was observed corresponding to bradycardia and vasopressor response which remained unaltered by atropine while antagonised by bilateral vagotomy. The extract when administered intra-arterially did not modify the apnoea induced by veratridine; but phenyldiguanide potentiated the bronchoconstriction, indicating the involvement of pulmonary vagal afferents. The extract produced a dose- and time-dependent blockade of indirect muscle twitch recorded from gastrocnemius muscle. The neuromuscular blockade was potentiated by neostigmine but unaltered by DTC.

1. INTRODUCTION

The occurrence of toxic waterblooms of freshwater cyanobacteria (blue-green algae) has posed a menacing threat to animal and human health across the globe[1]. This poisoning results due to the consumption of water contaminated with potent toxins liberated from different species of freshwater and brackish water cyanobacteria, such as *Anabena flosaquae*, *Microcystis aeruginosa*, *Oscillatoria aghardii* and *Aphanizomenon flosaquae*[2]. Toxins of these genera include hepatotoxic hepta- or pentapeptides and neurotoxic alkaloids. The neurotoxins[3] isolated are aphanotoxins produced by *Aphanizomenon flosaquae*, anatoxin-a (ANTX-A), saxitoxin, and neosaxitoxin by *A. flosaquae* NRC 44-1, homoanatoxin-a by *Oscillatoria formosa* and anatoxin-a(s) by *A. flosaquae* NRC-525-173.

As cyanobacterial waterblooms become more common in reservoirs, rivers, lakes, ponds, and swimming water due to accumulated wastes from industry, the likelihood of affected human population will also grow. The runoffs from detergents and chemical fertilisers enriched with nitrates and phosphates promote the formation of dense growths of cyanobacteria, called waterblooms[4]. The most toxic species so far studied is *A. flosaquae* which is responsible for the killing of domestic pets as well as livestock. The toxicity of *A. flosaquae* is attributed to the production of harmful secondary metabolites known as toxins. Many countries have shown growing interest in toxin warfare (TW) during the past several years. The toxins are considered to be suitable materials for use as a weapon of mass destruction because of their tremendous lethality and low cost of production. The principal toxic component of *A. flosaquae* is anatoxin-a which has already been recognised as a potent TW agent due to its lethal anticholinesterase activity.

The present investigation is therefore designed to study the changes in various physiological variables in

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rats evoked by cell-free extract of *A. flosaquae* UTEX-2383 because, in the field condition, the aqueous media contains admixture of different toxins instead of a single chemical entity.

2. MATERIALS & METHODS

2.1 Organism & Growth Conditions

The axenic culture of *A. flosaquae* UTEX 2383 was obtained from University of Texas Culture Collection, USA. Preliminary screening of the various chemically defined media BG-11 had shown that CB medium supported growth of this organism better than the other media[5]. Hence, for subsequent studies, only CB medium was used. In all experiments, 150 ml of culture media was put into a 750 ml Erlenmeyer flask. Replicate static cultures were maintained at 26 ± 1 °C under 12:12 h light:dark regime under fluorescent light illumination of 1000 lux intensity.

2.2 Bioassay for Toxicity

Cell-free extract of *A. flosaquae* UTEX 2383 from batch culture was prepared by the method of Ohtake *et al*[6]. Briefly, the cells grown in CB medium at late exponential growth were harvested by centrifugation. After three consecutive freeze-thaw cycles, the cells were disrupted by sonication at 50 W for 3 min and centrifuged at 30,000 g for 15 min. The resultant supernatant was filtered through a membrane filter (0.45 µm) and after lyophilisation, the extract was dissolved in 0.9 per cent saline and used in all experiments. The LD_{50} was determined by Dixon's up and down method[7].

2.3 Animals

Male Wistar rats (150-200 g) were used. The animals were housed in propylene cages and were provided food (Lipton diet) and water *ad libitum*. A total of 120 rats (6 in each group) were used for the present investigation.

2.4 Chemicals & Drugs

The doses selected for the drugs were based on available literature and experimental findings. The drugs, dissolved in physiological saline, were injected intravenously through a catheter in the jugular vein. The drugs used were: physostigmine salicylate, atropine sulphate, hexamethonium bromide, phenyl-diguanide from Sigma, USA; veratridine from Koch-Light,UK and the others from usual commercial sources.

2.5 Physiological Parameters

The rats were anaesthetised with pentobarbitone sodium (40 mg/kg,i.p.) for recording various physiological parameters on a Grass Polygraph (Model 7-16P-35). To assess the influence of vagus especially on the cardiorespiratory system, bilateral vagotomy was performed. The animals were maintained on positive pressure ventilation as and when required, with the help of a rodent ventilator (Ugo Basile Model 7025, Comerimo, Italy). A bronchospasm transducer (Ugo Basile Model 7020, Italy) was used to measure the tracheobronchial constriction/spasm, i.e., tracheobronchial response (TBR). The signals generated were fed to a low level DC preamplifier (Model 7P1) to record TBR. The carotid artery was cannulated to record the blood pressure using a low level DC preamplifier (Model 7P1). The pulse signals were also fed into the tachograph preamplifier (Type 7P4) to record the heart rate (HR). The neuromuscular (NM) transmission studies were carried out to record the twitch responses as described earlier[8]. After dissection, the gastrocnemius muscle was stimulated via sciatic nerve with supramaximal voltage (1-10 V) of 0.2 ms duration at a frequency of 0.2 Hz through Grass Stimulator (Model S 88) using force transducer (Model FT 03).

2.6 Statistical Analysis

Data in text and figures are Mean ± SEM. Statistical analysis was carried out by using analysis of variance (ANOVA) with repeated measures and ANOVA with Dunett's tests.

3. RESULTS & DISCUSSION

The acute LD_{50} of the cell-free extract in rat was 22.7 mg/kg with fiducial limits 16.78-30.72 mg/kg (i.p.).

3.1 Effects on Cardiovascular Parameters

Various physiological parameters were recorded after i.v. administration of the toxin (1.5, 3.0, 4.5, 6.0 and 7.5 mg/kg). At lower dose (1.5 mg/kg), the cell-free extract produced a transient tachycardia and a vasopressor response. However, at higher doses (>1.5 mg/kg) the toxin produced a transient

vasodepressor followed by vasopressor response and bradycardia (Fig. 1, Figs 2a, 2b; Table 1).

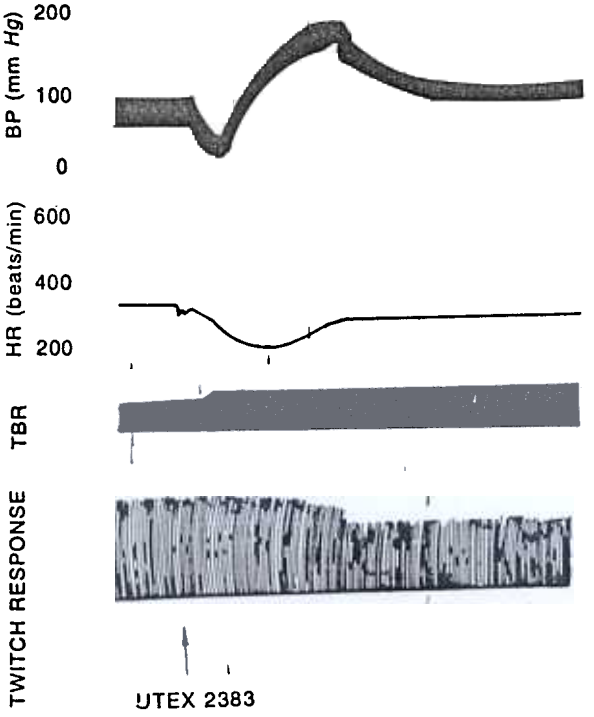

Figure 1. Effect of cell-free extract of *A. flosaquae* UTEX 2383 (4.5 mg/kg) on blood pressure (BP), heart rate (HR), tracheobronchial consrtriction (TBR) and twitch response in rats.

The vasodepressor effect was potentiated by physostigmine (20-50 µg/kg) and antagonised by atropine (0.5-4 mg/kg) suggesting the involvement of muscarinic receptors[9]. The hypertensive effect became prominent immediately after i.v. injection and reached its peak level within 2-3 min. This elevated blood pressure was maintained only with higher doses (6.0-7.5 mg/kg). At higher dose (7.5 mg/kg), the extract elicited bradycardia and the animals died of heart block. The change in systemic blood pressure was also observed in animals provided with or without artificial ventilation indicating that the cardiovascular changes are independent of respiratory movements. The pattern of ECG showed an elevation of ST segment, inversion of T wave and prolongation of Q-T interval suggesting

Table 1. Interactive effects of various pharmacological agents on mean arterial blood pressure (MAP) and heart rate (HR) in rats, 15 min after administration of cell-free extract (6 mg/kg) of *A. flosaquae*

Pretreatment	Dose (mg/kg)	MAP (% increase)	HR (% decrease)
Extract		88.0 ± 04.6	
Hexamethonium		26.4 ± 2.6*	
Hemicholinium-3		18.6 ±1.4*	
Prazosin		12.6 ±1.2*	
Physostigmine			82.0 ± 4.8*
Atropine			11.8 ±1.6*

The data are Mean ± SEM in terms of increase or decrease
n=6 in each group
* Denotes P <0.05 as compared to control.

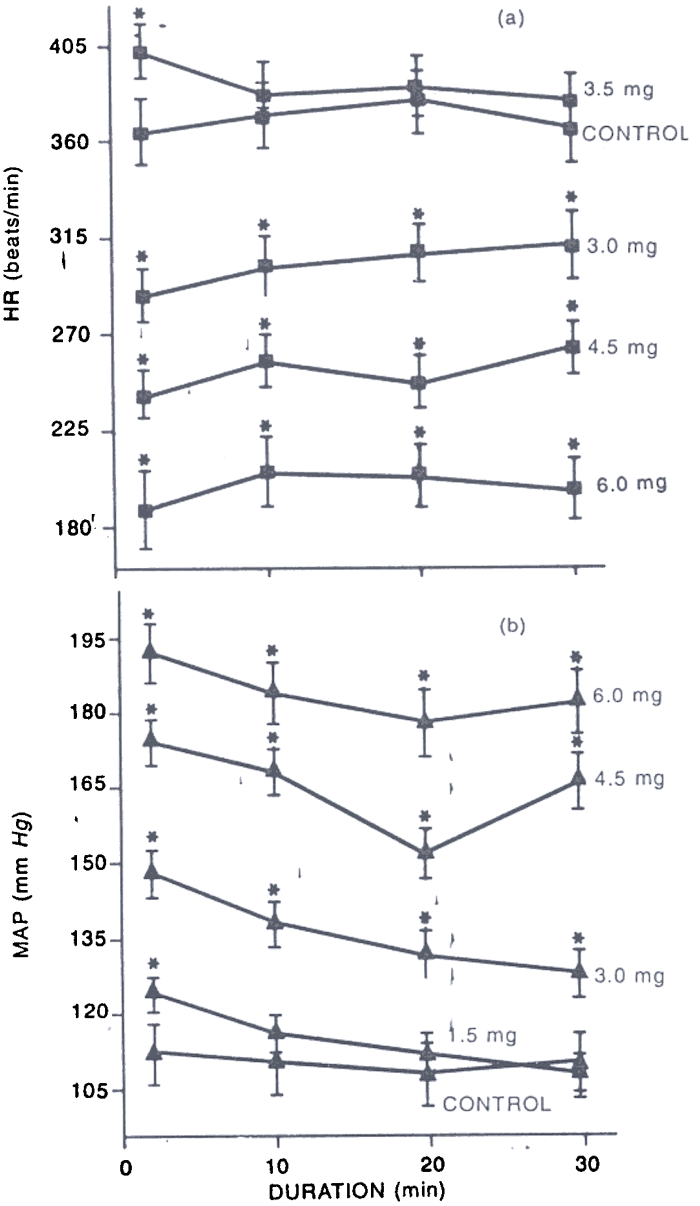

Figure 2. Dose response curve of *A. flosaquae* UTEX 2383 on (a) heart rate, and (b) mean arterial blood pressure in rats. Data are expressed as Mean ± SEM.

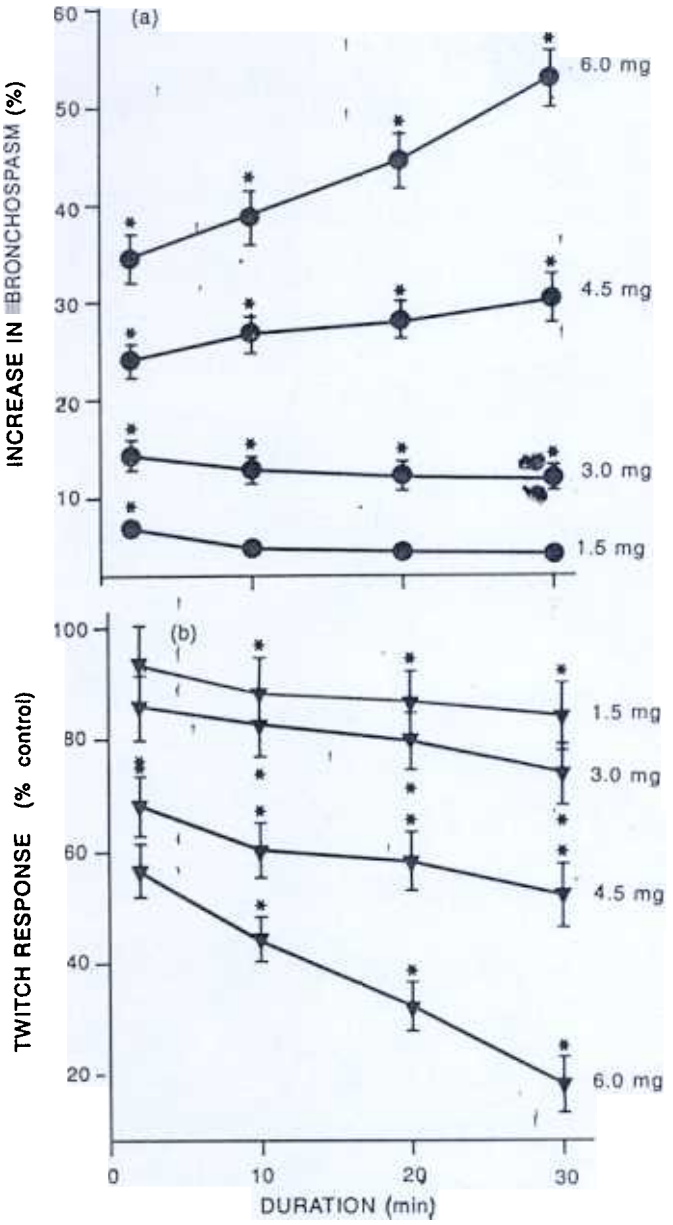

Figure 3. Effect of *A. flosaquae* UTEX 2383 on (a) bronchospasm and (b) twitch response in rats. Data are expressed as Mean ± SEM.

insufficient blood supply to the heart leading to myocardial ischaemia. The vasopressor effect was antagonised by hexamethonium (1-4 mg/kg), hemicholinium-3 and prazosin (0.2-0.5 mg/kg), suggesting the action of toxin through activation of autonomic ganglion[10]. Thus, the results presented here corroborate the *in vitro* data that anatoxin-a is a potent nicotinic cholinergic agonist.

Prazosin pretreatment reduced the extract (6 mg/kg) induced rise in blood pressure (Table 1). On

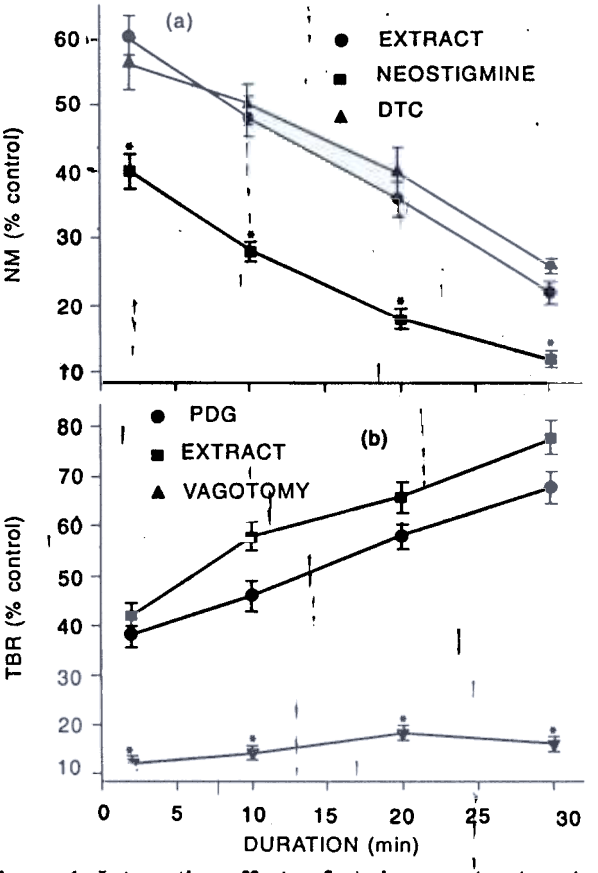

Figure 4. Interactive effects of various pretreatments on *A. flosaquae* UTEX 2383 induced changes on (a) neuromuscular transmission and (b) bronchospasm in rats. Data are expressed as Mean ± SEM.

the other hand, a_2-adrenoceptor antagonist yohimbine failed to alter the vasopressor effect of the extract. Combined pretreatment with prazosin and yohimbine antagonised the toxin-induced rise in blood pressure, but the degree of blockade remained similar to that of prazosin alone. Prazosin also blocked the tachycardic response induced by low dose of the extract but could not prevent the bradycardic effect of the higher dose. It seems that the vasopressor response of the extract was mediated predominantly via a_1-adrenoceptor.

3.2 Effects on Respiratory Movements

A transient apnea (Fig. 1, 3a) was observed corresponding to bradycardia and vasopressor response, which remained unaltered by atropine and hexamethonium. The toxin, however, failed to evoke apnea in bilaterally vagotomised animals (Fig. 4b). It has been found that pulmonary irritation (stimulation of afferent C fibers) evokes apnea followed by rapid shallow breathing, bradycardia, systemic hypotension and bronchoconstriction. The toxin(s) when

administered intra-arterially did not modify the apnea induced by veratridine, but phenyl diguanide altered these responses (Fig. 4b) suggesting the involvement of pulmonary C-fibers since phenyl diguanide per se induces pulmonary reflexes[11,12] of this nature and potentiates the action of the extract (Fig. 4b).

At low dose (1.5 mg/kg), the extract did not produce any change, whereas a higher dose evoked a dose-dependent sustained increase in bronchoconstriction unaltered by artificial ventilation indicating respiratory insufficiency (Fig. 3a). In fact, the function of airway smooth muscle contraction in airway defense is not clear, nevertheless an atropine-sensitive, parasympathetically-mediated broncho-constriction has been recognised as one of the principal components of pulmonary defensive reflexes[13].

3.3 Effects on Neuromuscular Transmission

The muscle twitch response of the sciatic-gastrocnemius preparation diminished in a dose- and time-dependent and irreversible manner (Fig. 1). The effect became apparent immediately after administration of the extract (Fig. 3b). The effect of the extract was potentiated by neostigmine, whereas the effect of DTC was not altered suggesting the depolarising type of blockade (Fig. 4a). Earlier, work with *in vitro* preparations like sciatic nerve-anterior tibialis and frog rectus abdominis also demonstrated a depolarising type of blockade[14]. The present study on *in vivo* neuromuscular transmission corroborates the *in vitro* findings[15]. But the rats pretreated either with mecamylamine or mechanical ventilation died after administration of the extract suggesting some other mechanism (s) of action in addition to respiratory paralysis.

4. CONCLUSION

The present study reveals that the cardiovascular responses to the extract (i.e. anatoxin-a being the principal component) bear close resemblance to those of a nicotinic cholinergic agonist after systemic administration in the rat. The respiratory changes seem to be mediated through pulmonary vagal afferents, but these changes were not responsive to atropine treatment suggesting involvement of noncholinergic system which requires further investigation.

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