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Experimental Myasthenia Gravis-like Neuromuscular Impairment with *Cleisthanthus collinus* Leaf Extract Administration in Rat

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Intraperitoneal administration of a sublethal dose (120 mg/kg body wt) of *Cleistanthus collinus* aqueous leaf extract (CCLE) was found to induce a neuromuscular disorder like myasthenia gravis in rats after 45 min. The neuromuscular junction (NMJ) blockade was analysed *in vivo* in the sciatic nerve–anterior tibialis muscle prpearation. The pattern of nerve evoked compound muscle action potentials (NCMAP) at various frequencies of supramaximal electrical stimulation from single to 100 Hz were recorded. Administration of CCLE showed a sequential decremental response in NCMAP resembling the established EMG patterns of myasthenia gravis (MG). The diagnostic tests, namely a transient improvement and repair of the decremental response in NCAMPs after administration of 4-aminopyridine (1 mg/kg), a characteristic decrement in the 4th or 5th potential on lower frequencies of stimulation, an increased sensitivity to d-tubocurarine (120 μg/kg) and with least or marginal effect on CMAPs on direct muscle stimulation suggested an MG-like neuromuscular disorder which occurs due to ACh receptor blockade. Further, with prior administration of antisnake venon serum (AVS) — an antiserum factor for krait alpha bungarotoxin, the NCMAP decremental response in both control and in CCLE administered rats was rectified transiently and also delayed the onset of NMJ blockade. It is suggested that the prolonged and irreversible effect of the leaf extract, unlike the reversible effect of curare, confers a sustained NMJ blockade required for inducing a MG-like condition.

Keywords: Myasthenia-like disorder; electromyography; *Cleisthanthus collinus;* NMJ blockade; antisnake venon serum; 4-aminopyridine; muscle action potentials.

INTRODUCTION

Patrick and Lindstrom (1973) observed that repeated immunization of rabbits with Acetylcholinereceptor (AchR) purified from the electric organ of *Electrophorus electricus* (eel) caused the development of muscular weakness and a decremental electromyogram (EMG) response to repeated nerve stimulation (RNS), reversible by anticholinesterases like neostigmine; this was termed experimental autoimmune myasthenia gravis (EAMG) (Lennon *et al.*, 1976; Lindstrom *et al.*, 1976). Recent reports also confirm the above immunological studies (Jacobson *et al.*, 1993). The characteristics of EAMG and myasthenia gravis (MG) include not only the nerve evoked decremental electromyographic response but also the absence of end plate potentials, but contrastingly the response to direct muscle stimulation and increased sensitivity to curare (Lambert *et al.*, 1976; Sanders *et al.*, 1976). Thus the decremental EMG response is a diagnostic characteristic of both the *in vivo* (Grob, 1971; Desmedt, 1973; Ozdemir and Young, 1971; Grob and Namba, 1976; Drachman *et al.*, 1976; Stalberg *et al.*, 1974, 1991) and *in vitro* (Elmqvist and Quastel, 1965; Pagala *et al.*, 1990) human intercostal muscles. Earlier investigations in our laboratory by Nanda Kumar *et al.* (1988, 1989) and Vijayalakshmi *et al.* (1994) revealed an irreversible decremental response in *in vitro* phrenic nerve–diaphragm muscle preparation with *Cleisthanthus collinus* leaf extract (CCLE). The plant leaves are highly poisonous and are abused for suicidal and homicidal purposes (Modi, 1977; Nanda Kumar *et al.*, 1988, 1989). A detailed electromyographic study *in vivo* by Vijayalakshmi *et al.* (1995) in rats confirmed a typical NMJ blocking action of CCLE. However, in the present report a detailed study on electrodiagnosis and confirmation with use of antisnake venom (AVS), 4-aminopyridine and d-tubocurarine (dTC) suggested that *Cleistanthus collinus* induced a Myasthenia Gravis (CCIMG)-like NMJ impairment. The EMG pattern induced by CCLE was compared with earlier reports of EAMG, as well as with MG. It is hoped that induction of sustained neuromuscular impairment with CCLE, unlike curare, might be another approach to postsynaptic receptor blocking. This work might throw further light on the exploration of *Cleistanthus collinus* as a new promising NMJ blocker and studies on the reversal of NMJ toxicity might be useful for clinicians treating MG or NMJ disorders.

MATERIALS AND METHODS

The CCLE was prepared from shade-dried leaf powder (Vijayalakshmi *et al.*, 1994). Albino Wistar male rats weighing 100–150 g were anaesthetized with an appropriate amount of sodium thiopentone (40 mg/kg body wt). A sublethal dose of 120 mg/kg of CCLE (LD_{50} = 190 mg/kg body wt) prepared in mammalian Ringer(w/v) was injected intraperitoneally to induce an

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acute neuromuscular impairment. After a latency phase of 30 min the acute phase of MG-like impairment commenced.

Recording of nerve evoked compound muscle action potentials (NCMAP). In the anaesthetized rat, the sciatic nerve and anterior tibialis muscle were exposed to cause least injury. Nerve stimulation was delivered at room temperature by applying supramaximal square wave pulses of 0.01 ms duration of 6 V through a pair of platinum electrodes from a Grass Electrical Stimulator (Model S-88, Grass Instrument Co., MA, USA) through a Stimulus Isolation Unit (Model SIU5, Grass Instrument Co., MA, USA). The NCMAP responses to a single supramaximal stimulation and to a train of 0.1 ms nerve evoked stimulations at 10, 30, 50 and 100 Hz were recorded in a Tektronix Storage Oscilloscope (Tektronix, Oregon, USA, Model 5131). Muscle evoked muscle action potential (MCMAP) responses to a single stimulus and to a train of direct muscle stimuli at 10, 30, 50 and 100 Hz (0.5 ms duration) were also recorded. For observation of nerve and muscle evoked tension, an Isometric Force Transducer (Harvard Apparatus, UK, Model 529503) was employed and observed on an oscilloscope. After recording the nerve evoked and muscle evoked compound muscle action potentials, the same rat was used for inducing neuromuscular impairment by intraperitoneal administration of CCLE.

Effect of antisnake venom serum (AVS). In a separate set of experiments prior to the administration of CCLE, antisnake venon serum (Sii antisnake venom serum – polyvalent lyopolized antisnake venom serum powder from *Naja naja, Bungarus caetuleus, Vipera russelle* and *Echis carinatus* (Serum Institute of India, Pune, India) was administered (i.p.) in mammalian Ringer (70 mg/kg w/v). The CCLE was administered after 45 min or when the normal decremental responses at various frequencies of stimulation were least or abolished due to AVS administration. Nerve evoked compound muscle action potentials (NCMAP) were recorded in rats prior to and after AVS administration followed by CCLE administration (Table 2). d-Tubocurarine chloride (Sigma, MO, USA) 120 μg/kg (w/v) body weight and 4-aminopyridine (Sigma, MO, USA) 1 mg/kg (w/v) body weight of rat, prepared in mammalian Ringer were administered intraperitoneally after CCLE administration in some experiments. MCMAP (direct muscle stimulation) were also recorded at the end of each experiment to determine whether or not muscle contractility and muscle excitation were affected.

RESULTS AND DISCUSSION

The sciatic nerve evoked compound muscle action potentials (NCMAP) were recorded at 30, 45 and 60 min from the anterior tibialis muscle before (normal) and in the same rat after administration of 120 mg/kg of CCLE intraperitoneally. The single NCMAP recorded 30 mV ± 1.01 amplitude and it was 21 mV ± 0.98 60 min after administration of CCLE. There was no decremental response at 10 Hz stimulation in the control (Fig. 1, Table 1). At lower frequencies of stimulation, namely at 10 Hz stimulation in the CCLE administered rat, a marked decremental response was observed in NCMAP. The decremental response at lower frequencies (10 Hz) showed a characteristic EMG pattern as a function of time. After 30 min, the 2nd, 4th and 5th potentials showed significant decrements and after 1 h there was a progressive decrement from the 1st to last potential (Fig. 1, Table 1). These EMG patterns show similarities to a MG disorder. At 30 Hz stimulation the decrement was significantly higher than observed at 10 Hz amounting to

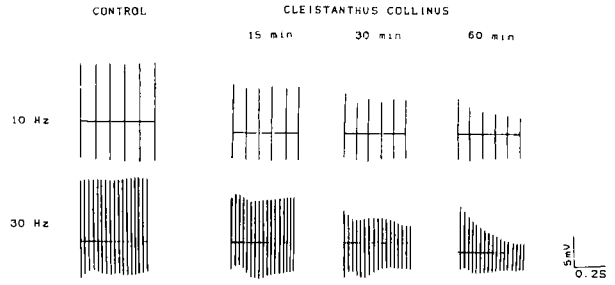

Figure 1. Electromyographic patterns recorded from anterior tibialis muscle on repetitive nerve stimulation (10 and 30 Hz) in control and *Cleistanthus collinus* administered rats. The figure shows electrodiagnostic pattern at 10 Hz stimulation. Note after 30 min a decrement in 2nd and 4th potential and after 60 min a greater decrement in the last action potential resembling the patterns described in the text for neuromuscular disorders.

Table 1. Responses of nerve-evoked compound muscle action potentials in anterior tibialis muscle in normal and *Cleistanthus collinus* leaf extract administered rats

Frequency of stimulation	EMG Parameter	Normal	*Cleistanthus collinus* 30	45	60 min
			Percent Decrement		
Single		0.0	0.0	13.33 ± 1.10 < 0.001	30.00 ± 0.98 < 0.001
10 Hz	A		0.0	26.13 ± 2.03 < 0.001	34.20 ± 3.19 < 0.001
	B	0.0	0.0	23.08 ± 1.99 < 0.001	46.67 ± 4.30 < 0.005
30 Hz	A		0.0	9.64 ± 0.84 < 0.001	19.28 ± 1.11 < 0.001
	B	0.0	0.0	29.41 ± 2.03 < 0.001	61.11 ± 5.79 < 0.005
50 Hz	A		22.62 ± 2.08 < 0.001	53.57 ± 4.97 < 0.005	55.92 ± 5.02 < 0.005
	B	0.0	40.00 ± 3.97 < 0.001	71.43 ± 6.01 < 0.005	87.50 ± 8.13 < 0.005
100 Hz	A		31.71 ± 0.28 < 0.001	62.86 ± 5.99 < 0.005	62.86 ± 5.99 < 0.005
	B	62.50 ±6.13	56.25 ± 5.30 < 0.005	86.96 ± 8.34 < 0.005	94.74 ± 9.25 < 0.005

Values are mean of 8 observations ± SD. Single normal NCMAP amplitude: 30 mV.
A: % decrement by area measurement method in experimental over normal.
B: % decrement in last potential over the first potential of the same train.

9.64% ± 0.84% to 19.28% ± 1.11% and 29.41% ± 2.03% to 61.11% ± 5.79% when determined by EMG parameters 'A' and 'B' respectively. At 50 and 100 Hz, the decrement observed was much higher when compared with 30 Hz stimulation (Table 1, Fig 1). Ozdemir and Young (1976) demonstrated three types of electromyographic (EMG) pattern that were characteristic of myasthenia gravis (MG). They are: (i) most commonly, the amplitude (and area) of the evoked CMAP derease sequentially until a minimum is reached at the 4th or 5th response and then the NCMAP increases. Because of this increment the CMAP may not uncommonly reach a level greater than the initial response; (ii) in the 2nd pattern the size of the NCMAP also continues to decrease until the 4th or 5th response and then the decrement ceases. The CMAP levels off without further continual decrement and with no appreciable increment; (iii) the 3rd pattern is characterized by the 2nd NCMAP which is smaller than the first and often very markedly so, but the subsequent responses do not show any further decrement. In the present report these patterns appeared as a function of time, for example, the pattern (i) described by Ozdemir and Young (1976) can be seen after 30 min of CCLE administration at 10 and 30 Hz stimulation (Fig. 1). The pattern (ii) described by Ozdemir and Young (1976) can be seen after 15 and 30 min of administration of CCLE at 30 Hz stimulation where from the 4th or 5th potential onwards the levelling off takes place at 10 and 30 Hz stimulation (Fig. 1). The third pattern can be seen in Fig. 1, on 10 Hz stimulation after 30 min. It is suggested that the different MG patterns observed by Ozdemir and Young (1976) in different patients might be due to varying degrees of severity of MG, or in other words the degree of binding of the number of ACh receptors due to autoimmunity. All these types of EMG patterns either separately or together were observed in 120 rats. Pagala *et al.* (1990) showed such a pattern in the intercostal muscle of a MG patient and Seybold *et al.* (1976) in EAMG (experimentally acquired myasthenia gravis) animals.

The few diagnostic tests which have been conducted in MG patients also support the MG-like NMJ impairment caused by CCLE. For example, Grob and Namba (1976), Lambert *et al.* (1976), and Drachman *et al.* (1976) described the high sensitivity of MG patients to curare. In the CCLE administered rat dTC (120 μg/kg) also caused a 90% to 95% decrement in CMAP (data not given). Vijayalakshmi *et al.* (1994) demonstrated the similarity of the CCLE pattern to dTC. This test confirms the similarity of CCLE to dTC, which is an established non-depolarizing NMJ blocker (Taylor, 1980; Standaert, 1984). This suggests the possible binding of CCLE to ACh receptors. Further confirmation was obtained by the nerve evoked EMG patterns at 10, 30, 50 and 100 Hz after 60 min of administration of CCLE alone, with prior administration of antisnake venom serum (AVS) followed by CCLE in the same rat. The decremental response was calculated by the area weight measurement method — parameter 'A' — and also by calculating the percent decrease in amplitude of the last NCMAP over the first NCMAP — a conventional EMG parameter 'B'. With prior administration of AVS (7 mg/100 g rat) intraperitoneally, the normal and usual decremental responses observed in the control were significantly reduced or absent (Table 2, Fig. 2). The decremental response observed by EMG parameter 'A' was 0%, 6.79%, 5.88% and 36.67%, whereas on AVS administration the percent decrement observed was much less and was 0%, 0%, 11.69% and 0% with parameter 'A', with parameter 'B' it was 0%, 0%, 6.67% and 33.33% at 10, 30, 50 and 100 Hz stimulation respectively (Table 2, Fig. 2). This suggests the ability of AVS to reduce the decremental response. For example in the rats administered with CCLE alone after 60 min (Fig. 1, Table 1) the percent decrement by parameter 'B' was 46.67%, 61.11%, 87.50%, 94.74%, whereas with prior administration of AVS followed by CCLE, the values were 0%, 10.27%, 15.0%, 44.05% at 10, 30, 50 and 100 Hz respectively (Fig. 2, Table 2). Such a decrement was also observed with EMG parameter 'A'. Thus in the AVS plus CCLE administered rat there was a delayed onset of NMJ blockade and a characteristic decremental EMG pattern and the Ozdemir and Young (1976) pattern observed with CCLE administration was also rectified. A diagnostic repetitive nerve stimulation with 100 Hz three times successively with a 2 s interval and recording EMG pattern at 10 Hz also demonstrated AVS rectification (see 5th vertical trace in Fig. 2).

The significance in selecting AVS to counteract the effects of CCLE is as follows: The Krait toxin, namely alpha bungarotoxin, selectively and irreversibly binds to the ACh receptors (Lee, 1972; Miledi *et al.*, 1971) causing NMJ blockade. Keesey *et al.* (1976) following reports by Chang and Lee (1963) demonstrated a method for detection of humoral NMJ blocking substance in MG employing alpha bungarotoxin binding ability. Based on the above observations, AVS, which consists of antiserum factor for Krait (alpha bungarotoxin) was employed as an antidote for CCLE and whose action specifies CCLE action at ACh receptor sites indirectly. It is likely that AVS might protect the ACh receptor sites, thereby causing less NMJ impairment and allowing normal depolarization, which was otherwise inhibited by CCLE. AVS may be suggested for patients who take CCLE for suicidal or homicidal purposes in India. However, when CCLE was administered to the rat first, followed by AVS there was no reversal or reduction in the decremental response. This suggests the possibility of an irreversible and stronger binding ability of CCLE than AVS as a function of time and the highly toxic mechanism of action of CCLE. AVS might act more as a preventive than curative agent. Further confirmation of the action of CCLE at ACh receptor sites might be interpreted from a significant reduction in the decremental response with administration of 4-aminopyridine (Fig. 3). There was a transient reversal in the NCMAP which lasted 40 min (Fig. 3) and thereafter a gradual decrement was observed. For example at 100 Hz nerve stimulation the NCMAP with CCLE showed 53% ± 4.65% and after 4-aminopyridine administration the percent decrement was 8.16% ± 0.65% by the area measurement method (Fig. 3). 4-aminopyridine increases the chemical transmission at central and peripheral synapses by increasing transmitter release and the efficacy and safety of diaminopyridine in Lambert-Eaton Myasthenic Syndrome and MG was also reported by Sanders *et al.*, (1993), and in a different context by Stalberg (1991).

Contrastingly, the muscle evoked muscle action potential on direct stimulation in the CCLE adminis-

Table 2. Nerve-evoked compound muscle responses in anterior tibialis muscle with antisnake venom serum (i.p.) followed by *Cleistanthus collinus* leaf extract

Frequence of stimulation	EMG parameter	Normal	Antisnake venom serum 30	Antisnake venom serum 60	Antisnake venom serum + *Cleistanthus collinus* 30	60	90	120 min
			Percent decrement					
Single			0.0	0.0	0.0	0.0	10.53 ±0.27 <0.001	47.37 ±3.80 <0.001
10 Hz	A		14.09 ±1.49 <0.001	0.0	6.90 ±0.29 <0.001	3.45 ±0.18 <0.001	28.33 ±0.15 <0.001	31.03 ±2.60 0.001
	B	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30 Hz	A		15.00 ±1.20 <0.001	0.0	6.63 ±0.58 <0.001	12.70 ±0.94 <0.001	4.76 ±0.58 <0.001	25.40 ±1.65 <0.001
	B	6.79 ±0.55	0.0	0.0	3.57 ±0.32 <0.001	10.27 ±0.61 <0.001	7.14 ±0.54 <0.001	5.00 0.45 <0.001
50 Hz	A		16.56 ±1.12 <0.001	11.69 ±0.65 <0.001	16.45 ±1.09 <0.001	28.57 ±2.35 <0.001	31.17 ±3.13 <0.001	50.65 ±4.95 <0.005
	B	5.88 ±0.29	4,00 ±0.18 <0.001	6.67 ±0.34 <0.001	0.0	15.00 ±1.05 <0.001	45.02 ±4.25 <0.001	30.00 ±3.01 <0.001
100 Hz	A		18.84 ±1.41 <0.001	0.0	18.84 ±1.40 <0.001	8.70 ±0.42 <0.001	39.13 ±3.45 <0.001	41.30 ±3.82 <0.001
	B	36.67 ±3.31	26.74 ±2.33 <0.001	33.33 ±3.11 <0.001	31.33 ±3.01 <0.001	44.05 ±4.02 <0.001	79.92 ±7.49 <0.005	63.64 ±6.08 <0.005

Values are mean of 8 observations; ± SD.
Single normal NCMAP amplitude: 38 mV; 'A' & 'B': See Table 1.

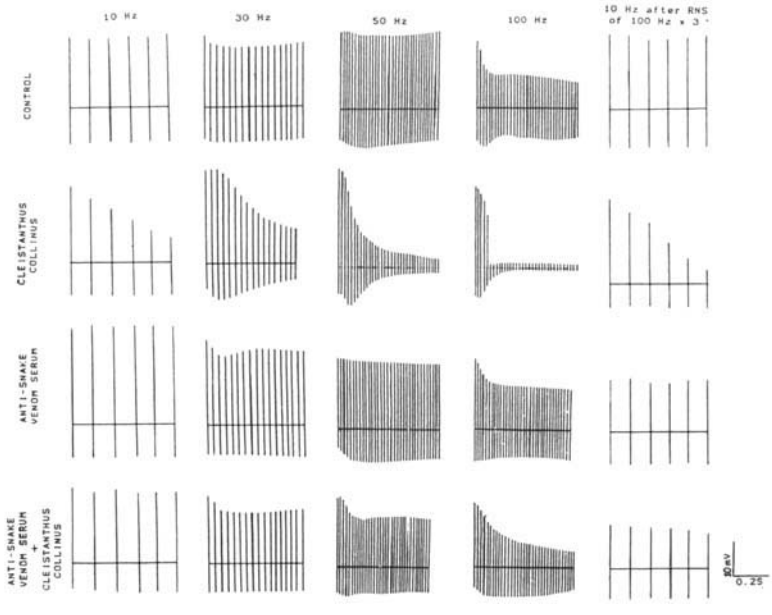

Figure 2. Electromyographic and electrodiagnostic patterns of nerve evoked compound muscle action potentials recorded from anterior tibialis muscle in rat in control, after administration of *Cleistanthus collinus* leaf extract (CCLE) alone, with antisnake venom serum alone and CCLE with prior administration of antisnake venom serum. CMAPs on 10 Hz nerve stimulation after repetitive nerve stimulation of 100 Hz for 3 times with 2 s interval is also presented in the last vertical trace. See diagnostic decremental pattern in CMAPs on 10 Hz stimulation with CCLE and its rectification with prior administration of antisnake venom serum. (For 3rd and 4th horizontal traces control is not presented in the figure and control values are presented in Table 2.) All recordings were made after 60 min of administration of CCLE or antisnake venom serum and/or CCLE.

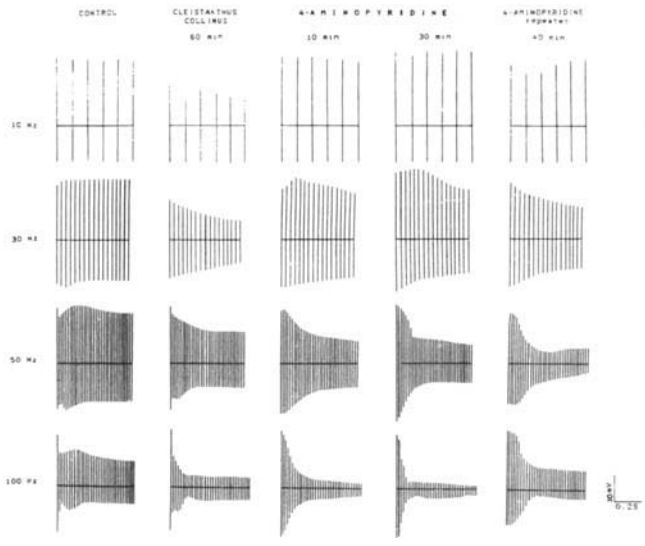

Figure 3. Electromyographic pattern (compound muscle action potential) recorded from anterior tibialis muscle on repetitive sciatic nerve stimulation at 10–100 Hz in control rat, in CCLE administered rat after 1 h and followed by 4-aminopyridine. Repetitive dose of 4-aminopyridine was administered after 30 min and was recorded at 40 min also.

tered rat showed no decremental response in the muscle evoked compound muscle action potentials (MCMAP), even after 2 h of administration of CCLE as reported by Vijayalakshmi *et al.*, (1995) *in vivo* and also *in vitro* by Nanda Kumar *et al.* (1988, 89). Lambert *et al.*, (1976) and Pagala *et al.* (1990) also demonstrated in the acute phase of EAMG, and in MG intercostal muscle, the least decrement on direct stimulation. It may be concluded that CCLE might be inducing an acute MG-like NMJ defect. It is suggested that the prolonged and irreversible nature of the leaf extract, like alpha bungarotoxin, but unlike the reversible effects of curare, confirms a sustained neuromuscular defect required for inducing a MG-like NMJ disorder. The above experimental condition may be further explored in view of the easy and economic procurability of *Cleistanthus collinus* leaves from South India (Nanda Kumar *et al.*, 1988).

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