Some Neuropharmacological Effects of the Crude Venom Extract of *Conus musicus* in Mice

K. BALAMURGAN*, D. AKALANKA, S. RAJU AND A. SHARMA

Department of Pharmacy, Annamalai University, Annamalai Nagar, Chidambaram, 600 802, Tamil Nadu, India.

This study reports some neuropharmacological effects of the crude venom extract of C*onus musicus* (family Conidae) in mice using various experimental models. The crude venom was found to significantly increase tail flick reaction time in mice. The effects of the venom on the central nervous system were studied by observing spontaneous motor activity, gross behaviour, rota-rod performance and potentiation of pentobarbitone sleeping time in mice. Preliminary acute toxicity evaluation was also carried out and the LD_{50} was found to be 460.23 µg/kg following intraperitoneal administration. At a dose of 200 µg/kg i.p., the extract produced a reduction in spontaneous motor activity, altered gross behavior and motor coordination and prolonged pentobarbitone-sleeping time. A liquid chromatography mass spectroscopic study has indicated the presence of ω-conotoxin in the crude venom extract.

Keywords: *Conus musicus*, sedation, spontaneous motor activity, gross behavior, motor coordination

INTRODUCTION

Predatory marine snails of the genus *Conus* (family Conidae) with over 500 species may comprise the largest single genus of marine animals living today. These species inhabit tropical reef environments throughout the world. Depending on their prey preference, cone snails can be classified into three major groups. The piscivorous group comprising among other species *Conus striatus* and *C. geographus* preys upon fish. The group that feeds on mollusks (molluscivorous) includes *C. textile* and *C. pennaceus*. A third group, the vermivorous snails, preys upon *Polychaete* annelids and includes *C. imperialis* and *C. vexillum*. All cone snails are venomous predators and have developed a sophisticated biochemical arsenal to rapidly immobilize their prey. Their venoms are complex mixtures of small, disulfide-bridged polypeptide toxins (conotoxins) that inhibit the functioning of ion channels and neurotransmitter receptors. In addition to their vital role in prey capture and defence against predators, conotoxins are useful tools in neuroscience in the characterization of receptors and receptor subtypes due to their high binding affinity and specificity. Conotoxins also offer great potential as leads in drug development. Indeed the N-type calcium channel blocker from *Conus magus* ω-conotoxin is currently undergoing clinical trials for the treatment of stroke and chronic pain. It is anticipated that the discovery of new toxins displaying characteristically high specificities will increase our understanding of the physiology, pharmacology, biochemistry and structure of the receptors they bind to, and may provide leads to the development of new pharmaceuticals [1-7].

MATERIALS AND METHODS

Preparation of crude venom extract: Specimens of *Conus musicus* were collected from Portonova, Chidambaram, Tamil Nadu, Southern India, dissected and a crude extract prepared from the venom duct material as previously described [8].

*Author to whom correspondence may be addressed.

Ground dried ducts were extracted with a 30% acetonitrile/water mixture acidified with 0.1% trifluoroacetic acid, centrifuged and the supernatants retained. Crude venom extract was lyophilized and stored at -20 °C.

Animals: The pharmacological experiments were conducted using Swiss albino mice weighing 20-25 g. The mice were maintained under standard nutritional and environmental conditions of 50 ± 10% r.h. and an alternating 12 h light and dark cycle throughout the experiment. The animals were used after an acclimatization period of at least 5 days to the laboratory environment and provided with standard food pellets and water *ad libitum*. The animals were deprived of food 24 h prior to experimentation. The animal ethical committee clearance was obtained from the institution for the present study.

Acute toxicity test: The mice were divided into groups of ten and the crude venom extract of *Conus musicus* (CMV) was injected i.p. in doses ranging from 50 to 500 µg/kg. The number of deaths within 24 h of injection was recorded. The LD_{50} was estimated from the graph of percent mortality against log-dose of the crude venom using the Miller and Tainter method [9].

Analgesic activity: To evaluate the central analgesic effects of the extract the tail flick test was performed. In this test, the time taken for a mouse to withdraw its tail when immersed in water maintained at 55±0.5 °C was recorded [10]. The first group was treated with normal saline while groups 2, 3 and 4 received the venom extract at doses of 50 µg/kg, 100 µg/kg and 200 µg/kg i.p. respectively. The fifth group was treated with pentazocine (10 mg/kg, i.p.) as a standard drug.

Spontaneous motor activity (SMA): Spontaneous motor activity was measured using an actophotometer (Techno LE3806, Lucknow, India). Mice were grouped in sixes and treated with normal saline or the CMV extracts (50, 100 and 200 µg/kg i.p.) or diazepam 4 mg/kg i.p. Activity was automatically recorded 30 min after treatment. The experiments were repeated at an interval of 30 min for a total of 60 min. The results obtained were compared with those of the control group at each time interval [11].

Gross behavioral pattern: After intra-peritoneal administration of the same test doses of the CMV extract as in the earlier experiments to a group of 6 mice, each animal was observed for gross behavioral effects. The behavior of the animals was continuously observed for 3 h after administration of the CMV extract and then after every 30 min for the next 3 h. The study was carried out for 6 h, 12 h and 24 h [12].

Motor coordination: A rota-rod (Techno 3C, Lucknow, India) biological research apparatus was used for this test. The instrument (a horizontal rotation device) was set at a rate of 16 rpm. Mice were placed on the rod and those that were able to remain on the rod for longer than 3 min were selected for the study. The first group was treated with normal saline, while groups 2 to 4 received intraperitoneal CMV extract at doses of 50, 100 and 200 µg/kg. The fifth group received diazepam 4 mg/kg i.p. Mice unable to remain on the rod for at least three min were considered as a positive result and the time taken to fall was recorded [13].

Pentobarbitone sleeping time: The mice were divided into 4 groups of six each. Group 1 received normal saline while groups 2, 3 and 4 received 50, 100 and 200 µg/kg i.p. respectively, of the CMV extract. The mice in group 5 received diazepam 4 mg/kg i.p. as the standard drug. The animals were administered with 40 mg/kg i.p. of sodium pentobarbitone 30 min later. The index of hypnotic effect was recorded. For this purpose, the time lapse between the administrations of pentobarbitone and the loss of righting reflex was recorded as the

onset of sleep. The duration of sleep was taken as the time between the loss and recovery of the righting reflex [14].

Statistical analysis: The data obtained was expressed as mean ± standard error. Differences in means were estimated by use of ANOVA followed by Dunnet's *post hoc* test. Results were considered significant at $p< 0.05$.

RESULTS AND DISCUSSION

Acute toxicity studies: The LD_{50} of the CMV extract following i.p. administration was found to be 460.23 µg/kg. While conducting the toxicity studies, animals were observed continuously for any gross behavioral changes and significant reduction of spontaneous locomotor activity, drowsiness and remarkable quietness were observed [15].

Analgesic activity: Analgesic activity was investigated by the tail flick test. The average tail flick latency before and after treatment in the normal saline treated group was 3.72 ± 0.31 (Table 1). The CMV extract treatment induced dose dependent related changes in tail-withdrawal latencies when compared to control group. The maximum analgesic effect was reached 60 min after administration. A cut-off time of 10 s was taken as the maximum analgesic response to avoid injury to the tail due to heat.

Spontaneous motor activity: The CMV extract produced a significant decrease in spontaneous motor activity in mice. This effect was dose dependent and was observed within 30 min of drug administration, persisting for 60 min (Table 2).

Motor coordination: The results of motor coordination test are presented in Table 3. The CMV extract elicited a marked reduction in motor coordination rendering the mice unable to hold onto the rotating rod. This effect was dose dependent with the reaction time decreasing with dose.

Pentobarbitone induced sleeping time: Prior administration of CMV extract significantly increased pentobarbitone-induced sleeping time in mice. Table 4 shows sleeping time in mice treated with pentobarbitone with or without extract. The average sleeping time was found to be 40.48 ± 1.92 min in mice treated with pentobarbitone alone. Prior administration of the CMV extract significantly altered the onset and increased the duration of action of pentobarbitone-induced sleeping time. The maximum duration of sleep was observed at a dose of 200 µg/kg of CMV extract, 92.95 ± 1.54 min [16].

Gross behavior pattern: A significant difference in response was observed when the animals that received the CMV extract were compared with the control (Table 5). In CMV treated mice, there was strong respiratory depression, severe writhing, tremors, convulsions and hind limb paralysis in the initial 3 h after drug administration, which became mild in subsequent 3 h followed by no above responses at the end of the 24 h of the study. But there was a mild to strong stimulation of salivary secretions with increased sense of touch and sound perceptions. No signs of diarrhea and mortality were observed in the study [17].

The results presented heretofore report some pharmacological activities of the crude venom extract of C*onus musicus* in mice. Results indicated that the CMV extracts significantly increased tail flick response. The tail flick response is usually considered suitable for testing central analgesic activity. The CMV was found to produce alteration in general behavior pattern, significant reduction of spontaneous motor activity, changes in gross behavior pattern, motor coordination and prolongation of pentobarbitone-induced sleeping time. The present findings suggest that CMV possesses CNS-depressant effects.

Table 1: Effect of *Conus musicus* crude venom on tail flick response in mice

Drug	Dose (mg/kg)	Mean reaction time (min)	Mean reaction time after administration of drug			% increase in reaction time after 60 min
			15 min	30 min	60 min	
Normal saline	0.2 ml	3.72 ± 0.31	3.50 ± 0.34	3.39 ± 0.26	3.73 ± 0.19	2.6
CMV extract	0.05	3.79 ± 0.31	6.50 ± 0.34	7.16 ± 0.31	8.00 ± 0.37	115.05
CMV extract	0.1	4.00 ± 0.26	7.00 ± 0.26	8.30 ± 0.21	9.00 ± 0.26	141.93
CMV extract	0.2	3.67 ± 0.33	8.17 ± 0.31	9.00 ± 0.26	9.67 ± 0.21	159.94
Pentazocine	10	3.83 ± 0.31	7.17 ± 0.31	8.50 ± 0.22	9.30 ± 0.21	150.00

Values are mean ± SEM, n=6 in each group, CMV = *Conus musicus* venom. Percentage increase in reaction time when compared to control is significant at $p < 0.05$.

Table 2: Effect of *Conus musicus* crude venom extract on spontaneous motor activity

Drug	Dose (mg/Kg)	Mean reaction time (min)	Mean reaction time after administration of drug	
			0 min	30 min
Normal saline	0.2 ml	408.78 ± 5.77	433.50 ± 6.49	418.39 ± 5.16
CMV extract	0.05	404.22 ± 5.09	150.72 ± 6.36	95.13 ± 1.31
CMV extract	0.10	414.70 ± 6.37	85.25 ± 1.21	60.49 ± 1.44
CMV extract	0.20	421.72 ± 6.15	40.55 ± 1.28	33.40 ± 1.06
Diazepam	4.00	419.99 ± 5.14	33.8 ± 1.91	19.07 ± 0.57

Values are mean ± SEM, n = 6 in each group, CMV = *Conus musicus* venom. Significantly different at $p<0.05$.

Table 3: Effect of *Conus musicus* crude venom extract on motor coordination

Drug	Dose (mg/Kg)	Mean reaction time (min)	Mean reaction time after administration of drug	
			0 min	30 min
Normal saline	0.2 ml	217.50 ± 4.17	226.50 ± 4.83	201.36 ± 6.09
CMV extract	0.05	212.50 ± 7.32	128.30 ± 4.10	145.27 ± 4.15
CMV extract	0.10	217.17 ± 6.05	90.21 ± 2.71	76.50 ± 2.51
CMV extract	0.20	214.06 ± 7.89	78.17 ± 3.68	65.67 ± 2.04
Diazepam	4.00	215.99 ± 5.14	33.8 ± 1.91	19.07 ± 0.57

Values are mean ± SEM; n = 6 in each group, CMV = *Conus musicus* venom. Significantly different at $p<0.05$.

Table 4: Effect of *Conus musicus* crude venom extract on pentobarbitone induced sleeping time

Treatment	Dose (mg/kg)	Onset of sleep (min)	Duration of sleep (min)
Pentobarbitone	40.0	3.01 ± 0.17	40.48 ± 1.92
CMV extract	0.05	2.92 ± 0.18	60.79 ± 1.04
CMV extract	0.10	1.86 ± 0.15	75.78 ± 1.32
CMV extract	0.20	1.51 ± 0.11	92.95 ± 1.54
Diazepam	4.00	2.02 ± 0.31	95.48 ±1.27

Values are mean ± SEM; n = 6 in each group, CMV = *Conus musicus* venom. Duration of sleeping when compared to control is significantly different at $p<0.05$.

Table 5: Effect of *Conus musicus* crude venom extract on gross behavioral pattern

Gross activity	Time (h)								
	3	3½	4	4½	5	5½	6	12	24
Respiratory depression	++	++	+	+	+	+	-	-	-
Writhing	++	++	+	+	+	-	-	-	-
Tremors	++	++	+	+	+	+	-	-	-
Convulsions	++	++	+	-	-	-	-	-	-
Hind limb paralysis	+	+	++	++	++	++	++	+	-
Sense of touch and sound	High	High	High	High	-	Low	-	-	-
Salivation	High	High	-	-	-	-	-	-	-
Diarrhea	-	-	-	-	-	-	-	-	-
Mortality	-	-	-	-	-	-	-	-	-

+ = Mild effect, ++ = Strong effect, - = No Effect.

The CMV extract significantly reduced spontaneous motor activity. This activity is a measure of the level of excitability of the CNS and the observed decrease may be closely related to sedation resulting from depression of the central nervous system. Previous studies have related prolongation of barbital hypnosis to pentobarbital metabolic inhibition or action on the CNS centers involved in the regulation of sleep. It is generally accepted that the sedative effect of drugs can be evaluated by measurement of spontaneous motor activity and pentobarbitone-induced sleeping time in the laboratory animal model [18]. These results corroborate previous reports that the

enhancement of barbital hypnosis is a good index of CNS depressant activity. Results of the gross behavior test (Table 5) further support the sedative effect of the extract and its possible application in anxiety management.

Present findings of analgesic activity are similar to those reported for pentazocine [19]. It has been reported that ω-conotoxin has potent sedative activity when tested in similar models and also inhibits spontaneous motor activity in mice [20]. Therefore, the ω-conotoxin content of the crude venom extract may be partially responsible for the observed pharmacological effects. Further studies should be carried out to establish the mechanism of CNS depressant action of the CMV extract.

REFERENCES

[1] B.M. Olivera, J. Liver, C. Clark, C.A. Ramilo, G.P. Corpouz, F.C. Abogadie, E.E. Mena, S.R. Woodward, D.R. Hillyard and L.J. Cruz, Science 249 (1990) 257-263.

[2] S.M. Maillo, M.B. Aguiler, E. Lopez-Vera, A.G. Craig, G. Bulaj, B.M. Olivera and E.P.H. de le Cotera, Toxicon. 40 (2000) 401-407.

[3] L.J. England, J. Imperial, R. Jacobsen, A.G. Craig, J. Gulyas, M. Akhtar, J. Rivier, D. Julius and B.M. Olivera, Science 281 (1998) 575-578.

[4] I.A. Sharpe, J. Gehrmann, M.L. Loughnan, L. Thomas, D.A. Adams, A. Atkins and E. Palant, Nat. Neurosci. 4 (2001) 902–907.

[5] R.M. Jones and G. Bulaj, Curr. Pharm. Des. 6 (2000) 1249–1285.

[6] G.S. Shen, R.T. Layer and R.T. McCabe, Drug Discov. Today 5 (2000) 98–106.

[7] C.E. Heading, Curr. Opin. Investig. Drugs 3 (2002) 915–920.

[8] C.X. Fan, X.K. Chen, C. Zhang, L.X. Wang, K.L. Duan, L.L. He and Y. Cao, J. Biol. Chem. 278 (2003) 12624–12633.

[9] J.M. McIntosh, A. Hasson, M.E. Spira, W.R. Gray, W. Li, M. Marsh and D.R. Hillyard, J. Biol. Chem. 270 (1995) 16796–16802.

[10] P. Safo, T. Rosenbaum, A. Shcherbatko, D.Y. Choi, E. Han, J.J. Toledo-Aral and B.M. Olivera, J. Neurosci. 20 (2000) 76–80.

[11] P.J. West, G. Bulaj, J.E. Garrett, B.M. Olivera and D. Yoshikami, Biochemistry 41 (2002) 15388–15393.

[12] Y. Ozturk, S. Aydini, R. Beis, K.H.C. Baser and H. Berberoglu, Phytomedicine 3 (1996) 139-146.

[13] P.N. Kaul and S.K. Kulkarni, J. Pharm. Sci. 67 (1978) 1293-1296.

[14] L. Ming-Chin, J. Ethnopharmacol. 59 (1998) 161-165.

[15] C. Miller and M.L. Tainter, Proc. Soc. Experimental Biol. Medicine 57 (1944) 261-264.

[16] H. Fujimori and D. Cobb, Psychopharmacology 7 (1965) 374-377.

[17] R.A. Turner, Screening Method in Pharmacology, Academic Press, New York and London. 1965, pp 100-116.

[18] R.M. Perez, J.A. Perez, L.M. Garcia and H.M. Sossa, J. Ethnopharmacol. 62 (1998) 43-48.

[19] L.C. Di Stasi, M. Cotal, L.S. Sigrid and M. Mendacolli, J. Ethnopharmacol. 24 (1988) 205-211.

[20] W. Rajendra, A. Armugam and K. Jeyaseelan, Toxicon. 44 (2004) 1-17.