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Analgesic effect of Persian Gulf *Conus textile* venom

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ABSTRACT

Objective(s): Cone snails are estimated to consist of up to 700 species. The venom of these snails has yielded a rich source of novel peptides. This study was aimed to study the analgesic effect of Persian Gulf *Conus textile* and its comparison with morphine in mouse model.

Materials and Methods: Samples were collected in Larak Island. The venom ducts were Isolated and kept on ice then homogenized. The mixture centrifuged at 10000 × g for 20 min. Supernatant was considered as extracted venom. The protein profile of venom determined using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Venom was administered intraperitoneally (IP) to evaluate the LD_{50} in Swiss albino mice. Different concentrations of *Conus textile* venom were injected intrathecally to mice to evaluate their analgesic effect in comparison to morphine. Injection was carried out between the L5 and L6 vertebrae. Differences between groups in the first and second phase were tested with Two-Way analysis of variance (ANOVA).

Results: SDS-PAGE indicated 12 bands ranged between 6 and 180 KDa. Finally, ten ng of *Conus* crude venom showed the best analgesic activity in formalin test. No death observed up to 100 mg/kg. Analgesic activity of crude venom was more significant (P<0.05) in acute pain than inflammatory pain. The analgesic effect of 10 ng Conus venom was the same as morphine for reduction of inflammatory pain (P=0.27).

Conclusion: The venom of Persian Gulf *Conus textile* contains an analgesic component for reliving of acute pain which can lead to find an analgesic drug.

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Introduction

Cones are a type of sea snails that belong to the *Conus* genus, which is a widespread genus of sea snails. Cone snails are mostly tropical in distribution. The first *Conus* venom peptides were isolated and characterized two decades ago (1), and the systematic investigation of cone snail toxins has continued at an accelerating pace. There are 700 different species of cone snails. All of them are venomous with different toxicity. These unique marine organisms deliver their venom through a specialized radular tooth that serves as both a harpoon and disposable hypodermic needle.

The venom of each species contains up to 200 pharmacologically active components that mainly target different voltage- and ligand-gated ion channels (2, 3). Conotoxins from cone snails are interesting molecules with a diverse human therapeutic potential, such as analgesic, antiepileptic, cardio- and neuro-protective activity (4, 5). With respect to the venom action on the prey, the various conopeptides are classified according to their biological role for the immobilization of prey (6-8).

Conotoxins are generally classified as α, μ, δ, κ, and ω classes (9). Different conotoxins are processing to produce drugs. ω-MVIIA (ziconotide) is FDA approved and the other conopeptides like Conantokin-G, A-Vc1.1 and CGX-1204 are candidates as pharmaceutical drugs (4).

The present study was aimed to find analgesic activity in the venom of Persian Gulf *Conus textile*. Analgesic effect was investigated in acute and persistent pain, and compared with morphine.

Materials and Methods

Specimens, animals and reagents

The *Conus textile* samples were obtained from Persian Gulf - Larak Island, from depth of 7m because of their great abundant in this area in the south of Iran, in May 2012 (Figure 1). Coordinates of sampling place was N 26 52' 25.41", E 56 20' 01.02" (Figure 2). The length of specimens ranged from 3-7 cm (Figure 3). Living snails were frozen and stored at -70°C.

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Figure 1. Study area in Larak island, south of Iran (26° 51'23"N, 56° 21' 3"E)

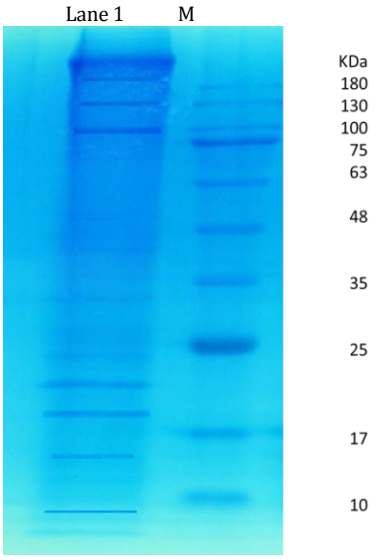

Figure 3. Electrophoretic profile of *Conus textile*
M: Molecular weight marker (fermentas). Lane1. *Conus textile* crude venom

Swiss albino mice weighing 20 to 25 g were chosed after an acclimatization period of at least 7 days in the laboratory environment and standard food pellets and water was provided. Morphine purchased from DaruPakhsh Pharma Chem. Co., Iran. Formalin was obtained from Merck Chemical Company.

Sample preparation and venom extraction

Specimens were dissected on a petri dish on ice and the venom ducts were removed and the extraction of venom was performed same as previously published method with some modifications (10, 11). The venom ducts were homogenized (Silent crasher, Heidolph-Germany) at 16000 × rpm for 5 min with 200 µl of cold sterile water. This blend was centrifuged at 10000 × g for 20 min at 4˚C. Finally, the supernatant was lyophilized in a freeze dryer (2-Alpha, Christ- Germany) and stored at -20˚C.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard method (12).

The venom samples were loaded onto a 15% polyacrylamide gel (13), at 100 V for 90 min. Solutions for preparing 15% resolving gel included H2O (1700 µl), 30% acrylamide mix (3750 µl), 1.5 M Tris (pH 8.8, 1950 µl), 10% SDS (75 µl), 10% ammonium persulfate (75 µl) and N,N,N,N-tetramethyl ethylene diamine (5 µl).

Determination of median lethal dose (LD_{50})

LD_{50} of a toxin is the dose required to kill half the members of a tested population after specified test duration. In this study, 36 mice were divided into six groups and *Conus textile* crude venom was injected IP in dose ranges 260 µg/kg, 300 µg/kg, 425 µg/kg, 3 mg/kg, 25 mg/kg, 50 mg/kg, 60 mg/kg and 100 mg/kg. The number of dead mice and clinical symptoms were recorded during 48 hour after injection (14).

Analgesic activity

To evaluate the central analgesic effects of crude venom in mice, formalin test was carried out as previously described (15). The formalin test is useful to investigate drug effects on both acute and

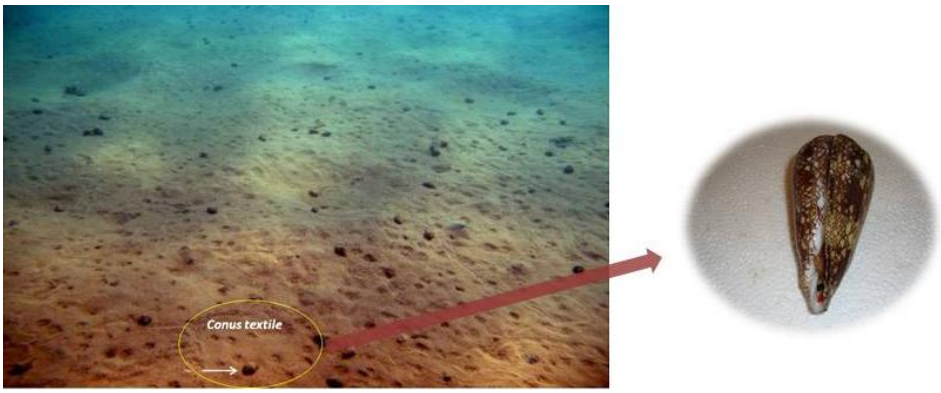

Figure 2. Persian Gulf *Conus textile* isolated from waters of Larak island (7 m depth)

Table 1. Nociceptive behavior of crude venom of *conus textile* and controls (expressed as mean ± SD, N=7)

Dose (ng)	mean ± SD			
	Crude venom		Morphine	
	Acute (5 min)	Chronic (40 min)	Acute (5 min)	Chronic (40 min)
5	37.4 ± 2.70	72.6±3.97	28.7±6.70	37.5 ± 9.7
10	35.2 ± 2.58	*47.4± 3.64	30.25± 2.50	*43.5 ± 4.65
20	42.6 ± 8.29	51.6± 5.68	28.5± 3.10	30.25 ± 2.21
60	45.8 ± 8.07	77.4 ± 5.50	27± 3.55	28.87 ± 3.42
100	70 ± 8.91	94 ± 11.93	22.5±7.7	14.75 ± 6.3
Negative control	49.6 ± 9.09	77 ± 4	49.6 ± 9.09	77 ± 4

persistent pain because it produces two phases of nociceptive behavior (5). Normal saline in the volume of 5 µl injected intrathecally as negative control, 5 min before formalin test. Intrathecal drug injection means the use of a therapeutic substance by injection into the subarachnoid space of spinal cord. This method is an alternative route of delivery. Normal saline was injected into lumbar spinal cord of mice (16). Mice were shaved on the lower back to help visualize the lumbar region. A 30-gauge needle attached to a 100µl insulin syringe was inserted between the L5 and L6 vertebrae and then injection was carried out. Thereafter, 10 µl of 5% formalin in saline were subcutaneously injected into the plantar surface of the left hindpaw (n=7), (17), and the nociceptive behaviors were observed. The elicited behaviors including licking, biting, scratching or shaking were observed but licking behavior counted. Throughout this experiment, the mice were observed in a transparent observation chamber. The time period for first phase was from 0-5 min (acute pain) after injection, while the second phase was from 20-40 min (inflammatory pain) after injection. Morphine (5 ng/5 µl, 10 ng/5 µl, 20 ng/5 µl, 60 ng/5 µl and 100 ng/5 µl) injected intrathecally as positive control, 5 min before formalin test. Then, 10 µl of 5% formalin in saline were subcutaneously injected at the same way, and the licking behavior was recorded. The mice in each test group (n=7) were intrathecally injected with 5, 10, 20, 60, and 100 ng of crude venom (5 µl). Then formalin test performed as described above.

Statistical analysis

All the data were expressed as mean ± standard deviation. In-group differences tested for all groups by *Fisher's* exact test. Results were significant at $P<0.05$. Differences between groups in the first and second phase were tested with Two-Way analysis of variance (ANOVA). The results of similar doses between two phases were compared by student t-test.

Results

SDS-PAGE

The extracted venom of *Conus textile* demonstrated 12 separate bands in the gel. Protein bands observed between 6 to 180 KDa (Figure 3).

LD_{50}

No death was observed up to 100 mg/kg. The recorded signs included reversible strong muscular paralysis at left hindpaw and drooping of both eyelids.

Analgesic activity of crude venom

Nociceptive behaviors induced by crude venom and controls showed as mean±SD in Table 1.

Two-Way ANOVA was calculated to determine the significant differences between groups in first and second phase, which no significant activity differences was observed at 5, 10 and 20 ng of crude venom concentrations in the first phase ($P>0.05$). Results of both phases of 60 and 100 ng were similar to control ($P=0.00$).

Comparison between the control and crude venom doses 5, 10 and 20 showed significant differences in both phases. Significant differences ($P<0.05$) observed between 100 ng and the other doses of crude venom (5 and 10 ng). Results of 10 ng of crude venom and morphine were similar in second phase ($P=0.27$).

Ascending amounts of morphine ranged from 5-100 ng reduced pain significantly and 100 ng had the most analgesic effect. Unlike the crude venom, morphine was more effective in high concentration. All morphine doses except for 10 ng were significantly more analgesic than crude venom in both phases.

The analgesic effect of 10 ng Conus venom was the same as morphine for reduction of chronic pain ($P=0.27$), the results are shown in Table 1 by *.

Discussion

The cone snail (genus Conus), amarine gastropodlives mainly in the tropical habitat of shallow waters near coral reefs. All species are venomous with different toxicity (19). Despite the potential of conopeptides as therapeutic agents, small numbers of them have been characterized in detail.

The venom of each species contains many pharmacologically active components that some of these components such as CTx-MVIIA, SO-3, ACV1, CVID, and GVIA have been identified (20-23). From the first report on analgesic activity of Conus genus (1975), many conotoxins with analgesic activity have been documented (24, 25). CTx-MVIIA was purified

from the venom of *Conus magus* (26) and was approved by the U.S. FDA (27) under the trade name of ziconotide for the treatment of refractory pain (28). Although ziconotide is able to reduce pain and improve the quality of life in patients with neuropathic pain; its therapeutic window is narrow with severe dose-limiting side effects (18).

In present study, no death observed up to 2.5 mg/mouse (100 mg/kg), which is 250,000 times higher than the effective dose (10 ng). LD_{50} result of this study was similar to published paper that documented no discernible changes in general behavior of examined mice at 100 mg/kg, IP (29). Buenaflor *et al* (1981) reported the LD_{50} of *Conus magus* at 57.5 mg/kg in mice (30) which demonstrates more lethality activity than Persian Gulf *Conus textile* venom. This result indicated that Persian Gulf *Conus textile* venom could be a favorable species for tracing new analgesic drugs with very low toxicity. Mechanism of analgesic effect of *Conus textile* venom show that when the electrical impulse generated along axon, sodium ions rush in and potassium ions rush out. Sodium ions accumulation cause to open calcium ion channels. Then influx of calcium causes acetylcholine to be inserted to synaptic junction. Acetylcholine bindings with receptor proteins alter the shape of the ion channel. This opens the sodium ion channel to let the sodiumin. Sodium ions set off an electrical impulse along the next nerve. Finally, the pain signal will work. Blocking channels by conotoxins lead to inhibit pain signals so that the peptides relief the sensation of pain (31). Conotoxins are powerful analgesic drugs that have a special mechanism of action including selective block of N-type calcium channels that limit neurotransmission at some synapses.

The effect of all crude venom doses were more marked during the acute phase than the chronic pain that it is unlike to the results previously reported by Lee *et al* (2010) and Wen *et al* (2006). Wen *et al* showed that the prohibitive effects of SO-3 and MVIIA on HVA I_{Ca} were both reversible. However, the dissociation from block by MVIIA was more rapid than SO-3. So SO-3 is more analgesic in the chronic pain. From the point of possible mechanism for this result, it seems that the affinity between the ligand and target receptor is not as high as expected.

On the basis of the results, 10 ng of Persian Gulf Conus venom showed the best analgesic activity in both phases comparing to the other doses (5, 20, 60, 100 ng). This finding is similar to the previously reported results (5). In this case, it seems that 10 ng of the venom is the best dose to induce balance between the number of ligands and receptors. Crude venom at 10 ng binds to their receptors as rapid as morphine but dissociate faster than it. Because morphine is more analgesic in inflammatory pain that it means morphine dissociate slower from receptors. But *Conus textile* venom is better to reduce acute pain that it demonstrates *Conus textile* venom separates from pain receptors faster.

In drug delivery systems, the best dose is that, it is more effective for longer time and drug can bind to receptors at the most (32-35). So, drug dosage should consume properly because of the defined receptors. When dose of drug is optimum, negative competition does not happen between drug and receptors. In this study, the best reaction between ligands and receptors was observed in 10 ng dose of venom.

Comparing the analgesic results between 10 ng and the other doses of crude venom (5, 60, and 100 ng) showed significant differences (P<0.05) in second phase. Results demonstrated that the amount of 5 ng was not efficient for pain reduction in both phases. Optimum dose for pain relieving in both phases was 10 ng. No significant differences observed between the doses 10 ng and 20 ng but 10 ng was more efficient than 20 ng. The results of both phases for 60 ng and the second phase for 100 ng were similar to control.

Comparison between the first and second phases showed significant differences in all doses (P<0.05). 100 ng morphine had the most analgesic effect. Unlike the crude venom, morphine was more effective in high concentration. All morphine doses except for 10 ng were significantly more analgesic than crude venom in both phases.

Statistically, significant differences observed between 100 ng morphine and the other morphine doses (5, 10, 20 and 60 ng), and also between all morphine doses and control. There was no significant difference between 10 ng of crude venom and morphine in second phase. Comparing of CTX-FVIA (Conus fulmen venom) and ziconotide showed that separation of CTX-FVIA from receptors occurs faster than ziconotide (5). So the toxicity and side effects will be reduced in the patient's body. Therefore CTX-FVIA can be a more appropriate drug for chronic pain reduction.

Whereas this study results indicated that the Persian Gulf *Conus textile* venom is more effective in acute pain reduction. It seems that binding and separation of *Conus textile* venom from receptors occurs faster than ziconotide. So *Conus textile* venom with low toxicity is an excellent candidate for acute pain treatment.

Regarding to similar analgesic potency of morphine and crude venom at 10 ng, it seems that purified peptide will show more analgesic activity than crude venom. This study is pending to purify the target conopeptide from Persian Gulf *Conus textile* and characterization of the crude venom.

Conclusion

Based on the results, analgesic activity of crude venom was more significant in acute phase. It is supposed that crude venom contains a rapid

analgesic conopeptide, which would be applicable for treatment of acute and refractory pain comparing to morphine with side effects such as addiction.

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