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Original Article

Persian Gulf Cone Snail Venom (*Conus coronatus*): The First Report as a Potential Source of Antagonist Conotoxins

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Abstract

Introduction: Conus is the genus of toxic gastropods with pharmacologically active compounds in its venom that mostly lives in marine environments. Conus venom consists of a rich source of analgesic peptides. In the current study, the analgesic effects of *Conus coronatus* venom from the Persian Gulf were investigated in mice models.

Materials and Methods: The venom ducts were extracted and homogenized. Deoxygenated cold aqueous acetonitrile solution (40%) was used in this study for conotoxin extraction. Purification was carried out using Sephadex G-25. Purified fractions were injected intraperitoneal (IP) in both formalin and hotplate tests with different doses. Following the pain response assessment, nicotine was used as the agonist of the acetylcholine receptor, and pain response to the co-injection of nicotine and conotoxin was calculated. Tricine-SDS-PAGE was used for molecular weight determination.

Results: Findings revealed that the action of purified fraction of *C. coronatus* venom (C2) at a dose of 0.1 mg/kg was comparable with morphine as a positive control (2.5 mg/kg). The analgesic potential of this fraction was observed in the hot plate test. However, the co-injection of nicotine and C2 decreased the analgesic effect.

Conclusions: According to findings, it can be stated that conotoxins isolated from *C. coronatus* had analgesic effects and could be used for discovering and producing novel medicines. Moreover, the peptides observed in this study with less than 6.5 kDa probably are members of the antagonist conotoxins which have been reported for the first time in this study.

Keywords: Analgesic, Antagonist, Conotoxin, *Conus coronatus*, Persian Gulf
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Introduction

Pain is a warning signal in many diseases and is linked with environmental stimuli. It can be generated biologically due to injury or exacerbation of a specific disease.[1] Different kinds of stimuli (chemical, physical etc.) are able to activate pain receptors to send signals for specialized sensory neurons in ion channels, and cause depolarization and other responses.[2] Some compounds such as conotoxins can relief pain. Conotoxins are peptides with analgesic properties that are extracted from marine cone snails.[3] These peptides are secreted in their venom ducts, and have high potency to induce a variety of ion channels on nerve cells which are involved in pain responses.[4-5] Therefore, the conotoxins are valuable candidates for physiological pain studies. Some have FDA approval, such as Prialt and Ziconotide. Ziconotide contains the MVIIA ω–conotoxin which has been isolated from *Conus magus*,[6] and is used to treat acute pain on incurable diseases.[7]

Conotoxins are suitable for pharmacological research and novel drug discovery. Briefly, they are small (typically <5 kDa) and cost-effective to synthesis. Generally, peptides with molecular weight between 500 Da to 5 kDa have been recommended as drug design. Smaller peptides (<500 Da) have low target properties specificity but they can be synthetized easily, and have high oral bioavailability and membrane permeability. However, the larger peptides (>5 kDa) are generally metabolized more rapidly than small peptides, and require intravenous administration, and are expensive for production.[8] So, conotoxins have both the stability and the selectivity of the larger peptides, in addition to easy synthesis, and could be potential candidates for novel drug leads.[9]

Interestingly, some conotoxins are described as antagonists of the nicotinic acetylcholine receptors (nAChRs), and previous studies on other species have reported that characterized Conus species have at least one antagonist conotoxin in their venoms.[6,9,10,14] Natural products have an inevitable role in the field of modern pharmacology. In the current study, the analgesic effects of *Conus coronatus* venom from the Persian Gulf were investigated in mice models. Additionally, we evaluated the possibility of the antagonist conotoxins in

this specie for the first time by defining the nicotine as a nicotine receptor agonist. Several conotoxins have been reported from Conus species until now but few of them have been characterized for their potential pain-relieving actions, and only few of these species are accessible. This is while *C. coronatus* is one of the dominant species in the Persian Gulf, and is accessible for researchers.

Materials and Methods

Conotoxins Extraction and Purification

Specimens were collected from coastal waters of the Persian Gulf (Zeyton Park of Qeshm Island), Location: N 26055.631; E 056015.209. Conotoxins were extracted and purified as described by previous studies.[16,17] Venom ducts were isolated and were at first kept in deoxygenated/cold acetonitrile solution (40%), then homogenized at 16,000 rpm for 5 min (Homogenizer Silent Crusher, Heidolph, Germany). The mixture was then centrifuged briefly and lyophilized using freeze dryer (Model Christ, 2 alpha, Germany) for 24 h at -56 °C.

Conotoxins were purified by Sephadex G-25 with the following method. Dry powder (10 g) was dissolved in Tris buffer (50 mM, pH 8.5) before packing in the column with total size about 2.5×90 cm. The flow rate was adjusted at 0.3 ml per min.[18]

Analgesic Test

Male mice weighing 20-25 g were obtained from the animal house of Ahvaz Jundishapur University of Medical Sciences.

Formalin Test

Lyophilized crude extract and purified fractions were used for intraperitoneal (IP) injection. Normal saline and morphine (2.5 mg/kg) were considered as negative and positive control, respectively. The strongest fraction (C2) that was identified in a previous study,[17] was used in different doses (0.01, 0.1, 0.25, 0.5 mg/kg) and compared to morphine.

Pain response was considered according to the flinches and licking numbers for the first phase (0-5 min) and second phase (15-60 min) after a subcutaneously injection of 10 µl formalin (2.5%) into the right paw.[19-20]

Hot Plate Test

The strongest fraction was used in the hot plate test. The temperature was kept constantly at 55 ± 1 °C on hot plate (RB200, Chengdu Taiment echonology Inc, China) and the first licking or jumping up was considered as response to the painful stimuli. After 30 min of nicotine or conotoxin treatment, a mouse was placed on hot plate and pain reaction was measured at intervals 0, 15, 30, 45, and 60 min. Cut off time was taken as 45 s.[19,21]

In a next experiment, nicotine (Merk) was used as the nicotinic receptor agonist of acetylcholine. At First, Nicotine was injected and after that, C2 was injected intraperitoneally. After half an hour, the mouse was placed on the hot plate and its reaction time was recorded.[19,20,22]

The groups examined in this test include:

Ineffective dose of nicotine (IN) + effective dose of C2 (EC)

Ineffective dose of nicotine (IN) + ineffective dose of C2 (IC)

Effective dose of nicotine (EN) + effective dose of C2 (EC)

Tricine–SDS-PAGE

Tricine–SDS-PAGE is commonly used for separating low molecular weight proteins. The acrylamide-bisacrylamide solution was prepared by dissolving 48 g acrylamide and 1.5 g bisacrylamide in 100 ml water. For cathode buffer (121 g Tris, 179 g Tricine, 10 g SDS in water (1 L) with pH 8.25) the inner electrode buffer was used and for the anode buffer (121 g Tris with pH 8.9) the lateral electrode buffer was used. Three layers of polyacrylamide gels were utilized (stacking gel (5%), spacer (10%) and resolving (16%)). Also, 0.5 µl conotoxin was mixed with 4.5 µl protein loading buffer as a loading sample. The mixture was loaded under the cathode buffer after 5 min incubation in 95 °C. The electrophoresis was operated at 60 V initially and the next voltage steps were set at 100 V.[23]

Statistical Analysis

Statistical analyses were performed using Graph-Pad Prism software, version 6. Values were expressed as mean ± SD. All the data were statistically calculated by using one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test (p<0.05).

Results

The Best Dose of C. coronatus venom (C2) Compared to Morphine

The strongest purified fraction (C2) of *C. coronatus* venom resulting from gel filtration were administered to the animals for formalin test at different doses (0.01, 0.1, 0.25, 0.5 mg/kg) and were compared with morphine. The administration of 0.25 and 0.5 mg/kg doses was able to inhibit the pain with more potency than morphine (p<0.05). The dose of 0.1 mg/kg morphine had almost the same analgesic effect (p<0.05) (Figure 1). The effect of this dose observed in hot plate test constantly remained until 30 min (Figure 2). This fraction had low molecular weight peptides as shows in Figure 3.

Measurement of IC_{50}

The IC_{50} values of the Au NPs for K562 cell lines were 81.62 µg/ml according to the normal curve (Figure 3).

Co-injection of Nicotine and C. coronatus venom (C2)

Co-injection of nicotine [0.1 (EN) and 0.05 (IN) mg/kg] and C2 [0.1 (EC) and 0.05 (IC) mg/kg] did not show analgesic

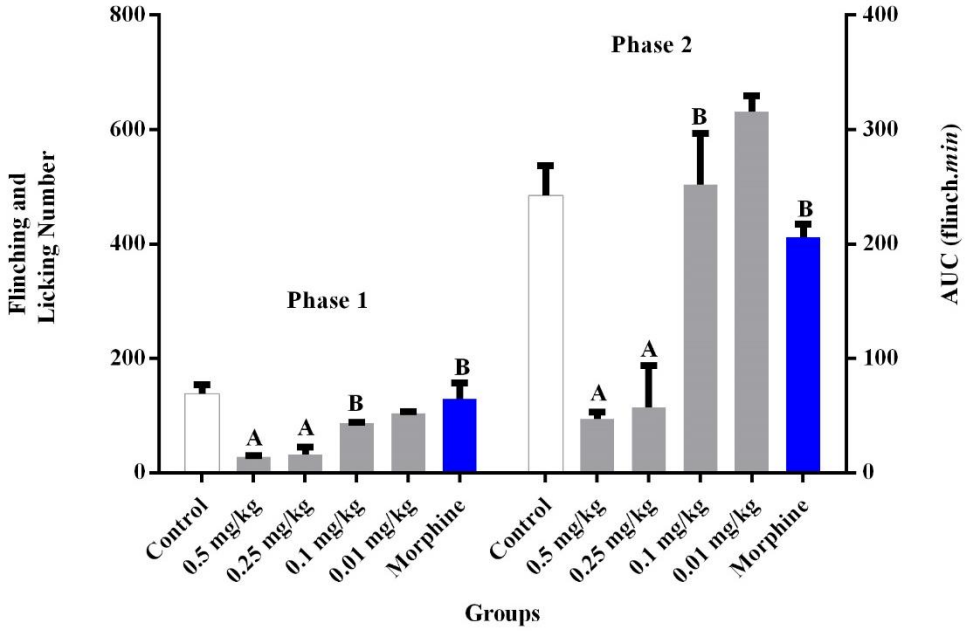

Figure 1. Comparison of Different Doses of C2 in Formalin Test. Flinching and licking numbers was calculated for the first phase and AUC (the area under curve) was determined for the second phase. Different letters (A and B) indicate significant differences with normal saline as negative control, Mean ± SD (n = 7), (p< 0.05).

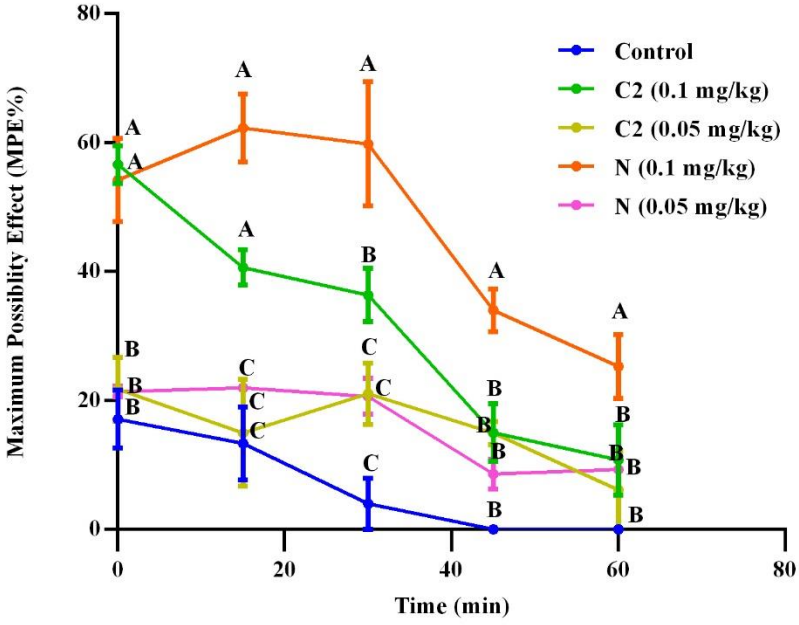

Figure 2. Analgesic Effect of C2 and Nicotine, in Hot Plate Test. The comparison was performed at intervals 0, 15, 30, 45, and 60, and different letters (A, B, and C) indicate significant differences with normal saline as negative control, (Mean ± SD (n = 7), (p< 0.05).

effects on the first [(IN) + (EC)] and the second [(IN) + (IC)] groups (p<0.05). However, the third group [(EN) + (EC)] had a significant analgesic effect for a period of 30 min (p<0.05) (Figure 4). Injection of the active dose of nicotine (0.1 mg/kg) separately showed the strongest analgesic effect that remained until the end of the experiment (p<0.05).

Discussion

C. coronatus is one of the marine cone snails that lived in coastal waters of the Persian Gulf. In a previous study, the analgesic effect of *C. coronatus* venom was investigated and the strongest fraction (C2) was detected by formalin and hot plate tests,[17] which are predominantly used with rats and mice as behavioral models.[9,20,24]

In the current study, the administration of different doses of C2 fraction have revealed that a dose of 0.1 mg/kg had an analgesic effect similar to morphine (p<0.05) (Figure 1) and μ-conotoxin KIIIA from *C. kinoshitai*.[25] In drug delivery

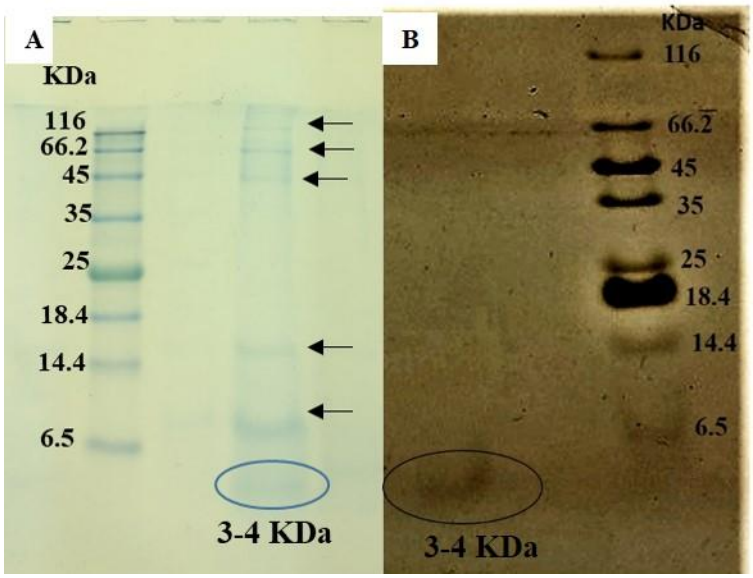

Figure 3. Polyacrylamide Gel Electrophoretic of *C. coronatus* venom. (A) Multiple bands on gel Before purification (B) Single band of analgesic fraction after purification.

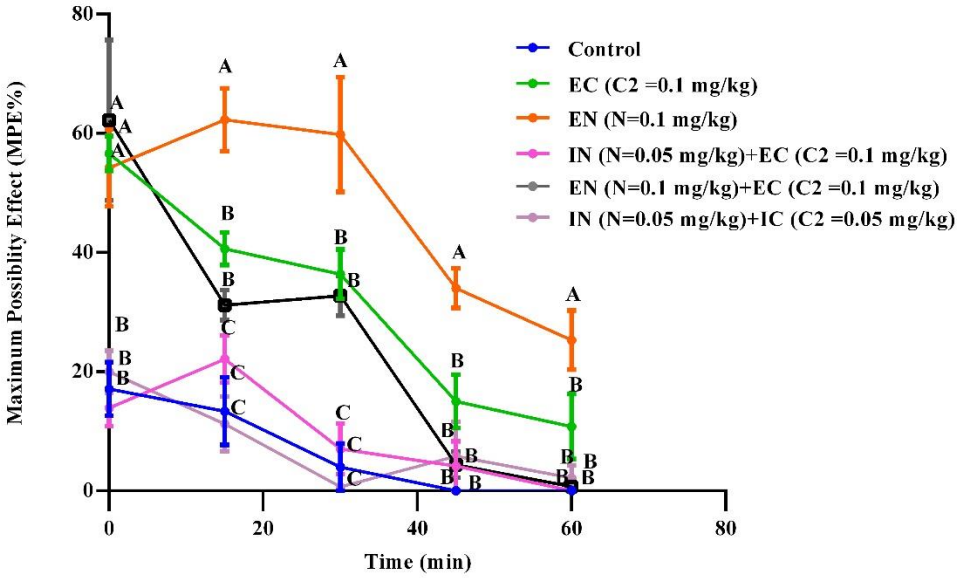

Figure 4. Comparison of Co-Injection of Nicotine and C2 Fraction, in Hot Plate Test. The comparison was performed at intervals 0, 15, 30, 45, and 60 min and different letters (A, B, and C) indicate significant differences with normal saline as negative control, (Mean ± SD (n = 7), (p< 0.05).

systems, the best dose or effective dose is the one that lasts longer with a smaller amount than others, and causes the drug to bind to the receptors.[20,26] Additionally, the dose of 0.5 mg/kg was stronger than morphine (p<0.05), and probably the conotoxins of this dose was able to bind the receptors more rapidly than morphine in order to reduce pain,[26] which is similar to conotoxin extracted from *C. flumen*.[19] In another study the effective dose of μ-conotoxin, SmIIIA from *C. stercusmuscarum* was 0.9 mg/kg inhibited both phases of formalin test which was higher than the effective dose of our study (0.1 mg/kg).[27]

Hot plate test has shown that analgesic effects of C2 remained for 30 min (Figure 2) and was stronger than the analgesic effects of *C. regularis* conotoxin that just remained for about 15 min (at dose of 0.85 mg/kg).[20] Moreover, the same dose of nicotine had more analgesic effect (p<0.05). Compounds such as nicotine, which are agonists of the nAChRs activities, are able to relief the pain in an appropriate dose. This is while at higher doses, they induced adverse effects in the behavioral models.[15,28]

It can be assumed that co-injection of nicotine and C2 led to competition between them, and reduced the analgesic effect (Figure 4). The C2 fraction probably contained antagonist conotoxins of nAChRs, which was similar to the study on

MIJ conotoxin of *C. magus*.[29] These peptides have low molecular weight (<6.5 kDa) on tricine–SDS-PAGE (Figure 3) and generally, have been recommended for drug design.[8,9] Dyachenko et al. (2022) recently discovered that α-Conotoxin RgIA and oligoarginine R8 in the mice model alleviate long-term oxaliplatin induced neuropathy and showed a relatively high affinity for the α9/α10 nAChR.

Unlike *C. magus* (which is piscivorous), *C. coronatus* is a vermivorous and usually feed on worms. Most studies have been conducted mainly on fish-eating species that are highly poisonous and hemolytic.[31] This is while *C. coronatus* showed extremely good potential in our studies. It is recommended to identify the structure of this conotoxin to synthesize and design them as a new drug leads. Ma et al. (2021) synthesized a series of variants of α-conotoxins GI and MI and identified their inhibitory activity against muscular nAChRs as well as their toxicity in mice. They displayed potent inhibitory activities toward muscular nAChRs and low toxicity in mice.

Based on the results of previous studies, nAChRs are activated by acetylcholine. Nicotine increases the release of acetylcholine in the brain by stimulating nicotine receptors.[22,28] Therefore, nAChRs is the target for many conotoxins (α-conotoxins) that despite their differences in their original sequences, are extremely similar in their structure described by NMR or X-ray studies.[14,29,33] Dutertre et al. suggested that the venom of each cone snail species investigated until now had at least one nAChR antagonist.[34] Besides, these peptides inhibit neurotransmission by acting on the postsynaptic membrane by binding to nicotinic acetylcholine receptors, thereby preventing the transmission of pain to the central nerve system and can have a powerful analgesic effect.[14,33] In summary, the present study demonstrated that the conotoxin C2 fraction, at a dose of 0.1 (mg/kg), with 25 times less concentration than morphine, had a similar analgesic effect (p<0.05), and doses of 0.5 and 0.25 (mg/kg) were stronger than morphine (p<0.05). In addition, it was nine times stronger than the other μ-conotoxin, such as SmIIIA from *C. stercusmuscarum* (0.9 mg/kg)[27] to inhibit both phases of formalin test, and *C. regularis* conotoxin (at dose of 0.85 mg/kg)[20] in the hot plate test.

It can be assumed that this fraction of cone snail venom is a highly potent fraction to reduce pain. It should be noted that due to the low amount of conotoxin, especially after purification, it is recommended to use intrathecal injection instead of IP injection. This could allow us to do more experiments and tests.

Conclusion

Based on the results of the present study, it can be concluded that *C. coronatus* conotoxins are probably members of nAChR antagonists. This indicates the good potential of these peptides with low molecular weight for pain relief. Therefore, they can be used as a candidate for the development of new drug lead.

Authors' Contributions

All authors contributed equally to this study.

Ethics Approval

All animal protocols were approved by the ethics committee of Abadan University of Medical Sciences with Ref: IR.ABADANUMS.REC.1399.130.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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