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ANTINOCICEPTIVE EFFECT OF N. OXIANA EICHWALD VENOM

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Abstract. Pain is a protective response of an organism forming in answer to the harmful effects of internal and/or external environment. In treatment of pain multiple medications are used; prolonged use of the most of them produces adverse side effects or causes addiction. Generation of now generation medications devoid of the adverse side effects is sure to be a topical problem. To solve the problem, the venom of cobra was fractionated into 7 fractions by chromatography on the Superose 12 column to study their analgesic activity using standard hot plate and acetic acid-induced writhing tests. Some physical-chemical characteristics were determined for fractions S-6 and S-7.

Key words: cobra venom, fractions, peptides, pain, analgesics

Introduction

Today, the pain relief for a human being remains a most significant medical problem. A wide spectrum of analgesics is available, but the marked side effects are known to accompany the intake of most potent medications with high analgesic activity. To generate medications with potent analgesic effect but devoid of addictiveness is topical today. In this respect, some animal venoms known for their analgesic effects for quite a long time, turned out very attractive.

Still, changes in functional activity of endogenous antinociceptive systems under effects of zootoxins are underexplored for today thus limiting chances for generation of novel effective medications based on animal venoms, but increasing the risk of adverse side effects. Precise and detailed vision of physiological mechanisms underlying antinociceptive actions of animal venoms is obviously necessary for successful generation of zootoxin-based medications. Today, only fragmentary information on some mechanisms underlying antinociceptive actions of the Central Asian cobra venom is available.

Pain makes suffer million people worldwide; reducing life quality and adversely affecting social sphere and economics. Quality and findings from the pharmacological treatment of chronic pain are far from optimal, but novel methods of treatment are arising all the time [1]. Recently, the analgesic action of venoms from the Elapids has been found to be mediated by neurotoxins they contain; their synthetic analogues are widely used in the clinical settings [2]. Generation of novel medications eliminating chronic pain is the main goal in works with biotoxins, since the worldwide scope of pain is immense, but medications seem adequate and satisfying demand of pharmacological methods are unacceptably scarce. Medications generated on the basis of components isolated from venoms seem to be a serious foundation for generation of novel selective analgesics; clinical trials for some of them are under way at the moment. Biotoxins are a potent tool in elucidation of pain mechanisms, experimental checkup of conception featuring expression and activation of receptors, generation of neurotransmitters, as well as for understanding of signaling pathways [2]. Most potent medications prescribed in the pain syndrome are known to have the marked side effect manifesting in addiction (opioids), tolerance (paracetamol, fenazol), limitations for use

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(papaverin, ibuprofen, diclofenac, aspirin, indometacin) and absence of any antinociceptive effect in chronic pains of various geneses. The necessity for medications devoid of addictiveness and those with other mechanisms of analgesia is evident as well. The animal venoms, including those of snakes with their antinociceptive actions known for quite a long time, turned out one of sources for novel analgesics [3].

The animal venoms contain a cocktail of bioactive agents including proteins and peptides potentially to be used as medications in many medical spheres. Peptide biotoxins are a fertile source for analgesics; their effects are targeted at the wide spectrum of ion channels, participants to metabolic pathways for pain signal transfer. Properties of deeply studied biotoxins allow their using in treatment of some painful states, as well as of chronic pains [4]. Of vast diversity, biotoxins as potential medications are underexplored; mainly, peptides seem to be well studied [5]. Venomous animals were always considered as the sources of medications for treatment of diseases, what is more, quite long before mechanisms of action of peptides they contain became clear [8].

Snake venom contains rich mixture of peptides and other agents producing various pharmacological effects on CNS, muscular and vascular systems [6]. Antinociceptive, analgesic and anti-inflammatory effects of snake venoms are an object for permanent attention of scientists and pharmacologists [7]. Cobrotoxin is α-neurotoxin isolated from the venom of the Chinese or Taiwan cobra (*Naja atra*) [8]. Effects of cobrotoxin (found in the hot plate test and acetic acid-induced writhing test) demonstrated dose-dependent analgesia upon intraperitoneal administration, while injections into brain showed antinociceptive effect accomplished apart from the muscarinic acetylcholine or opioid receptors (atropine and naloxone were found to produce no effects) [9]. Cobrotoxin is used for treatment of patients with cancers to relieve chronic pains, but further studies for its safety are necessary [6]. In patients with inoperable cancers crotoxin was found to relieve pains [10, 11]. Per oral administration of venom from the Chinese cobra (*Naja atra*) was demonstrated to produce antinociceptive and anti-inflammatory effects in the rheumatoid arthritis model [12]. Studies on venom-based medications are presently intensifying, and seem to be prospecting, particularly in generation of analgesics from agents found in animals previously not studied [2].

Thus, analysis of publications on antinociceptive actions of snake venoms and components isolated from them suggests that the snake venoms of our region contain appropriate components with nociceptive action as well.

Materials and methods

To achieve the purposes of the study, the venom samples were acquired from Uzzoob'edinenie self-financing enterprise and stored at -18°C hermetically packed. Components with the target activity were obtained from the cobra venom chromatographically with the Superose 12 column (Pharmacia, Sweden)), desalinated on the column with Sephadex G-10 (Pharmacia, Sweden)), as packing material, freeze- dried and stored in the refrigerator. Purity and molecular masses of the venom fractions were determined by SDS-PAGE by Laemmli [13] and reversed phase high performance liquid chromatography.

The pain threshold and potential analgesic effect was assessed using hot plate test [12]. The time period from placing a mouse female on hot surface (54±0.5°C) to the appearance of behavioral response to the nociceptive stimulation, such as licking of front and hind paw pads and jumping, was registered [3]. The model makes possible determination of analgesic effect

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of future analgesics, peak time and duration of analgesia. Analgesic activity of materials under study was presented as mean latent time for suppression of pain response in the experimental group of animals. Specific pain response to chemical stimulation was assessed in the acetic acid-induced writhing test after intraperitoneal administration of the acetic acid solution (0.75%) in the volume of 0.1 ml per 10 g of body mass by calculation of writhings within 15 minutes after injection to each animal. Analgesic effect manifested by reduction in number of writhings in the presence of materials under study to be expressed in % to the control [3, 4].

The control animals in both models were administered with isotonic solution; for comparison of analgesic action ketonal was injected in the concentration of 20 mg/kg. Analgesic activity of materials under study was presented as mean latent time in the group of animals (n= 5-6).

All reagents used in the study were of reagent and analytical grade. Naloxone hydrochloride dihydrate (Moscow endocrine plant Federal State Unitary Enterprise, Moscow, Russian Federation), atropine (Dalhimfarm Open Joint Stock Company, Khabarovsk, Russian Federation) and ketonal for injections (LEK D D, Slovenia) were acquired in store chain. White inbred mice with body mass of 18-20 g were used for measurement of hemolytic activity of fractions by hemoglobin output of the washed erythrocytes from donor blood, phospholipase A_2 [19] activity by the inhibition of coagulation time of egg yolk, and toxicity [20] and analgesic effects of the materials under study in compliance with ethical principles for handling laboratory animals set at the Institute of Biophysics and Biochemistry under Mirzo Ulugbek National University of Uzbekistan (protocol No.4, 2021.25.03).

Statistical significance of differences between control and experimental values, determined for a data series using a paired t-test, where control and experimental values are taken together, and an unpaired t-test, when taken separately. A p-value <0.05 and <0.01 indicates a statistically significant difference. The results obtained are statistically processed in Origin 8.6 (Origin Lab Corporation, USA).

Results and Discussion

Snake venom has been known for its antinociceptive action since time immemorial; it was used to treat inflammation of the trigeminal nerve and tumor-caused pains. Venoms were obviously used without adequate knowledge about their sources and mechanisms underlying their analgesic effects. Recently, the analgesic action of snake venoms belonging to various taxonomic groups, particularly, the elapids including the Central Asian cobra (*N. oxiana Eichwald*) has been identified to be accomplished by neurotoxins they contain; their synthetic analogues found usage in the clinical settings [5, 6].

Traditional medicine mainly uses the venom of *N. oxiana Eichwald* as an external analgesic being used for injections extremely rare due to its high toxicity. At the same time, attempts to find any references to usage of fractions or components of this venom failed. Thereupon, it was interesting to assess potential analgesic effect of some components of the Central Asian cobra.

To search for and characterize protein-peptide components with novel, previously unexplored antinociceptive activity variants and conditions for chromatographic separation of the cobra venom were first selected. Superose 12 and weakly alkaline (pH 7.6) tris-HCL (50mM) turned out to be the most acceptable. As the result, whole cobra venom was separated in 7 protein peaks (S-1 – S-7); most of them being presented as polypeptides with molecular mass of 15-6.5 kDa **(Figure 1)**.

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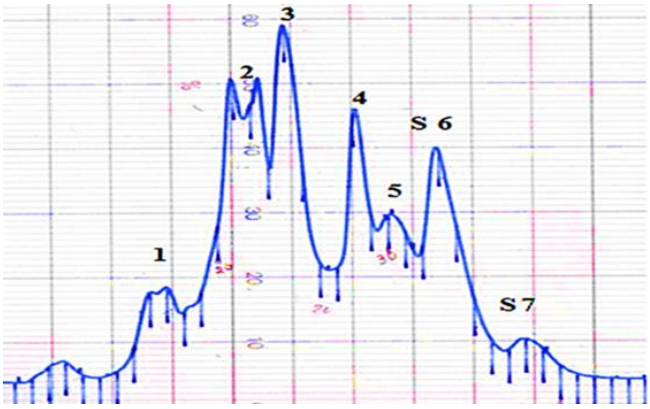

Figure 1. Fractionation of cobra venom on Superose 12. The cobra venom (50mg) was dissolved in 0.5 ml of 0.05M tris-HCl (pH 7.6), centrifuged for 10 min, the supernatant was applied on the column (15x80cm). Elution rate – 30 ml/h. Fractions (S-1 - S-7) were collected in the volume of 1.43 ml.

Electrophoretic analysis of the fractions demonstrated that in the first protein peak there were two main by mass components with molecular mass ranging from 55 to 65 kDa and some minor components (of < 94 and 30 kDa). The other peak fractions were found to contain mainly low molecular mass proteins and peptides with molecular mass ranging from 6.5 to 15 kDa **(Figure 2)**.

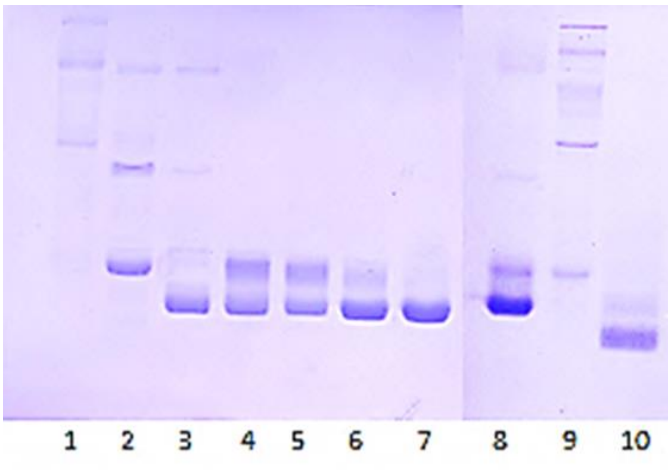

Figure 2. SDS electrophoresis of the cobra venom and its fractions on Superose 12. 1. S-1; 2. S-2; 3. S-3; 4. S-4; 5. S-5; 6. S-6; 7. S-7; 8. whole venom, 9. Markers with various molecular masses in kDa (94, 67, 43, 30, 20.1, 14.4); 10. melittin (2.84 kDa).

Problems in study on pain and nociception, as well as on medical analgesia are the topical directions in up-to-date biomedicine [2-4, 7]. Components with analgesic activity were obtained from venom of cobras inhabiting various regions of the world [9], but the components with similar activity in the venom of the Central Asian cobra were not studied ever. That is why, next stage aimed at studying potential antinociceptive activity of the venom and fractions S-1 – S-7 obtained. Antinociceptive effects were assessed in accordance with appropriate recommendations [2, 3] on the models of thermal and chemical pain stimulation in laboratory mice [7]. In hot plate test (thermal model) nociceptive response manifested in the hind paw-licking and jumping, while the prolongation of jump latent period at high temperature of the plate (54°C) was the evidence for effect of agents with lower analgesic potential [10].

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The materials were used in low doses (0.4 μg/kg) were dissolved in the physiological solution and administered to mice intraperitoneally in the volume of 150 mcL per animal. Responses of animals were registered at 30, 120 and 180 min after administration **(Table 1)**. The cobra venom (S-0) and fractions S-1 – S-7 can be seen to produce approximately equal analgesic effects in 30 min after administration increasing the latent time of response two times more than the one for the control; effectivity of the venom remains practically unchanged during the experiment. Among fractions under study, S-4 – S-7 peptide fractions were of the greatest interest as their effect was progressing by time (S-4, S-5 and S-7); the maximum target effect could be seen for S-7 fraction. In prospect, more detailed study on S-6 fraction similar to S-7 fraction by composition (Figure 2, lanes 6 and 7) is needed, as the dynamics of its effect was quite different.

Study on antinociceptive action of cobra venom and fractions obtained by chromatographic separation continued on the model of chemical stimulation widely used for screening. Its selectivity is relatively low, but there was positive correlation between effective doses of analgesics in the model and doses of medications producing the desired effect in the clinical settings [11].

Table 1. Analgesic activity of *N. oxiana Eichwald* venom and its fractions**,** including ketonal in the dose of 20 mg/kg and S-0 – S-7 in the dose of 0.4 μg/kg in 30, 120 and 180 min after administration. (M±m; n=6).

Agents	Animals (n)	Dose (μg/kg)/ mg/kg	Latent time (s)		
			In 30 min	In 120 min	In 180 min
Control	6		9.3±0.7	9.3±0.7	9.3±0.7
Ketonal	6	20	18.3±2.0*	17.6±1.0*	20.0±1.6*
S-0	6	0.4	16.4±1.2*	18.0±1.2*	11.0±1.0*
S-1	6	0.4	18.0±1.4*	37.0±1.4*	22.0±1.4*
S-2	6	0.4	40.0±3.0*	35.0±3.0*	35.3±3.0*
S-3	6	0.4	20.3±1.7*	58.0±1.7**	54.0±1.7**
S-4	6	0.4	18.0±1.2*	29.0±1.2*	≥60.0±1.2**
S-5	6	0.4	22.0±1.7*	58.0±2.3**	≥60.0±2.3**
S-6	6	0.4	27.0±2.3*	≥60.0±2.3**	≥60.0±1.2**
S-7	6	0.4	19.6±1.8*	41.0±1.8*	≥60.0±2.6**

Note: *p<0.05; **p<0.01

The whole venom (S-0) reduced number of writhings in 30 and 120 minutes while in 180 minutes the effect was not registered, that is, the analgesic effect of low venom doses is the short-term one (120 min) (Table 2). Fractions S-1 – S-3 demonstrated the tendency towards the suppression of responses to the pain stimulant, but the significant reduction in writhings by 2 and more times, as the criterion of analgesic effect [4], was registered only after administration of S-6 at 30 and 120 minute and of S-7 at 120 and 180 minute. Intensity of writhings is known to reduce by time, according to some opinion [12], serving as the obstacle for assessment of effect duration of the analgesic under study. Data in Table 2 for fraction S-7 appear all the more interesting as its analgesic effect increased with time.

Table 2. Analgesic activity of *N. oxiana Eichwald* venom and its fractions**,** including ketonal in the dose of 20 mg/kg and S-0 – S-7 in the dose of 0.4 μg/kg in 30, 120 and 180 min after administration in the acetic acid-induced writhing test. (M ± m; n=6).

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Agents	Animals (n)	Dose (μg/kg)/ mg/kg	Number of writhings within 15 min		
			In 30 min	In 120 min	In 180 min
Control	6		32.0±0.0	32.0±0.0	32.0±0.0
Ketonal	6	20	31.0±0.7*	26.0±1.0*	19.0±2.4*
S-0	6	0.4	27.0±1.0*	22.0±2.0*	32.0±2.0*
S-1	6	0.4	21.0±1.0*	29.0±1.0*	23.0±1.0*
S-2	6	0.4	22.7±1.0*	22.0±1.0*	25.0±1.0*
S-3	6	0.4	23.0±1.0*	21.0±1.0*	23.0±1.0*
S-4	6	0.4	18.0±1.2*	17.0±1.0*	22.0±1.0*
S-5	6	0.4	18.0±1.0*	17.0±1.0*	16.0±1.0*
S-6	6	0.4	15.7±1.0*	15.5±1.0**	16.5±1.0*
S-7	6	0.4	16.5±1.0*	15.5±1.1**	13.0±1.0**

Note: *p<0.05; **p<0.01

To sum up, fractions S-6 and S-7 arguably demonstrated the greatest analgesic action among materials under study. This action was accomplished due to the prolongation of the latent time for pain response development (by 4-5 times as compared to the control group) and reduction in number of writhings in both models used (Table 1 and Table 2, respectively). Acute toxicity for fractions S-6 and S-7 was determined using the probit analysis by Litchfield and Wilcoxon [13]. LD_{50} of S-6 and S-7 was determined to be 2.05 and 10.3 mg/kg, respectively.

Thus, fraction S-7 demonstrating the highest analgesic activity in both models turned out the least toxic one. It is pertinent to note that LD_{50} of the whole venom from the Central Asian cobra and particular neurotoxins NT-1 and NT-2 isolated from the venom is 0.48, 0.56 and 0.9 mg/kg, respectively. The findings suggest that fractions S-6 and S-7 possess low cytotoxicity as well. The suggestion was checked up in the experiments in suspension of the washed erythrocytes from donor human blood. Thus, fractions S-6 and S-7 towards the end of the 3rd hour (maximum time period recommended for determination of analgesic activity in the models used [3, 4]) demonstrated moderate hemolytic activity. Similarly low hemolytic activity was demonstrated by fractions S-1 and S-5 (data not presented), while the whole venom under the similar conditions demonstrated the laky blood effect, that is, caused the 100% lysis of erythrocytes [14].

Upon testing of the materials under study for activity of phospholipase A_2 in the egg yolk coagulation test it was found in fractions S-1 and S-2 (Figure 1). Upon introduction of fraction with the highest phospholipase activity (S-2, 10μg) into the incubation medium only S-6 and S-7 caused practically complete hemolysis (data not presented) demonstrating the synergic effect characteristic of cytocardiotoxins from cobra venom.

Neural networks involved into the reception and transfer of pain signal, as well as in processing and formation of response in the brain are still underexplored. Synaptic (sensor) connections of the spinal cord (nociceptive pathway) were identified and studied using neuro-, cytocardiotoxins and “short” peptides from the venoms of cobra and rattlesnakes interacting with one of 8 known types of alfa-subunits of nicotine acetylcholine receptors or cationic channels of synapses [15]. Thus, polypeptide with molecular mass of 6,714 Da and LD_{50} 2.69 mg/kg called najanalgesin demonstrating analgesic effect in both thermal and chemical models of pain was generated from the venom of *Naja atra* [16].

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To assess potential mechanisms underlying analgesic action of S-6 and S-7, fractions from the Central Asian cobra, blockers of pain opioid receptors (naloxone) and pain cholinergic receptors (atropine) were used.

The data summed up in **Figure 3A** demonstrated that naloxone administered to animals in the dose of 3 mg/kg increased the latent time for responses in the hot plate test insignificantly, while under conditions of the experiment S-6 and S-7 fractions increased it by 2.6 and 3.6 times on average, respectively. Administration of naloxone to mice suppressed analgesic effect of S-6 and S-7 (nearly by 29%) indicating the moderate contribution of opioid receptors to their antinociceptive action.

Administration of atropine, a blocker of cholinergic pain receptors, resulted in significant prolongation of latent time (**Figure 3B**) of response; effect of atropine reduced the parameter for S-6 by 1.6 times in 1 hour, while in 3 hours there was no effect of atropine registered. Administration of atropine and S-7 produced no effect on the action of the latter in 1 hour, while in 3 hours their total effect increased by 1.7 times.

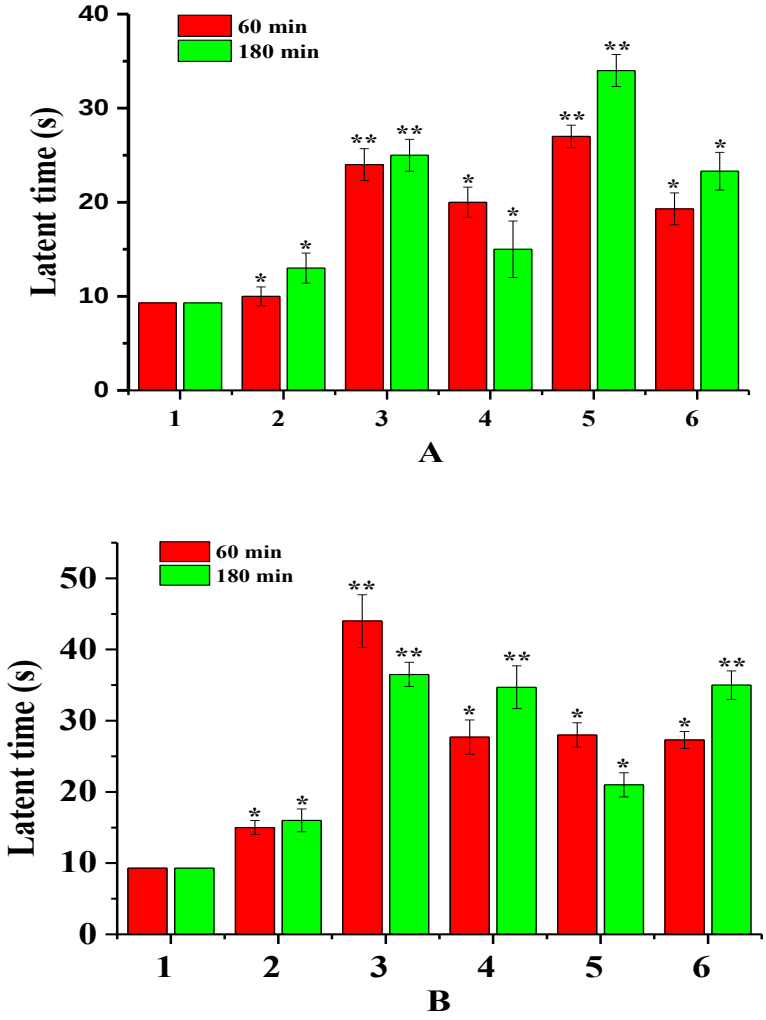

Figure 3. Effect of S-6 and S-7 on the latent period in the hot plate test after administration of naloxone (A) and atropine (B). A1 - Control; A2 - naloxone; A3- S-6; A4 – S-6+ naloxone; A5

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– S-7; A6 – S-7+ naloxone; B1 – Control; B2 - atropine; B3 – S-6; B4 – S-6+ atropine; B5 – S-7; B6 – S-7+ atropine.

Similar data were obtained in the acetic acid-induced writhing test (**Figure 4A**); naloxone produced no marked effect. Atropine reduced number of writhings in 3 hours increasing the effect of S-6 in 1 hour but producing practically no effect in 3 hours; while it produced no significant effect on the effect of S-7 at all (**Figure 4B**).

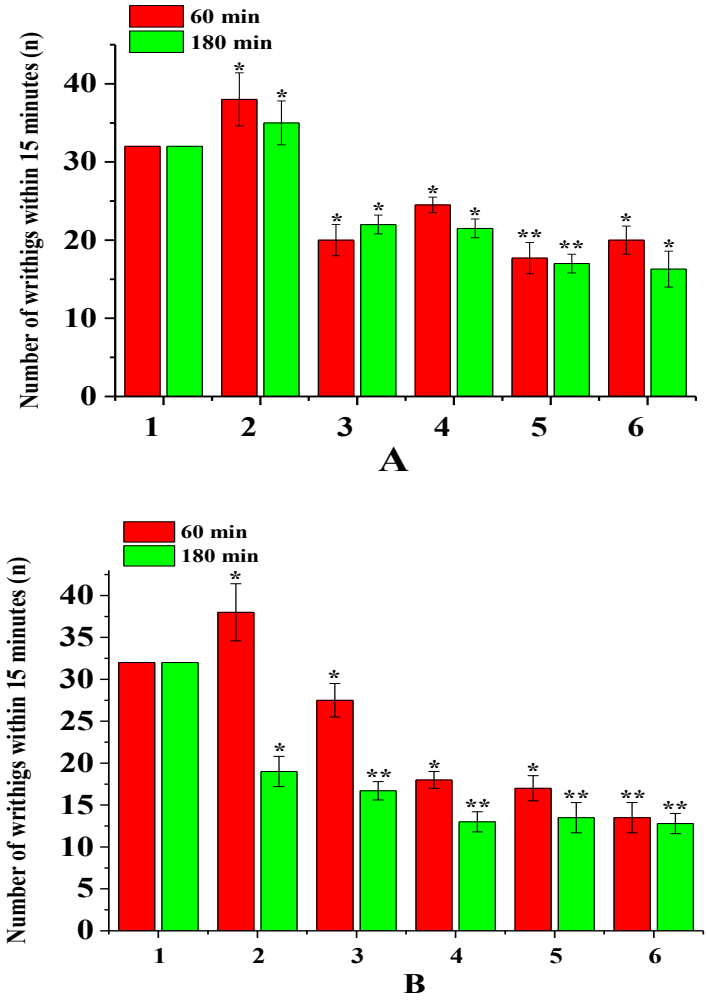

Figure 4. Effect of S-6 and S-7 on number of the acetic acid-induced writhings after administration of A1 - Control; A2 - naloxone; A3- S-6; A4 – S-6+ naloxone; A5 – S-7; A6 – S-7+ naloxone; B1 – Control; B2 - atropine; B3 – S-6; B4 – S-6+ atropine ; B5 – S-7; B6 – S-7+ atropine.

Thus, our findings suggest that analgesic effects of fractions under study could be accomplished by various mechanisms. For example, in the venom of the Chinese cobra (*Naja atra*) several peptides with analgesic activity were identified, to name najalgesin with antinociceptive effect by inhibition of c-Jun HN_2- kinase [4] and a "short" neurotoxin producing central analgesic action in low doses (25 μg/kg), but effect of hyperalgesia in high doses (100 μg/kg) interacting with adenosine receptors A_1 and A_{2a} [18]. Participation of other

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mechanisms differing from the receptor ones, including those of ionic channels (mambalgin 1 and 2, producing effect on H+-sensitive channels (ASICs)) of neurons [18] or direct effect on some links of nociceptive signaling pathways cannot be excluded [16]. Our findings suggest participation of central and peripheral nociceptive pathways in accomplishment of analgesic action of fractions S-6 and S-7. However, mechanisms of their analgesic action are sure to be further studied.

Conclusions

Our findings demonstrate presence of peptide components with low toxicity and marked antinociceptive action in the venom of the Central Asian cobra, *N. oxiana Eichwald.* Acting in low doses (0.4 - 4.0μg/kg), peptide-analgesics with molecular masses ranging from 6.5 to 15 kDa are characterized by low hemolytic and phospholipase activities and, as per acute toxicity data, cannot be neurotoxins. The peptide components with analgesic action can be considered as pharmacological agents in development of new generation analgesics with specific effects on mechanisms underlying generation and transfer of pain signal.

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