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ACTIVITY OF DIZOCILIPINE AND ITS COMBINATIONS WITH DANTROLENE AND LISURIDE ON THE LD50 PERIOD IN MICE WITH EXPERIMENTAL TETANUS

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ABSTRACT
Tetanus intoxication is a result of combined tetanus toxin binding in the organism: centrally in the spinal cord at the level of inhibitory synapses and peripherally at the level of the neuromuscular junction and muscle cell. Although acute intoxication is dominated by the central action of tetanus toxin, it is considered that, for the purpose of successful implementation of therapy, peripheral activity of the tetanus toxin should be also antagonized. Experimental tetanus was induced by intramuscular application of tetanus toxin. Application of substances on mice in experimental groups was performed after the occurrence of local tetanus in right leg, approximately 24 hours after administration of tetanus toxin. In this research, we attempted to normalize disorders caused by tetanus toxin using dizocilipine maleate (at doses of 0.01; 0.1; 1.0 and 2.0 mg/kg b.w.), alone and in combination with dantrolene (at dose of 2.0 mg/kg) and lisuride (at dose of 50.0 µg/kg) on the LD50 period in mice with experimental tetanus in the trial. Through our research, we found that dizocilipine had the best effect at a dose of 0.1 mg/kg b.w. Additionally, combination of dizocilipine and lizuride had no effect on the LD50 period, as compared to the control group.

KEYWORDS: Tetanus, Tetanus Toxin, Dizocilipine, Dantrolene, Lisuride.

INTRODUCTION
Tetanus is caused by the action of neurotoxin formed in the body by *Clostridium tetani*, a motile, Gram-positive, anaerobic, spore forming, rod-shaped bacteria. The toxin is produced during vegetative growth of the microorganism in suitable environment.[1,2] The toxin DNA is contained in a plasmid and it is antigenically homogenous. The microorganism's resistant spores are ubiquitous, with the natural habitat in moist, fertile soil, however they can survive indefinitely in dusty indoor environments. The spores can be incompletely destroyed at boiling temperature or eliminated by autoclaving at 120°C with pressure of 1 atmosphere during 15 minutes. *Clostridium tetani* can be isolated from the faeces of domestic[3-5] and laboratory animals[6,7] and also in humans, but presence of the microorganism does not indicate tetanus infection due to a lack of a plasmide in all strains.[1]

Tetanus develops when microorganism spores are introduced into the wounds of different origin such as superficial skin or penetration injuries, post parturition or ovariohysterectomy infection, ect.[8,9], but occasionally no obvious wound could not found (cryptogenic tetanus).[5-10] Tetanus toxin is one of the most potent known poisons on a weight basis because of its absolute neurospecificity and enzymatic function at the site of action. As little as 1 ng/kg will kill a mouse; 0.3 ng/kg will kill a guinea pig. The estimated minimum human lethal dose is less than 2.5 ng/kg (11). Humans and horses are most susceptible to tetanus, and so widespread vaccination against the disease is performed in these species. Disease occurs occasionally in dogs and is rare in cats. The development of tetanus in dogs and cats usually follows introduction of spores into a penetrating wound, but more than one third of affected dogs have no known wound history.[5] The global incidence of tetanus has been estimated at approximately one million cases annually. Mortality rates from tetanus vary greatly across the world, depending on access to healthcare, and approach 100% in the absence of medical treatment.[12]

Under anaerobic conditions in necrotic or infected tissue, the *Clostridium tetani* producing two toxins: tetanospasmin and tetanolysin.[1,5] Tetanolysin damages local tissue and provides optimal conditions for bacterial multiplication.[13-15] and does not appear to have clinical significance.[5]

Tetanospasmin is responsible for appearance of clinical signs of disease and it is consists of two sub-units, heavy (H) and light (L) chains, which are connected by disulphide bonds.[11-16] The carboxyl terminal portion of the H chain, termed HC, mediates attachment to gangliosides (GD1b and GT1b) on peripheral nerves, and subsequently the toxin is internalised[17] It is then moved from the peripheral to the central nervous system by retrograde axonal transport and trans-synaptic spread. The entire toxin molecule is internalised into presynaptic cells and, in a process requiring the HN fragment, the L chain is released from the endosome. The L chain is a zinc metalloprotease, which cleaves synaptobrevin.[11-13] A single base pair mutation in the L chain abolishes this proteolytic activity.[11-18] Synaptobrevin is an integral membrane component of synaptic vesicles and it is essential for the fusion of synaptic vesicles with the presynaptic membrane.[19] Outside the nervous system, synaptobrevin is found in endocrine cells.[20] Cleavage made by tetanus toxin L chain prevents release of their contents, the inhibitory neurotransmitter γ-aminobutyric acid (GABA), into the synaptic cleft. The α - motor neurons are therefore under no inhibitory control and undergo sustained excitatory discharge causing the characteristic motor spasms of tetanus. The toxin exerts its effects on the spinal cord, the brain stem, peripheral nerves, at neuromuscular junctions, and directly onto muscles. The extent to which cortical and subcortical structures are involved, remains unknown. Certainly, the toxin is a potent convulsant when injected into the cortex of experimental animals.[11,13,20] Since tetanus toxin in the animal or human body acts centrally in the spinal cord by preventing the release of inhibitory transmitters from the end of inhibitory neurons[7,21] it's also act peripherally: at the level of neuromuscular junction and at the level of muscule cell.[13] The consequence of this combined effect of tetanus toxin is manifested in the skeletal muscle spasm.[6,12,13,22] For this reason, in our research we tried to investigate action of substances which antagonize central and peripheral effect of tetanus toxin.

MATERIAL AND METHODS

The complete survey was conducted on albino mice of both sexes weighing around 20-25 grams (strain Pasteur Institute Novi Sad, bred at the Department of Pharmacology and Toxicology of the Veterinary Faculty in Sarajevo). For the purposes of this experiment, we used a control group (5 animals per group) and 4 experimental groups (10 animals per group). Ethics Committee of the Veterinary Faculty approved the research and experimental procedures (Approval No. 2/17, date 05.09.2017). The handling and care of animals was performed in accordance with CPCSEA guidelines for the use and care of experimental animals. The animals were kept in conventional conditions and treated according to the Animal Welfare Regulations. The animals were maintained on standard diet with free access to water and housed in groups of 5 (control group) and 10 (experimental groups) mice per cage for seven days prior to the experiment. All substances (dizocilipine maleate, dantrolene an dlisuride) used in the experiment were commercially available, highest purity reagents, from Sigma-Aldrich (St. Louis, MO) and tetanus toxin from Institute of Immunology, Zagreb, Croatia.

Prior to its administration, tetanus toxin was diluted in water for injection and was then administered intramusculary (i.m.) in the *m. gastrocnemius* of the mouse's right leg, at a dose of 0.2 µg per animal. We used this dose of tetanus toxin on the basis of previously conducted studies[6,7] when it was used to determine the period of LD_{50} of tetanus toxin for each group. At that time, it was established that a dose of 0.2 µg per animal administrated i.m. represents the dose which kills 50% of experimental animals within 48 hours, while a dose of 0.1 µg per animal kills 50% of experimental mice within 7 days.

Specific doses of dizocilipine maleate, dantrolene and lisuride intended for use in this experiment were established in the preliminary study due to lack of bibliographic data. Consequently, chemicals were administrated in following doses: dizocilipine maleate at doses of 0.01; 0.1; 1.0 and 2.0 mg/kg b.w.), dantrolene (at dose of 2.0 mg/kg b.w.) and lisuride (at dose of 50.0 µg/kg b.w.).

All substances used were diluted in water for injection and were administered intraperitoneally (i.p.). Application of the above mentioned substances on mice in experimental groups was performed after the occurrence of local tetanus in right leg, approximately 24 hours after administration of tetanus toxin. The substances were administered once per day, which was continued until the animal died. Each experimental group had its own control group, with application of tetanus toxin and the solvent (water for injections) in an equivalent way as the experimental group.

Basic statistical data diagnostics was conducted using Microsoft Excel® (Microsoft Office package, Microsoft, USA).

Induction of experimental tetanus

First signs of local tetanus in the animal's right leg were registered 24 hours after application of tetanus toxin. Leg was stiff and extended, and during this period no death of experimental animals was recorded. In the next 48 hours, general tetanus started to develop, followed by animal's death. Time in hours, when 50% of experimental animals died, was marked as the LD_{50} period in mice with experimental tetanus.

RESULTS

Effects of N-methyl-D-aspartate (NMDA) receptor antagonist dizocilipine maleate (MK-801) and its combination with direct muscle relaxants dantrolene and lisuride on the LD_{50} period in mice with experimental tetanus, are presented in Tables 1 and 2, respectively.

Intraperitonealy administrated dizocilipine maleate at a dose of 0.01 mg/kg b.w. had a minimum effect on the LD_{50} period in mice with experimental tetanus, while a dose of 0.1 mg/kg b.w. slightly prolonged the LD_{50} period in mice in the experiment. Doses of 1.0 and 2.5 mg/kg b.w. shortened the LD_{50} period in mice with experimental tetanus, as compared to the control group (Table 1).

Table 1. – Effect of different i.p. doses of dizocilipine maleate (D) on the LD50 period in mice with experimental tetanus

The data presented in Table 2. showed that co-administration of dizocilipine maleate with dantolene and lisuride had no effect on the LD_{50} period in mice with experimental tetanus

Tabela 2. The effects of combined i.p. application of dizocilipine maleate (D) with dantolene (DA) and lisuride (L) on the LD_{50} period in mice with experimental tetanus

Table. 1: Effect of different i.p. doses of dizocilipine maleate (D) on the LD_{50} period in mice with experimental tetanus.

Group	Number of animals in group	T (µg/per animal) D (mg/kg b.w.)	LD_{50} period
Control group (T)	5	0.2	113.15 ± 7.51
Experimental groups			
T+D	10	0.2 + 0.01	118.7 ± 7.52
T+D	10	0.2 + 0.1	127.6 ± 6.98
T+D	10	0.2 + 1	102.3 ± 5.32
T+D	10	0.2 + 2.5	99.5 ± 4.75

T = tetanus toxin, D = dizocilipine maleate, b.w. = body weight

Table. 2: The effects of combined i.p. application of dizocilipine maleate (D) with dantolene (DA) and lisuride (L) on the LD_{50} period in mice with experimental tetanus.

Group	Number of animals in group	T (µg/ per animal) D (mg/kg b.w.) DA (mg/kg b.w) L (µg/kg b.w)	LD50 period
Control group (T)	5	0.2	113.15 ± 7.51
Experimental groups			
T+D+DA	10	0.2 + 0.1 + 2	112.3 ± 5.88
T+D+L	10	0.2 + 0.1 + 50	107.1 ± 3.45

T = tetanus toxin, D = dizocilipine maleate, DA = dantrolene, L = lisuride; b.w. = body weight

DISCUSSION AND CONCLUSIONS

The syndrome, which occurs in tetanus intoxication, is a result of tetanus toxin activity or its component tetanospasmin. This component of tetanus toxin has a strong affinity to inhibitory synapses in the ventral horn of the spinal cord, which prevents the release of inhibitory neurotransmitters.[11,13,20] This results in a prevail of excitatory (stimulatory) neurons of the spinal cord and uncontrolled stimulation of voluntary muscles, as reflected by their spasms.[22]

The previous studies have shown that tetanus toxin has a very poor effect on the postsynaptic membrane[23], so that the membrane function is preserved. This is supported by the positive results obtained in this experiment after administration of antagonists of excitatory neurotransmitters. If this was not the case, the substances examined would not be able to bind to receptors located on this membrane, so their effect would be absent.[24]

During the tetanus intoxication, there is a prevail of excitatory transmitters, such as aspartate, so we tried with application of dizocilipine maleate (MK-801), which is a N-methyl-D-aspartate (NMDA) receptor antagonist, to examine its effect in the treatment of illness. We were, however, unable to find data in the accessible bibliography on the use of dizocilipine maleate in the treatment of tetanus, so our results could not be compared with those of other authors.

The first part of the experiment was carried out to determine the effective dose of dizocilipine maleate (Table 1). In the experiment, dizocilipine maleate at doses of 0.01, 0.1, 1.0 and 2.5 mg/kg b.w. were administered to mice, in accordance with findings of our preliminary reseach. This research was carried out on a smaller number of experimental animals, and based on results obtained, our opinion was that the above mentioned doses will have an effect on the LD_{50} period in mice with experimental tetanus. The dose of 0.01 mg/kg b.w. had a minimum effect on the LD_{50} period in mice in the experiment. On the other hand, administrated doses of 1.0 and 2.5 mg/kg b.w. did not produce the expected results and had in fact shortened this period. Due to unsatisfactory results, these doses were not further used in our research. It was found that dizocilipne maleate had an effect only at a dose of 0.1 mg/kg b.w.,

which slightly prolonged the LD_{50} period in experimental group of mice, compared to the control group. Thus, application of dizocilipine maleate in this dose showed to be the only effective method (Table 1).

Since tetanus toxin also has peripheral activity at the level of the neuromuscular junction and the muscle cell itself, and that all this contributes to the development of muscular spasm, we tried, in addition to central, to antagonize the peripheral effects of tetanus toxin.

Therefore, we combined dizocilipine maleate with direct-acting skeletal muscle relaxants dantrolene and lisuride. However, from the obtained results, it can be clearly seen that combination of dizocilipine maleate with dantrolene and lisuride had no effect on the LD_{50} period in mice with experimental tetanus (Table 2).

According to these results, it can be concluded that dizocilipine maleate, in a dose of 0.1 mg/kg b.w. slightly prolonged the LD_{50} period in the experimental group compared to the control group of mice with experimental tetanus, in dose od 0.01 had a minimum effect, while doses of 1.0 and 2.0 mg/kg b.w. shortened the LD_{50} period compared to the control group. Therefore, the use of dizocilipine maleate for treatment of experimental tetanus in mice in doses 1.0 and 2.5 mg/kg b.w. is highly contraindicated; while use of 0.01 mg/kg b.w. provides week beneficial effect.

Co-administration of dizocilipine maleate with dantrolene and lisuride had no effect on the extension of this period.

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