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THE EFFECT OF COMPETITIVE AND NONCOMPETIVE ANTAGONIST OF ASPARTATE ON THE SURVIVAL TIME OF MICE WITH EXPERIMENTAL TETANUS

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ABSTRACT
Tetanus is dangerous, infectious, noncontagious, acute and mostly afebrile illness of humans and different animal species, which is caused by Gram positive, ubiqutarious anaerobic *Clostridium tetani.* It produces a specific neurotoxin, which consists of two components: tetanospasmin and tetanolysin. Tetanospasmin is a component of tetanus toxin which actually leads to emergence of clinical signs. Mechanism of action of tetanus toxin is still unknown, but it is clear that the main target site is the spinal cord, where it blocks the release of inhibitory transmitters which results in domination of excitatory transmitters. In our experiment, we tried to normalize the disorders which are results of tetanus toxin action by applying indol-2-carboxylic acid (at doses of 5, 10, 20, 30 and 50 mg/kg b.w.) and to investigate its combination with ketamine (at dose of 10 mg/kg b.w.) and aminooxyacetic acid (at dose of 20 mg/kg b.w). Experiments were conducted on albino mice of both sexes. Experimental tetanus was induced by intramuscular application of tetanus toxin at a dose of 0.2 µg per animal. Application of substances in experimental groups was performed after the occurrence of local tetanus in right leg, approximately 24 hours after administration of tetanus toxin. Through our research, we found that indol-2-carboxylic acid had the best effect at a dose of 20 mg/kg b.w. Additionally, combination of indol-2-carboxylic acid with ketamine and aminooxyacetic acid had weak effect on the half time survival period in mice with experimental tetanus in the trial, as compared to the control group.

KEYWORDS: Tetanus toxin, indol-2-carboxylic acid, ketamine, aminooxyacetic acid.

INTRODUCTION
Tetanus, also known as lockjaw, is a very dangerous infectious, acute, usually afebrile disease characterized by muscle spasms. The causative agent of the disease is bacteria *Clostridium tetani.* The *C. tetani* is a gram positive, obligate anaerobic bacillus, while mature microorganisms lose their flagella after the development of spores (Cato, 1986). These spores are extremely stable, and even though boiling temperature for 15 minutes kills most of microorganisms, some will survive unless autoclaved at 120°C, 1.5 bars, for 15 minutes, which ensures sterility. Tiny amounts of toxin are thought to be present in a typical infection. It is estimated that a lethal dose for adult humans is approximately 2.5 ng/kg body weight (b.w.), while a lethal dose for mice is 1.0 ng/kg b.w. (Gill, 1982). The bacteria usually enters the human or animal organism through damaged skin (Collee & van Heyningen, 1990). 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 (Hassel, 2013).

Clostridium tetani produces a specific neurotoxin - tetanus toxin (Atkinson, 2012). The toxin is produced during vegetative growth of the microorganism in suitable environment (Cook et al., 2001; Dupuy & Matamouros, 2006). Tetanus toxin consists of two components: tetanospasmin and tetanolysin (Schiavo et al., 1992). Tetanospasmin is responsible for appearance of clinical signs of disease, while tetanolysin damages the surrounding tissue and helps bacterial reproduction (Pinder, 1997). Tetanospasmin consists of the heavy (H) and light (L) chains (Doussau et al., 1999)

interconnected by disulfide bonds. The H chain is responsible for the specific binding of the synaptic vesicle membrane and facilitates the entry of a light chain into the vesicle interior, while the light chain is a carrier of toxic properties and is also responsible for specific proteolytic activity within the cell (Doussau i sar., 1999). The L chain cleaves synaptobrevin (Schiavo et al., 2000). Synaptobrevin is an integral membrane component of synaptic vesicles and it is essential for the fusion of synaptic vesicles with the presynaptic membrane (Hardman & Limbird, 1996). Outside the nervous system, synaptobrevin is found in endocrine cells (Baumert et al., 1989). Cleavage made by tetanus toxin L chain prevents the release of their contents, i.e. the inhibitory neurotransmitter γ-aminobutyric acid (GABA), into the synaptic cleft. The α-motor neurons are, therefore, under no inhibitory control and thus they 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, neuromuscular junctions, and directly onto muscles. The extent to which cortical and subcortical structures are involved remains unknown. However, it is well known that the toxin is a potent convulsant when injected into the cortex of experimental animals (Montecucco & Schiavo, 1994).

Therefore, by conducting this research, we attempted to examine the effect of indol-2-carboxylic acid (competitive antagonist of aspartate) and its combination with ketamine (noncompetitive antagonist of aspartate) and aminooxyacetic acid (precursor of γ-aminobutyric acid or GABA) on survival time in mice with experimental tetanus in the trial.

MATERIALS AND METHODS

Materials

Complete experiment was conducted on albino mice of both sexes weighing approximately 20-25 grams (strain swiss albino mice 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 3 experimental groups (10 animals per each group). Ethics Committee of the Veterinary Faculty had approved research and experimental procedures (Approval No. 1/17, date 20.05.2017). 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 10 mice per cage for seven days prior to the experiment.

Substances used in the experiment

- Tetanus toxin (Institute of Immunology, Zagreb, Croatia);
- Indol-2-carboxylic acid (Sigma-Aldrich, USA);
- Ketamine (Pharmaceutical Research Department, F. Hoffmann-La Roche CO., Ltd., Ch-4002 Basel, Switzerland);
- Aminooxyacetic acid (Sigma-Aldrich, USA).

Methods

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 (Hadžović et al., 1975; Muminović, 1983) when it was used to determine the period of time for LD_{50} of tetanus toxin for each group. At that time, it was established that a dose of 0.2µg 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.

Ketamine and amonioxyacetic acid were diluted in water for injection, and indol-2-carboxylic acid, due to its poor solubility, was diluted in 10 mM solution of NaOH. Specific doses of indol-2-carboxylic acid, ketamine and aminooxyacetic acid, 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: indol-2-carboxylic (at doses of 5, 10, 20, 30 and 50 mg/kg b.w.), and aminooxyacetic acid (at dose of 20 mg/kg b.w.) were administrated intraperitoneally (i.p.), whilst ketamine (at dose of 10 mg/kg b.w.) was administrated intramusculary (i.m.). 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 animal's death. 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 by using Microsoft Excel® (Microsoft Office package, Microsoft, USA).

RESULTS

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.

Application of substances

Effects of indol-2-carboxylic acid (competitive antagonist of aspartate) and its combination with ketamine (noncompetitive antagonist of aspartate) and aminooxyacetic acid (inhibits 4-aminobutyrate aminotransferase or GABA-T activity) on the survival

half time in mice with experimental tetanus, can be seen from the following results presented in the table 1.

Table 1: Effects of indol-2-carboxylic acid (I2CK), and combination of indol-2-carboxylic acid with ketamine (K), as well as combination of ketamine with aminooxyacetic acid (AOK) on the survival time of mice.

Groups	N	Substances	Doses (µg/kg, b.w.; mg/kg b.w.)	Survival Time (h)
Control	5	TT	0.2 µg/tj.m.	112.05 ± 10.05
Experimental	10	TT + I2CK	0.2 µg/kg, /tj.m. + 5 mg/kg, tj.m.	107.35 ± 6.67
			0.2 µg/kg, tj.m. + 10 mg/kg, tj.m.	118.85 ± 6.75
			0.2 µg/kg, tj.m. + 20 mg/kg, tj.m.	142.88 ± 9.51
			02 µg/kg, tj.m. + 30 mg/kg, tj.m.	132.43 ± 9.82
			0.2 µg/kg, tj.m. + 50 mg/kg, tj.m.	97.7 ± 10.74
	10	TT + I2CK + K	0.2 µg/kg, tj.m. + 20 mg/kg, tj.m. + 10 mg/kg, tj.m.	119.0 ± 11.19
	10	TT + K + AOK	0,2 µg/kg, tj.m. + 10 mg/kg, tj.m. + 20 mg/kg, tj.m.	102.7 ± 3.22

N = number of animals; b.w. = body weight

Intraperitoneally administrated indol-2-carboxylic acid at doses 5 and 10 mg/kg b.w. had no effect on the survival time compared to the control group. A dose of 30 mg/kg b.w., indol-2-carboxylic acid prolonged this period, whilst the dose of 20 mg/kg b.w achieved the best results. The dose of 50 mg/kg b.w. shortened the survival time of mice with experimental tetanus as compared to the control group.

Co-administration of indol-2-carboxylic and ketamine caused slight extension of survival time in mice with experimental tetanus.

Combination of ketamine with aminooxyacetic acid had no effect on the survival time compared to the control group mice with experimental tetanus.

DISCUSSION

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 (Doussau et al., Lalli et al., 1999). This results in a prevail of excitatory (stimulatory) neurons of the spinal cord and uncontrolled stimulation of voluntary muscles, which is reflected in their spasms (Schiavo et al., 2000).

The previous studies have shown that tetanus toxin has a very poor effect on the postsynaptic membrane (Halpern & Neale, 1995; Zamula, 1996), 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 (Montecucco & Schiavo G, 1994).

In researches conducted by Hadžović et al. (1976) and Muminović (1983), it was established that inhibitory transmission can be slightly increased by application of aminooxiacetic acid (precursor of GABA), as well as with combination of aminooxiacetic acid and glycine, substances which are used to stimulate inhibitory transmission. Using a combination of these substances, the above mentioned authors succeeded in significantly

prolonging the survival time of mice with experimental tetanus.

We were, however, unable to find data in the accessible bibliography on the use of indol-2-carboxylic acid 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 competitive antagonist of aspartate indol-2-carboxylic acid (Table 1). Through our research, we found that indol-2-carboxylic acid had the best effect at a dose of 20 mg/kg b.w. and slightly weaker in dose of 30 mg/kg b.w. With this doses, the survival time of experimental group was prolonged, when compared to the control group of mice with experimental tetanus. However, the application of doses of 5 and 10 mg/kg b.w. did not give the expected results, and dose of 50 mg/kg b.w. shortened the survival time of mice with experimental tetanus, so these doses were not further used in our research. Since we achieved the best results by using indole-2-carboxylic acid at a dose of 20 mg/kg b.w., this dose was further used in our experimental research.

In order to expand the research, we combined indol-2-carboxylic acid and ketamine as a noncompetitive antagonist of aspartate. We assumed that concomitant use of indol-2-carboxylic acid and ketamine could lead to summarizing of their effects. These combinations of substances gave a slight extension of survival time in mice with experimental tetanus, compared to the control group.

In this experiment, we also combined the application of ketamine with aminooxyacetic acid. Aminooxyacetic acid inhibits (GABA-T) activity *in vitro* and *in vivo*, leading to less ɣ-aminobutyric acid (GABA) being broken down. Subsequently, the level of GABA is increased in tissues. From the results obtained, we can conclude that combination of ketamine with aminooxyacetic acid had no effect on the survival time compared to the control group of mice with experimental tetanus.

CONCLUSION

Based on these results, it can be concluded that indol-2-carboxylic acid, in a doses of 20 and 30 mg/kg b.w. prolonged the survival half time in the experimental group compared to the control group of mice with experimental tetanus. However, the application of doses of 5, 10 and 50 mg/kg did not give the expected results. Co-administration of indol-2-carboxylic acid with ketamine led to slight extension of survival time in mice with experimental tetanus, whereas combination of ketamine with aminooxyacetic acid had no effect on the extension of this period.

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