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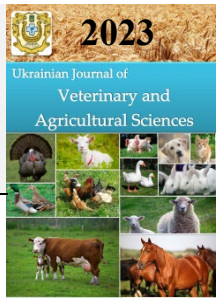

Pharmacological evaluation and preclinical studies of hypochlorous acid solution

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Abstract

The article presents materials on the electrochemical synthesis of hypochlorous acid and its pharmacological and toxicological evaluation. In the market of veterinary drugs, special attention has been paid to long-known, potent detoxifying antimicrobial agents based on active oxygen obtained by the electrolysis method. In addition to a broad spectrum of antimicrobial action, such drugs have several other advantages, especially the biogenic nature, which causes the absence of allergic reactions. New electrocatalysts were proposed for the electrochemical synthesis of hypochlorous acid, which was produced according to the following method using a combined electrochemical-pyrolytic method. VT1-0 technical titanium was used as a current collector. The current collectors were subjected to several preliminary preparation steps, such as NaOH degreasing and etching in 6 M HCl. Initial nanotubes were obtained by anodizing Ti foil in ethylene glycol with 0.3 wt.% ammonium fluoride and 2 vol.% water for 4 hours. The electrochemical reduction was carried out in 1 M $HClO_4$ by cathodic polarization for 1 hour. Later, a thin discontinuous layer of platinum or consecutive layers of platinum-palladium were applied to the base by electrodeposition. Nitrite electrolytes for platinization and phosphate-palladation were used for this purpose. Depending on the task, platinum, and palladium on the ground's surface varied from 0.1 to 2.0 mg/cm^2. The obtained material was heat-treated in an air atmosphere. At this stage, the surface layers of composites were formed due to the oxidation of the base and encapsulation of platinum and palladium particles in titanium oxide. It was established that the solution of hypochlorous acid, obtained by the electrolysis method, is a low-hazard substance that belongs to the fourth class of toxicity. Its half-lethal dose (DL_{50}) is not determined. The fact that, in nature, hypochlorite acid is formed by granulocytes of neutrophils involved in the last link of phagocytosis confirms that the resulting solution is low-toxic, environmentally safe, and incapable of causing side effects and distant consequences. The obtained results proved the perspective of using new technology for producing hypochlorite acid for veterinary medicine; its development is highly relevant, clinically expedient, and economically justified.

Keywords: hypochlorous acid solution; electrocatalyst; acute toxicity; sublethal dose (DL_{50}).

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1. Introduction

One of the main reasons that prevent the full realization of the genetic potential of animals is non-communicable diseases, while diseases of the digestive system accompanied by intoxication syndrome take one of the first places in terms of frequency, mass, and amount of economic damage. Detoxification agents occupy a special place based on the pathogenetic therapy of such diseases. Therefore, searching for and developing affordable, effective, safe, and environmentally friendly veterinary drugs is particularly important (Bacanlı & Başaran, 2019; Hunchak et al., 2020; Bigdan et al., 2020; Zhou et al., 2021; Brezvyn et al., 2021).

Given the above, special attention should be paid to long-known, potent detoxifying, antimicrobial agents based on active oxygen: solutions of weak hypochlorous acid (HOCl), sodium (NaClO), and calcium (Ca (ClO)2) hypochlorites obtained by electrolysis. In recent years, these solutions have begun to be widely used in practice, the scope of their application in human and veterinary medicine is expanding, and much positive experience has been gained in their use. In addition to a broad spectrum of antimicrobial action, such drugs have several other advantages, especially the biogenic nature, which causes the absence of allergic reactions (McKenna & Davies, 1988; Prütz, 1996; Rosen et al., 1998; Ono et al., 2012; Pelgrift & Friedman, 2013; Taharaguchi et al., 2014).

Hypochlorous acid is the predominant form of oxygen-containing compounds of chlorine (I) in aqueous solutions in the pH 4–8 range. At lower pH values, molecular chlorine is the main form; at higher pH values, hypochlorite ion is the main form. Hypochlorous acid is a potent oxidizing agent and readily reacts with various organic substances and biomolecules. As a result of the destruction of hypochlorite acid, chloride ions, and water are formed. Solutions of hypochlorous acid are colorless, the physical and chemical properties of which depend on the concentration. It is not considered harmful, as it exists in low concentrations in the body of humans and animals. Produced in the body of humans and animals by white blood cells to fight infections and has a broad spectrum of antimicrobial activity.

We proposed new electrocatalysts for the electrochemical synthesis of hypochlorous acid, which were manufactured according to the following method using a combined electrochemical-pyrolytic method. VT1-0 technical titanium was used as a current collector. The current collectors were subjected to several preliminary preparation steps, such as NaOH degreasing and etching in 6 M HCl. Initial nanotubes were obtained by anodizing Ti foil in ethylene glycol with 0.3 wt.% ammonium fluoride and 2 vol.% water for 4 hours. The electrochemical reduction was carried out in 1 M HClO4 by cathodic polarization for 1 hour. Later, a thin discontinuous layer of platinum or consecutive layers of platinum-palladium were applied to the base by electrodeposition. Nitrite electrolytes for platinization and phosphate-palladation were used for this purpose. Depending on the task, platinum and palladium on the surface of the base varied from 0.1 to 2.0 mg/cm^2. The obtained material was heat-treated in an air atmosphere. At this stage, the surface layers of composites were formed due to the oxidation of the base and encapsulation of platinum and palladium particles in titanium oxide.

The introduction and use in the practice of veterinary medicines require careful and detailed study of applied and theoretical aspects of their influence on the functioning of the body as a whole and its protective systems in particular. Particular attention is paid to the complex study of drugs, multifaceted and objective studies are conducted using many main and auxiliary criteria (determination of toxicity, species sensitivity, the presence of terato-, muta- and allergogenic effects, immunotoxicity, etc (Gutyj et al., 2017; Karpenko et al., 2022; Kushnir et al., 2022; Martyshuk et al., 2022).

Based on the above, our work aimed to provide a pharmacological and toxicological evaluation of the action of the obtained hypochlorite acid solution.

2. Materials and methods

The electrocatalytic activity of the obtained materials in the hypochlorite acid synthesis reaction was evaluated under the electrolysis of 0.05 M NaCl solutions in a duct cell with a Ti cathode at 25 ºC. The cathode area varied, so the current density was 40 mA/cm^2. The pH of the solutions was monitored with a pX-150MI universal ionometer. The volumetric current density in the channel cell was 2.4 A/dm^3, and the volumetric rate of solution supply was 9.5 l/h.

Determination of the content of hypochlorous acid was carried out according to the following method. 10 cm^3 of a 1 M solution of acetic acid, 2 cm^3 of the test solution, and 5 cm^3 of a 10 % potassium iodide solution were sequentially introduced into a 250 cm^3 titration flask. The solution was stirred, covered with a watch glass, and kept in a dark place for 5 minutes. The iodine released as a result of the reaction was quickly titrated with a 0.002 N sodium thiosulfate solution to a straw color, 1 cm^3 of a 0.5 % starch solution was added as an indicator, and the titration was continued until the blue color of the iodostarch complex disappeared, and the solution became colorless. Measurements were repeated at least three times. The content of hypochlorous acid in mg/dm3 was calculated according to the formula:

$$C(ClO^-) = \frac{[V_1 \cdot K \cdot C(Na_2S_2O_3)] \cdot M(1/2\,NaClO) \cdot 10^3}{V},$$

where V_1 – the volume of sodium thiosulfate solution used for titration of hypochlorous acid, cm ^{3};

M(1/2 NaClO) = 37,25 g/mol;

$C(Na_2S_2O_3)$ – the concentration of sodium thiosulfate;

K – correction factor for sodium thiosulfate concentration;

V – the volume of the sample used for analysis (2 cm^3).

Preclinical studies of the obtained hypochlorite acid solution were carried out on laboratory animals in the vivarium of the DNDKI of Veterinary Medicines and Feed Additives (Lviv), according to the methods and techniques described in the monograph "Preclinical studies of veterinary medicinal products" (2006) (Kotsiumbas et al., 2006). All manipulations with laboratory animals that were involved in the experiment were carried out following the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the "General Ethical Principles of Animal Experiments", adopted by the First National Congress on Bioethics (Kyiv, 2001).

Study of acute toxicity and establishment of the class of harmfulness of a solution of hypochlorous acid obtained by electrolysis on laboratory rats.

For the acute toxicity study, 40 purebred rats, aged 4–5 months, with a body weight of 110–150 g, were kept on a standard diet. All animals were housed and fed in the same conditions with free access to water and feed. According to the principle of analogs, four groups of 10 heads each were formed by random sampling, considering body weight as a determining indicator. The experiment lasted seven days. Rats of the I (control) group were injected intragastrically with 10 ml of distilled water during the day; rats of II, III, and VI groups – hypochlorous acid solution with a concentration of 1200 mg/l in 15, 20, and 25 ml, respectively; administration was carried out in small doses with an interval of 5 hours.

The toxic effect of the solution obtained in the electrolyzer on the body of rats was studied by observing the behavior of experimental animals and recording their clinical condition and death.

Study the effect of a solution of hypochlorous acid obtained by electrolysis on the body of intact rats under the conditions of repeated administration.

The study was conducted on 40 purebred white rats, aged 4–5 months, with a body weight of 180–200 g, which were kept on a standard diet with free access to water and food. According to the principle of analogs, four groups of animals, ten animals in each, were formed by random sampling, considering body weight as a determining indicator. The experiment lasted 20 days. The hypochlorous acid solution was administered intragastrically using a needle with a blunt end, 5 ml daily. Animals of the 1st group (control)

received 5 ml of saline. The rats of the experimental groups received 5 ml of hypochlorous acid solution of different concentrations: II group – 50 mg/l; III group – 100 mg/l; IV group – 1000 mg/l.

Daily clinical observations were carried out during the experiment, recording the timing of the possible development of toxicosis and the death of animals. On the 10th and 20th days, blood was taken from the rats for hematological studies from the femoral vein under light ether anesthesia, after which they were euthanized by the method of cervical dislocation, macroscopic analysis of internal organs was performed, and the relative weight of internal organs was determined (liver, spleen, kidney) by generally accepted methods (Kotsiumbas & Velichenko, 2009; Vlizlo et al., 2012; Kotsiumbas et al., 2013).

Statistical processing of the obtained results was carried out using the Student's t-test. The standard package of Microsoft Excel, a statistical analysis program, was used for the relevant calculations. The difference was considered significant at P values < 0.05.

3. Results and discussion

A necessary condition for the complex preclinical studies of new therapeutic agents is the study of the acute toxicity of the drug. These studies are a stage for obtaining information about the safety/dangerousness of the medicinal product, as well as a start for further conducting a series of experiments to study its effect on the body of laboratory animals under conditions of repeated administration and high doses. Such studies determine the successful development of further preclinical and clinical research and have a decisive impact on the possibility of creating a highly effective competitive veterinary drug.

At the experimental stage of developing a solution of hypochlorous acid by electrolysis, we conducted primary toxicological studies and gave a pharmacological assessment of the new medicinal substance.

The study of the toxicological parameters of the solution of hypochlorous acid obtained by electrolysis began with determining toxicity and the semi-lethal dose (DL_{50}) and establishing the class of harmfulness in laboratory rats.

The obtained observation results during the experimental period are shown in the Table 1.

Table 1

Clinical observations of rats in an experiment on the study of acute toxicity of hypochlorous acid solution (M ± m, n = 10)

Indicators	Group of animals			
	Control, H_2O	Concentration of HOCl* 1200 mg/l		
	I – 10	II –15	III – 20	IV – 25
Initial body weight, g	180.0 ± 2.7	180.4 ± 3.2	180.2 ± 3.1	180.1 ± 2.4
Final body weight, g	186.2 ± 2.7	184.9 ± 1.7	186.5 ± 5.7	182.3 ± 3.3
Body temperature, ºC	38.7 ± 0.1	38.5 ± 0.5	38.3 ± 0.3	38.5 ± 0.4
Blood pressure, mm Hg. Art.	90 – 130	90 – 130	90 – 130	90 – 130
Frequency of breathing, movements/min.	117 ± 4	115 ± 6	116 ± 7	114 ± 2
Disorders of gastrointestinal tract	Absent	Absent	Absent	Absent
Heart rate, movements/min.	387 ± 7	389 ± 9	388 ± 7	389 ± 5
Inadequate reactions	Absent	Absent	Absent	Absent
Death of animals	Absent	Absent	Absent	Absent

Note: *HOCl – hypochlorous acid obtained by electrolysis

During the entire observation period, inadequate reactions and death of animals were not observed. The experimental animals were active, outwardly tidy, had a good appetite, and responded to sound and light stimuli; reflex and neuromuscular excitability remained. Violations of breathing rate, heart rate, urination, and defecation were not noted. There were no spontaneous "atypical" behavioral reactions. The experimental rats did not differ from the animals of the control group in terms of physiological parameters, behavior, attitude to feed and water, the condition of the external mucous membranes, and the function of the gastrointestinal tract and urinary system.

During the experiment, the rats of the experimental and control groups experienced slight fluctuations in body weight gain during the observation period. Still, no significant difference was found between the control and experimental groups. Therefore, the solution of hypochlorous acid, obtained by the electrolysis method, is a low-hazard substance that belongs to the fourth class of toxicity. A half-lethal dose (DL_{50}) has not been determined because hypochlorous acid is a natural endogenous component in humans and animals. At the same time, it is an essential part of the innate immune system. Hypochlorous acid is produced by granulocytes of neutrophils involved in the last link of the oxidative pathway directly when the cell detects the arrival of a foreign substance that undergoes phagocytosis. This phagocytic phenomenon leads to the secretion of reactive oxygen species and hydrolytic enzymes (Kavros, S. F.). This fact characterizes the solution of hypochlorous acid obtained by electrolysis as a low-toxic, environmentally safe veterinary drug that is not capable of causing side effects and minor consequences.

Study of the effect of a solution of hypochlorous acid obtained by the electrolysis method on the body of intact rats under the conditions of repeated administration.

The study of toxicity under the conditions of repeated administration of sufficiently high concentrations of ginochloric acid was determined by detecting possible physiological and morphological changes in the body of intact rats and determining the dependence of these changes on the concentration.

Under the conditions of intragastric administration of various concentrations of hypochlorous acid solution to rats during the experiment, constant clinical observation of the animals was carried out. The rats of the experimental groups were mobile and willingly ate food; no deaths of animals were established. Only on the 20th day in the IV group rats, where the concentration of hypochlorous acid was 1000 mg/l, a slight decrease in activity and inhibition was observed in comparison with animals of the control group.

When examining the skin, mucous membranes, natural openings, and macroscopic examination of internal organs after dissection of animals of II (50 mg/l), III (100 mg/l), and IV (1000 mg/l) groups, no signs of intoxication or other manifestations of inflammation were found, pathological processes. During the pathological autopsy, the internal organs' size, color, consistency, and natural location did not exceed the physiological norm. From the study of the coefficients of the relative mass of the internal organs of the rats of the experimental groups, no changes were registered either (Table 2).

Due to its reactivity, constant movement, and performance of several functions, blood plays a crucial role in adapting to the effects of various toxic factors on the body (Gutyj et al., 2018; Varcholyak & Gutyi, 2019). Changes in peripheral blood under the influence of the studied solution are shown in the Table 3.

Table 2

Coefficients of the relative mass of internal organs of rats on the 20th day of the experiment, under the conditions of repeated administration of hypochlorite acid solution (M ± m, n = 5)

Organs	Group of animals / Concentration of *HOCl			
	I control	II 50 mg/l	III 100 mg/l	IV 1000 mg/l
Liver	3.92 ± 0.08	3.84 ± 0.07	3.87 ± 0.04	4.09 ± 0.09
Both kidneys	7.27 ± 0.37	7.38 ± 0.49	7.40 ± 0.12	7.15 ± 0.59
Spleen	2.68 ± 0.05	2.51 ± 0.78	2.69 ± 0.68	2.43 ± 0.18

Note: *HOCl – hypochlorous acid obtained by electrolysis

Table 3

Hematological and biochemical indicators in rats using different concentrations of hypochlorous acid solution (M ± m, n = 5)

Indicators	Group of animals	10th day	20th day
Erythrocytes, 10^{12}/l	I (Control)	6.34 ± 0.12	6.32 ± 0.32
	II 50 mg/l	6.29 ± 0.27	6.58 ± 0.71
	III 100 mg/l	7.28 ± 0.21	6.30 ± 0.48
	IV 1000 mg/l	6.52 ± 0.33	6.15 ± 0.46
Leukocytes, 10^{9}/l	I (Control)	13.28 ± 0.57	13.68 ± 0.66
	II 50 mg/l	14.86 ± 0.45	15.82 ± 0.47
	III 100 mg/l	15.16 ± 0.47	16.16 ± 0.56
	IV 1000 mg/l	15.50 ± 0.46	16.28 ± 0.78
Hemoglobin, g/l	I (Control)	134.28 ± 1.69	136.25 ± 2.81
	II 50 mg/l	129.33 ± 5.02	139.63 ± 3.18
	III 100 mg/l	131.71 ± 2.32	134.00 ± 2.49
	IV 1000 mg/l	140.85 ± 3,26	139.67 ± 1.92
Total protein, g/l	I (Control)	68.8 ± 0.69	69.5 ± 0.81
	II 50 mg/l	69.3 ± 0.02	69.6 ± 0.18
	III 100 mg/l	67.7 ± 0.32	69.5 ± 0.49
	IV 1000 mg/l	68.5 ± 0.26	69.7 ± 0.52
Albumin content, g/l	I (Control)	34.2 ± 0.69	36.5 ± 0.31
	II 50 mg/l	36.3 ± 0.02	39.6 ± 0.18
	III 100 mg/l	37.7 ± 0.32	39.5 ± 0.49
	IV 1000 mg/l	36.5 ± 0.26	39.7 ± 0.52

Repeated use of sufficiently high concentrations of the hypochlorite acid solution obtained by the electrolysis method led to inevitable fluctuations in the studied indicators, but they were within the physiological norm.

As can be seen from the results presented in the Table 3, the effect of the hypochlorous acid solution on hematological indicators is noticeable in animals of the III and IV groups, while in rats of the II group, these indicators were at the level of the control group. Thus, the number of erythrocytes, leukocytes, and hemoglobin concentration in the blood of rats of the III group increased on the 20th day, which may indicate the stimulation of hematopoietic function by the studied solution (100 mg/l). At the same time, in experimental rats of the IV group, the values of these indicators slightly decreased in comparison with the animals of the III group. Still, these indicators were within the physiological norm for this type of animal. Therefore, introducing high concentrations of hypochlorous acid did not affect the studied hematological parameters.

An essential indicator of the intensity of metabolism in the animal body is the level of total protein. This indicator of blood serum reflects the supply of nutrients to the body. During the observation period in the experimental rats, it did not undergo significant complications; it was within the physiological norm.

Albumins comprise the most significant part of blood proteins, play an essential role in maintaining oncotic blood pressure, and transport many biological substances: carbohydrates, lipids, certain hormones, and trace elements. As can be seen from Table 3, when high concentrations of hypochlorous acid were introduced, this indicator was also within the physiological norm.

Toxic reactions that can appear after the administration of high doses of drugs are due to the selective tropism of each component to different tissues of the body, as a result of which neuro-, hepato-, and nephrotoxic reactions occur. Such changes can be diagnosed after a comprehensive toxicity study, considering biochemical and morphological

changes. That is why, when studying the degree of metabolic and toxic load on the body of rats after introducing a solution of hypochlorous acid in high concentrations, an increase in the erythrocyte index of intoxication was found in separate groups of experimental animals.

As can be seen from the research results given in the Table 4, during the experiment, a probable increase in the erythrocyte index of intoxication was observed in rats of the IV group on the 10th day by 30 % ($P < 0.05$) and, especially on the 20th day, it was 57.3 times higher ($P < 0.001$), compared with a control group. In our opinion, such pronounced changes may indicate that the concentration of the hypochlorous acid solution (1000 mg/l) caused an increase in endogenous toxins that can cause changes in the permeability of erythrocyte membranes.

Table 4

Erythrocyte index of intoxication in rats under the influence of different concentrations of HOCl solution, % (M ± m, n = 5)

Indicator	Group of animals	Duration of the experiment	
		10th day	20th day
Erythrocyte index of intoxication, %	I (Control)	16.5 ± 0.12	17.6 ± 0.45
	II 50 mg/l	15.4 ± 0.36	17.8 ± 0.53
	III 100 mg/l	14.1 ± 0.29	13.4 ± 0.27
	IV 1000 mg/l	21.5 ± 0.16*	27.7 ± 0.43**

Note: *HOCl - hypochlorous acid obtained by electrolysis.
Probability to control: $^{*}P < 0.05$; $^{**}P < 0.01$.

The results of the research confirm that the solution of hypochlorous acid, obtained by the electrolysis method, at a concentration of 50 and 100 mg/l does not have a toxic effect on the body of rats, which is indicated by the absence of inflammatory processes and fluctuations of the studied hematological and biochemical parameters within the physiological norm. Using a solution of hypochlorous acid at a concentration of 1000 mg/l for 20 days caused a slight increase in endogenous toxins, which can cause changes in the permeability of erythrocyte membranes to the body of rats, as indicated by the obtained research results.

Thus, summarizing the conducted research and considering the low toxicity of the hypochlorous acid solution obtained by the electrolysis method, it is advisable to use it as a highly effective, environmentally friendly, cheap preventive, and therapeutic agent in veterinary medicine.

4. Conclusions

1. A new technique for the electrochemical synthesis of hypochlorous acid using electrocatalysts of the combined electrochemical-pyrolytic method is proposed.

2. Conducted preclinical studies of hypochlorite acid obtained in an experimental electrocatalyst established that the obtained substance is low-hazard and belongs to the fourth class of harmlessness.

3. The results proved the perspective of using new technology to produce hypochlorite acid for veterinary medicine. The development is exceptionally relevant, clinically appropriate, and economically justified.

The perspective of further research: Carrying out a series of toxicological studies to study the effect of hypochlorous acid, obtained by electrolysis, on the body of animals in a chronic experiment. Study of pharmacodynamics and pharmacokinetics of hypochlorous acid and its effect on hematological and biochemical indicators in laboratory animals. Development of control methods of oxygen-containing compounds and study of the stability of the obtained hypochlorous acid solution.

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Conflict of interest

The authors declare no conflict of interest

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