BIOLOGICAL RESEARCH

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CHOLINE MONITORING TO ASSESS INFLAMMATORY HEPATITIS

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The aim *of the study is to create a rapid and reliable biosensor for determining choline in biological media.*
Materials and methods. *Choline oxidase (EC 1.1.3.17, 17 U/mg) from Arthrobacter globiformis was purchased from Sigma. Hydrogen peroxide (30 %, v/v aqueous solution), choline chloride, and bovine serum albumin (V fraction) (BSA) were obtained from Sigma. Glutaraldehyde 25 % was obtained from Merck KGaA. Semipermeable terylene membrane, 12 μM thickness, 0.4 μM diameter of pore were obtained from the Institute Joint Institute of Nuclear Research. The body fluid samples from mice were obtained from the National University of Pharmacy, Ukraine.*
The layer consisting of 5 μL of a solution comprising ChOx, BSA, and glutaraldehyde was formed on the inner surface of the ring-fixed semipermeable terylene film (working area Ø 2.4 mm) by creating a membrane. Subsequently, it was maintained at 4 °C for a period of 12 hours. The enzymatic membrane was mechanically affixed to the Pt electrode's surface, forming a biosensor. Chronoamperometric measurements were conducted with a custom-made potentiostat (Vilnius University, Life Sciences Centre, Institute of Biochemistry), utilizing a conventional three-electrode electrochemical cell comprising a platinum auxiliary electrode, a saturated Ag/AgCl reference electrode, and the biosensor as the working electrode.
To simulate inflammation, we recreated the model of acute toxic tetrachloromethane hepatitis. Hepatitis is an acute or chronic inflammation of the liver, caused by various factors: intoxication with household substances, poisons, drugs, alcohol, autoimmune, and infectious processes. To simulate inflammation, we recreated the model of acute toxic tetrachloromethane hepatitis by the method of O. V. Stefanov. The study was conducted at the Biomedical Research Laboratory of the Educational and Research Institute of Applied Pharmacy of the National University of Pharmacy.
Results. *A reagentless amperometric choline biosensor was developed and characterized using the enzyme choline oxidase from Arthrobacter globiformis (ChOx). The biosensor showed rapid response, appropriate stability, and sensitivity to choline when acting in model and in real biologic media. Since choline is a product of esterase-catalyzed reactions, the activity of esterases can be evaluated via choline release. This study revealed the increased concentrations of choline in the samples of the model of acute toxic tetrachloromethane hepatitis compared to control animals.*
Conclusions. *The ChOx based biosensor is a reliable tool for the monitoring of choline in biological media, such as blood serum. The activity of esterases can be evaluated via choline release. Consequently, measured esterase activity by choline-type biosensors could serve as biomarkers for the assessment of hepatitis-type inflammation dynamics.*
This is also highly relevant for the study of the pharmacological action of drugs with the expected anti-inflammatory effect
Keywords: *choline biosensor, choline oxidase, esterase activity, hepatitis, inflammation monitoring*

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1. Introduction.

Inflammation is one of the leading pathogenetic components of a wide variety of diseases, both infectious and non-infectious in nature [1].

The mesenchymal inflammatory syndrome, which is one of the leading pathogenetic links in liver diseases, determines the activity of the pathological process and develops as a protective reaction, aimed at localizing and neutralizing the pathological factor (antigenic nature) [2, 3].

The inflammatory process in the liver is accompanied by a vascular reaction, changes in vascular wall permeability, phagocytosis, and exudate formation [4, 5].

As a rule, the inflammatory response involves several steps, including tissue destruction, disrupting blood flow, and the proliferation of cellular elements, each of which aims to repair the damaged area [6–9]. In recent decades, a clear connection between various inflammation processes and the activity of enzymes belonging to the esterase family has been demonstrated.

Esterases are enzymes that catalyze the hydrolytic cleavage of esters into alcohols and acids in cells with the participation of water molecules (hydrolysis). Esterases belong to the class of hydrolases. The group of esterases includes those that act on triglyceride carbon esters (lipase), phospholipids, acetylcholine, as well as on cholesterol esters or simple alcohols [10–12].

A typical esterase is cyclic phosphodiesterase, which catalyzes the hydrolysis of the phosphodiester bond in cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), tagging cAMP- and cGMP-mediated signal transduction. Cyclic phosphodiesterases are often targeted by drugs due to their different distribution in different cell types [13, 14].

Another common esterase is alkaline phosphatase, which is a homodimeric protein. Each monomer contains a magnesium ion, which is essential for catalytic function, and two zinc ions, and is most active under alkaline conditions. Alkaline phosphatase plays a key role in liver metabolism and bone development [15, 16].

There are others that are active on inorganic acid esters, such as phosphorus (phosphomonoesterase, phosphodiesterase, nuclease) or sulfur (sulfatase) [17]. They are usually involved in important phases of digestion and intermediate metabolism. Esterases are involved in the cleavage reactions of an ester bond in organic compounds. The exterases include numerous specific enzymes: cholinesterase, chlorophyllase, tannase, pectase, etc. [18, 19].

Esterases are enzymes also involved in drug metabolism, known to be reduced in frail older people. The mechanism for this is not well understood [20]. This relationship highlights the importance of their potential applications as markers for investigating inflammation processes. Determining the starting point of the inflammatory process and predicting its dynamics are very important, not only in acute circumstances involving organ transplantation or implantation but also for the investigation of the efficacy of drugs.

In the presented studies, cholinesterase plays a key role. Cholinesterase is an enzyme that breaks down choline esters and is usually used in relation to acetylcholinesterase, which breaks down the neurotransmitter acetylcholine into choline and acetic acid. Cholinesterase is present in all cholinergic synapses, where acetylcholine is rapidly broken down during the transmission of a nerve impulse. Cholinesterase is involved in the transport of ions across the membranes of blood cells and skeletal muscle, in the regulation of excitability and contractility of smooth muscle and myocardium [21–23]. Serum cholinesterase levels are used as an indicator of liver synthetic activity. A decrease in serum cholinesterase activity is usually accompanied by a decrease in albumin concentration and an increase in transaminase activity. Restoration of enzyme activity indicates normalization of liver function [24–26]. In turn, choline has a highly effective lipid metabolism, resulting in a decrease in the amount of adipose tissue deposits in the liver (lipotropic effect), which negatively affect the functional properties of hepatocytes, which can cause a violation of the stable functioning of the organ. Excessive amount of adipose tissue in the liver leads to fatty degeneration and even hepatosis. Choline increases the liver's ability to detoxify metabolic products and xenobiotics (toxic agents, drugs, etc.) [27–30].

This study presents an approach to a rapid esterase evaluation technique using a reagentless amperometric biosensor. The activity of esterase can be effectively assessed by quantifying the concentration of the product, choline, generated during the catalyzed reaction. The enzyme choline oxidase (ChOx), immobilised in the biosensor membrane, was an element that ensured high analysis selectivity. The biosensor's efficacy was examined to monitor esterase activity in a model type of inflammation - hepatitis. Modern electrochemical biosensors offer a simpler, cost-effective, and sensitive alternative to traditional methods [31]. The efficacy of the biosensor was assessed in monitoring esterase activity during a hepatitis model of inflammation.

The aim of the research was to create a rapid and reliable biosensor for determining choline in biological media.

2. Materials and methods

Choline oxidase (EC 1.1.3.17, 17 U/mg) from *Arthrobacter globiformis* was purchased from Sigma. Hydrogen peroxide (30 %, v/v aqueous solution), choline chloride, and bovine serum albumin (V fraction) (BSA) were obtained from Sigma. Glutaraldehyde 25 % was obtained from Merck KGaA. Semipermeable terylene membrane, 12 μM thickness, 0.4 μM diameter of pore were obtained from the Institute Joint Institute of Nuclear Research. The body fluid samples from mice were obtained from the National University of Pharmacy, Ukraine.

A 0.02 M phosphate buffer solution (PBS) was prepared by mixing stock solutions of KH_2PO_4 and Na_2HPO_4. All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared with distilled water.

The layer consisting of 5 μL of a solution comprising ChOx, BSA, and glutaraldehyde was formed on the inner surface of the ring-fixed semipermeable terylene film (working area Ø 2.4 mm) by creating a membrane. Subsequently, it was maintained at 4 °C for a period of 12 hours. The enzymatic membrane was mechanically affixed to the Pt electrode's surface, forming a bio-

sensor [32]. Chronoamperometric measurements were conducted with a custom-made potentiostat (Vilnius University, Life Sciences Centre, Institute of Biochemistry), utilizing a conventional three-electrode electrochemical cell comprising a platinum auxiliary electrode, a saturated Ag/AgCl reference electrode, and the biosensor as the working electrode [33]. These measurements were conducted in a 1.65 mL thermostated electrochemical cell at 20°C. The current-time responses of the biosensor to multiple concentrations of choline and H_2O_2 were monitored at a working potential of 0.6 V versus Ag/AgCl in a stirred PBS buffer solution (pH 7.2). In assessing body fluid samples of mice, 50 µL of samples were added to the cell.

To simulate inflammation, we recreated the model of acute toxic tetrachloromethane hepatitis. Hepatitis is an acute or chronic inflammation of the liver, caused by various factors: intoxication with household substances, poisons, drugs, alcohol, autoimmune, and infectious processes. To simulate inflammation, we recreated the model of acute toxic tetrachloromethane hepatitis by the method of O. V. Stefanov [34]. The animals were kept in the same conditions, on a standard diet in accordance with sanitary and hygienic requirements [35].

The study was conducted at the Biomedical Research Laboratory of the Educational and Research Institute of Applied Pharmacy of the National University of Pharmacy in autumn 2024 (September-November).

The study was conducted following the Law of Ukraine «On Medicinal Products» and in accordance with the requirements of the SEC of the Ministry of Health of Ukraine for preclinical trials, taking into account the provisions of Directive 86/609/EC of the European Parliament and of the Council of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States on the protection of animals used for experimental and other scientific purposes, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 «On the Protection of Animals Used for Scientific Purposes» (Protocol of the Commission on Bioethics of the National Academy of Sciences No. 14 of 10.04.2024) [36].

The experiments were carried out on white outbred male rats weighing 0.22-0.25 kg, divided into two days groups of 6 animals each: intact control - animals without pathology; pathology control – animals with model pathology (tetrachloromethane hepatitis).

Liver pathology was modeled by a single intragastric injection of a 50 % oil solution of tetrachloromethane at a dose of 0.8 ml per 100g of animal weight for two days with a 24-hour interval in the control pathology group. Animals were withdrawn from the experiment on the third day in compliance with the rules of bioethics, anesthetized by decapitation under thiopental anesthesia, and blood was collected for research.

3. Results and discussion

This study gave special attention to creating a reagentless amperometric biosensor using the enzyme choline oxidase (ChOx) from *Arthrobacter globiformis* (EC 1.1.3.17). Choline oxidase catalyzes the oxidation of choline to betaine aldehyde (Fig. 1). This reaction produces a secondary product, H_2O_2 [37]. The amount of H_2O_2 produced is directly proportional to the amount of choline and is a base of action of usual ChOx-based biosensors. As seen from the inset of Fig. 1, the biosensor responses are fast, which allows for a fast analysis.

In fact, an efficient H_2O_2 oxidation or reduction on the electrode surface of such biosensors is crucial since, finally, the anodic or cathodic current of H_2O_2, generated in this process, will be proportional to the esterase activity in the sample [38].

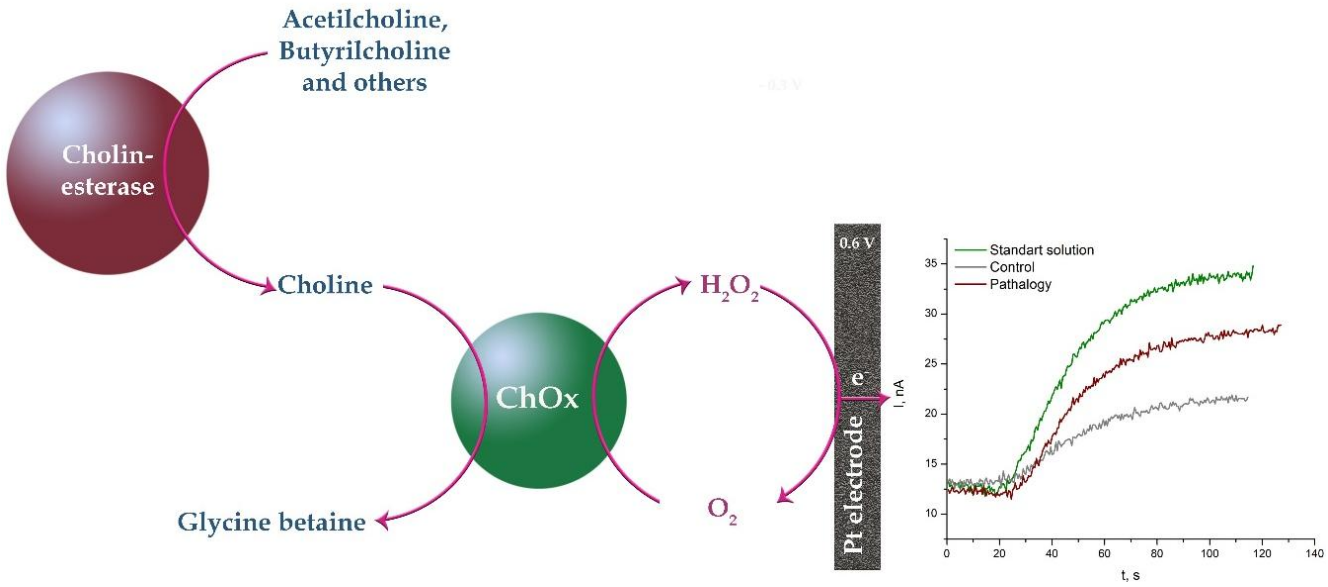

Fig. 1. Principle of detecting choline esterase activity based on the amperometric action of the choline biosensor and its current-time responses

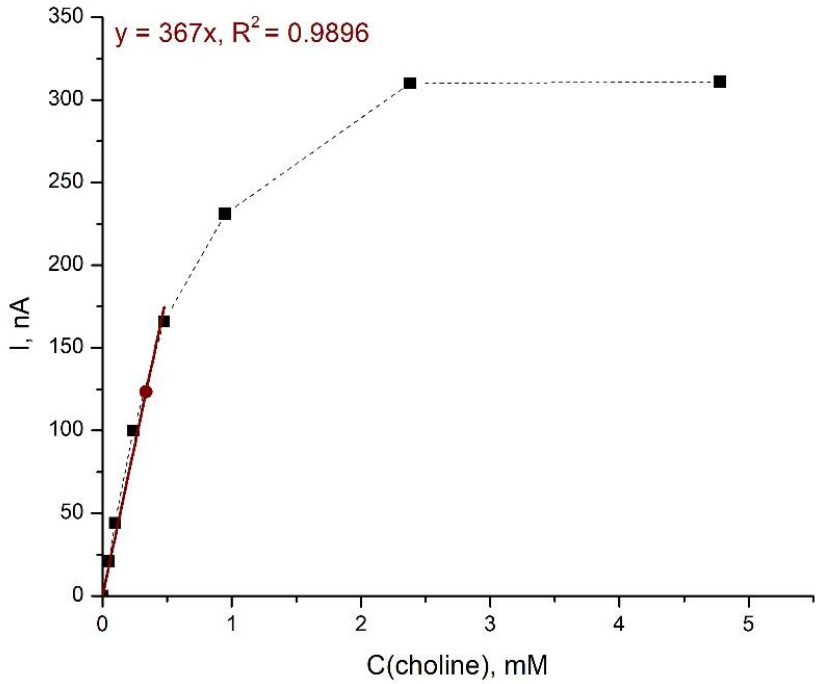

Fig. 2. Dependence of steady-state current density (I) on choline concentration, measured under potentiostatic conditions at + 0.6 V *vs* Ag/AgCl in a stirred PBS, pH 7.2, 20 °C

Therefore, at the beginning of the study, the effectiveness of H_2O_2 oxidation using the proposed biosensor was investigated. Fig. 2 illustrates the calibration curve of the biosensor for choline detection.

The data shows that H_2O_2 oxidation is highly efficient (sensitivity 367nA/mM) at a potential of 0.6 V in a PBS of pH 7.2. This pH value was chosen, so there would be no difference between the pH value of the medium in the electrochemical cell and the tested serum added. However, ChOx activity increases towards alkalinity (data not shown); therefore, if necessary, the sensitivity of the analysis can be increased by increasing the pH of the buffer solution [39].

Stability and repeatability studies are demonstrated in Fig. 3. The coefficient of variation (CV), calculated for responses to 0.1 mM of choline, was 0.5% (Fig. 3, left), which reveals the reliable performance of the biosensor over the course of two weeks (Fig. 3, right).

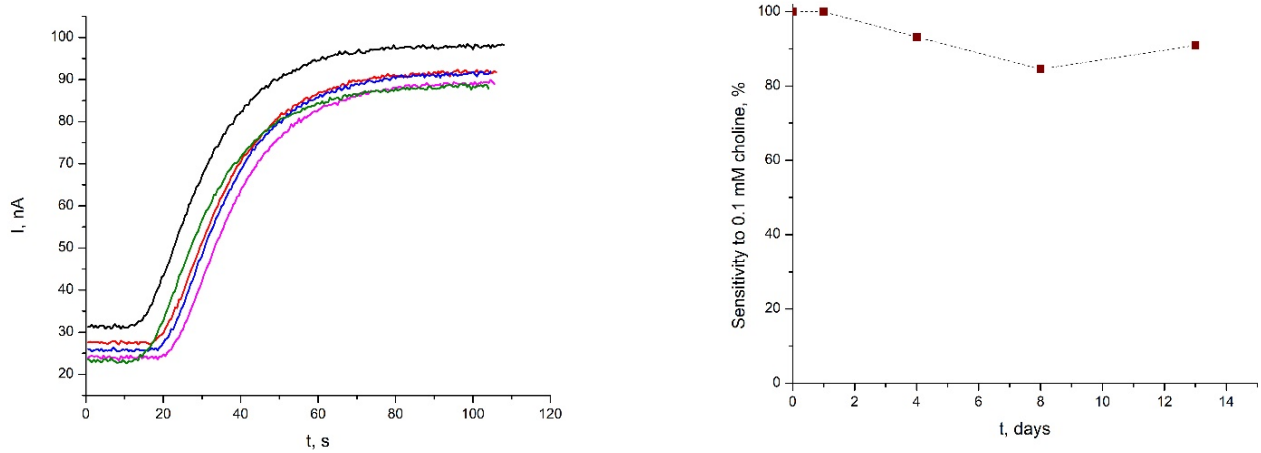

Fig. 3. Repeatability (left) and stability (right) of the choline biosensor

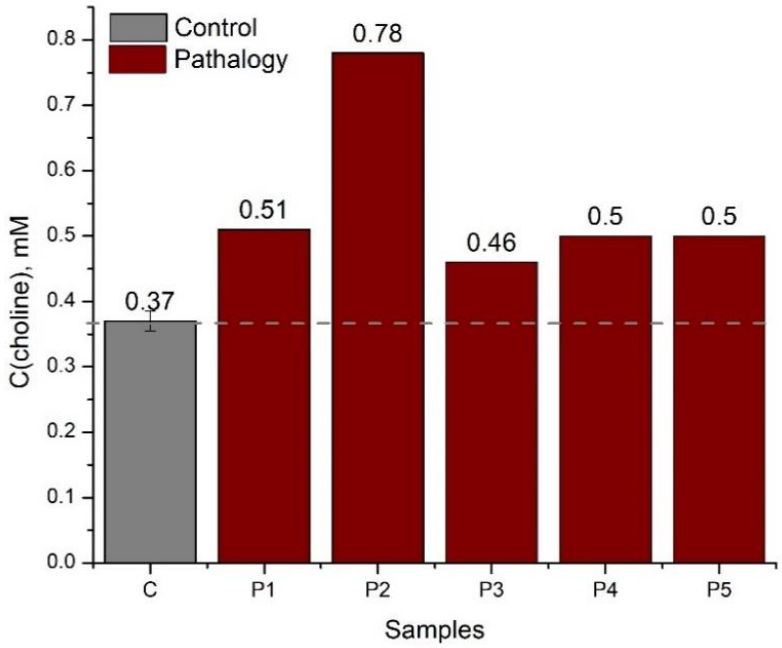

Fig. 4. Concentrations of choline in blood serum samples were obtained using a choline biosensor at + 0.6 V vs. Ag/AgCl in a stirred PBS, pH 7.2, 20 °C

Blood serum samples from control and hepatitis-infected rats were analyzed to clarify the differences in choline concentrations in the absence and presence of an inflammatory process. Hepatitis is an acute or chronic inflammation of the liver, caused by various factors: intoxication with household substances, poisons, drugs, alcohol, autoimmune and infectious processes [40]. Fig. 4 clearly demonstrates that lower choline concentrations were found in the serum of the control rats than in the diseased rats.

Preliminary studies show that all diseased rats had higher choline concentrations than controls. The choline amount varied from 0.46 to 0.79 mM for diseased rats, while in controls, choline concentration did not exceed 0.37 mM.

The analysis of biological samples, such as blood serum, has been reliably performed using this biosensor thanks to the additional discriminatory layer, used in its design, which prevents the penetration of substances other than H_2O_2 towards the electrode.

Prospects for using the research results. The device with the new biosensor, created based on the results of the work, should be competitive in the modern world market in the diagnosis of diseases, filling the niche of fast, inexpensive, easy-to-use diagnostic tools that are constantly evolving. The development of a method for determining the activity of esterases as an inflammatory marker based on an electrochemical biosensor will allow for rapid real-time analysis with the ability to work with opaque samples. The need for such devices is justified by the proper selectivity of the analysis, provided by the enzymes present in the biosensor, the low cost of analysis, and the high prospect of integrating the biosensor into a simple, low-volume electrochemical analysis.

Limitations of the study. The possibility of conducting research is limited by the sociopolitical situation in the country, the lack of opportunities to purchase reagents for a broad study of the antioxidant system of experimental animals, and difficult communication with a foreign partner institution.

Prospects for further research. Further studies are needed, including a bigger set of esterases acting in a wider variety of inflammations.

4. Conclusions

The ChOx biosensor showed rapid response, appropriate stability, and sensitivity to choline. Since choline is a product of esterase-catalyzed reactions, the activity of esterases can be evaluated via choline release. The choline-type biosensors that monitor esterase activity could be biomarkers for assessing inflammation dynamics.

This is also very relevant for studying the pharmacological action of drugs with the expected anti-inflammatory effect. To establish the anti-inflammatory effect of the drugs under study, it is desirable to monitor changes in the concentration of inflammatory markers in the biological fluids of laboratory animals with reproducible pathology in both acute and chronic inflammation. The new choline biosensor can quickly determine the concentration of inflammatory markers in biological fluids and evaluate and adjust treatment with a new investigational drug. The study requires a small volume of biological fluid for analysis, so laboratory animals are not withdrawn from the experiment to obtain test samples. Still, small portions of blood serum or urine are obtained for dynamic studies.

This study presents the first results of developing a rapid esterase evaluation technique using a reagentless amperometric choline biosensor. The enzyme choline oxidase, immobilized in the biosensor membrane, was an element that ensured high analysis selectivity. The biosensor's efficacy was demonstrated via choline content in hepatitis-type inflammation.

Conflicts of interest.

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this paper.

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Data availability

Data will be provided upon reasonable request.

Use of artificial intelligence tool

The authors confirm that they did not use artificial intelligence technologies in the creation of the presented work.

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