

## PEGylation of antibiotic enrofloxacin and its effects on the state of the antioxidant system in rats

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The antibiotic enrofloxacin's molecular structure has reactive carboxyl groups used for chemical modification and obtained a PEGylated form of enrofloxacin. For this, the nanopolymer polyethylene glycol-400 (PEG-400) fragments were introduced into the molecule of enrofloxacin at the carboxyl group by converting it to the anhydride group. PEGylated enrofloxacin has good solubility in water and was stable. Effects of PEGylated enrofloxacin on the antioxidant system were studied. Four groups of rats were formed: one control and three experimental. Rats of the control group were injected intramuscularly with saline. Rats of the first experimental group were injected intramuscularly with the antibiotic enrofloxacin, rats of the second experimental group were injected intramuscularly with PEG-400, and animals of the third experimental group of rats were injected intramuscularly with the complex of the antibiotic enrofloxacin with PEG-400. The rats were injected daily for four days. Biochemical studies of rat blood on 7, 14, and 21 days after the last injection showed that the blood TBARS content increased in animals injected with the antibiotic enrofloxacin compared to the control. The administration of enrofloxacin to animals resulted in a decrease of antioxidant enzymes in the blood. When animals were injected with the PEGylated form of the antibiotic enrofloxacin, the blood concentration of TBARS was the lowest, which indicates the absence of toxic effects on the cells of the body. Simultaneously, the activities of SOD, catalase, and GP in the blood of rats treated with PEGylated enrofloxacin were stable and corresponded to the formation of lipid peroxidation products. The activity of antioxidant enzymes was the highest in rats injected with PEGylated enrofloxacin. Therefore, the intramuscular administration of the newly developed PEGylated antibiotic enrofloxacin does not cause the excessive formation of lipid peroxidation products and does not harm the body's antioxidant state.

**Keywords:** rats, antibiotic enrofloxacin, PEG-400, TBARS (thiobarbituric acid reactive substances), superoxide dismutase, catalase, glutathione peroxidase

### Introduction

Enrofloxacin is a representative of the most successful group of synthetic antibiotics – fluoroquinolones. It has a wide range of activities against Gram-negative and Gram-positive bacteria (Tarushi et al., 2010). However, antibiotic enrofloxacin dissolves very poorly in water (Hewitt et al., 2009), which creates difficulties in obtaining optimal doses and limits this drug's bioavailability. Also, enrofloxacin is hygroscopic and has a bitter taste, which is undesirable for oral administration. Therefore, the search for new enrofloxacin compounds with improved characteristics is relevant. The development of new antibiotic compounds should be aimed at changing the molecular structure, which would make it insensitive to the action of protective enzymes of the body and not induce their synthesis. The development of new antibiotic compounds should be aimed at changing the molecular structure, making it insensitive to protective enzymes and not induce their synthesis. At the same time, new drugs should be provided with efficient transport carriers into the bacterial cell (Varvarenko et al., 2015; Chekh et al., 2017). The last must not lose their properties during chemical modifications to the original antibiotic (Posokhova & Viktorov, 2005).

Polyethylene glycol (PEG) is used as an ingredient in the pharmaceutical industry. PEG is biodegradable and biocompatible, does not form toxic metabolites, and is commercially available (Wang et al., 2018; Mozar & Chowdhury, 2018). Polyethylene glycol and the drug's active substance are covalently linked together, forming compounds with improved stability, good solubility in body fluids, and a long half-life (Chen et al., 2008; Dron et al., 2018). The conjugation of a native drug molecule to

polyethylene glycol is called PEGylation. PEGylation is one of the most successful ways to improve drug delivery (Bruce, 2001). In particular, the combination of PEG with various therapeutic biomolecules promotes the active substance's penetration into cells (Nikitin et al., 2005; Barry, 2007). Pegylated peptides are more protected from opsonization and active phago- and endocytosis. The PEG molecule's branched structure helps slow drug metabolism and prolong their blood circulation time (Kozłowski & Harris, 2001). The introduction of drugs into the body causes side effects (Chernushkin et al., 2020). In particular, antibiotics stimulate cellular respiration with the subsequent generation of reactive oxygen species (ROS) (Posokhova & Viktorov, 2005). The increase in ROS's body leads to the development of oxidative stress (Fruehauf & Meyskens, 2007; Vlizlo et al., 2014a; Vlizlo et al., 2014b; Gutyj et al., 2017; Slivinska et al., 2020), which, in turn, leads to the activation of the genes encoding antioxidant enzymes (SOD, CAT, GP) and rapidly mobilizing endogenous antioxidant defenses (Danchuk, 2006; Grymak et al., 2020).

The main purpose of our research is to investigate the effect of the PEGylated form of antibiotic enrofloxacin (E-PEG), the antibiotic enrofloxacin and polyoxyethylene PEG-400 individually on the content of TBARS (thiobarbituric acid reactive substances) and the activity of antioxidant enzymes (SOD, CAT, GPO).

## Materials and Methods

The research was conducted on white male Wistar laboratory 3-month-old rats weighing between 180 g and 200 g. Animals were housed under standard vivarium conditions and were fed with standard compound feed for laboratory rats with free access to drinking water. Four groups of rats were formed: control and three experimental, 12 individuals in each.

Rats of the control group were injected intramuscularly with saline (0.03 ml/animal). Rats of the first experimental group were injected intramuscularly with the antibiotic enrofloxacin (0.03 ml/animal), rats of the second experimental group were injected intramuscularly with PEG-400 (0.03 ml/animal), and the third experimental group of rats was injected intramuscularly with the complex of the antibiotic enrofloxacin with PEG-400 (0.03 ml/animal). The volume of administered drugs corresponded to the dose of enrofloxacin to treat animals (0.03 ml/200g). The rats were injected daily for four days.

Enrofloxacin (Sigma-Aldrich) was pegylated with polyethylene glycol chains PEG-400 of an average molecular weight of 400 Da. The results were compared with the original enrofloxacin and PEG-400, which were used for the synthesis. The animals sacrificed by decapitation on 7, 14, and 21 days after drug administration, blood and body tissues were taken from rats for laboratory tests.

To assess lipid peroxidation (LPO) in the blood plasma of rats, we determined the content of TBARS (thiobarbituric acid reactive substances) by the color reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA).

The status of the antioxidant system was assessed by determining the activity of antioxidant enzymes in the blood – superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP). SOD (superoxide dismutase, EC 1.15.1.1.) activity was measured in red blood cells based on the nitrotetrazolium reduction by superoxide radicals. CAT (catalase, EC 1.11.1.6) activity was tested by color intensity, decreasing the formed complex between hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and molybdenum salts. The rate of oxidation of GSH established GP (glutathione peroxidase, EC 1.11.1.9) activity before and after incubation with tertiary butyl hydroperoxide (Vlizlo et al., 2012).

All procedures were made to minimize animal suffering and were followed the guidelines of the European Convention "For the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and "Common Ethical Principles for Animal Experiments" (Kyiv, 2001). The experiments were carried out following humanity's principles set out in the European Union Directive (DIRECTIVE 2010/63/EU).

## Results

The molecular structure of the antibiotic enrofloxacin (Fig. 1) contains reactive carboxyl groups, through which it is possible to connect various substances and obtain new compounds.

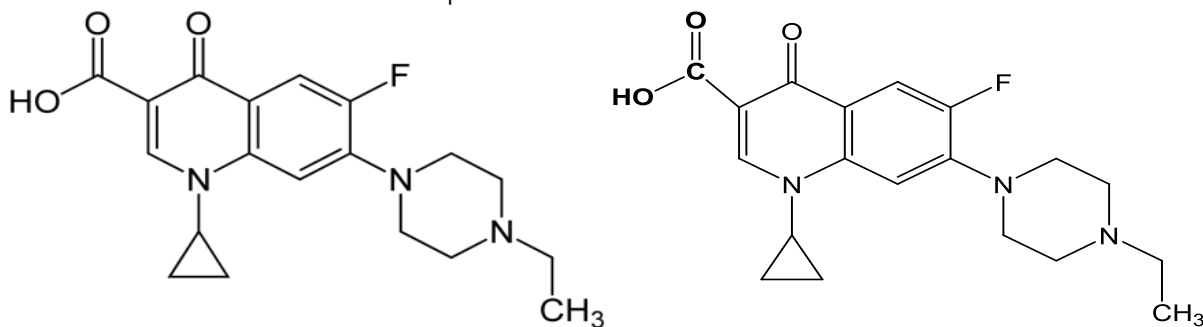
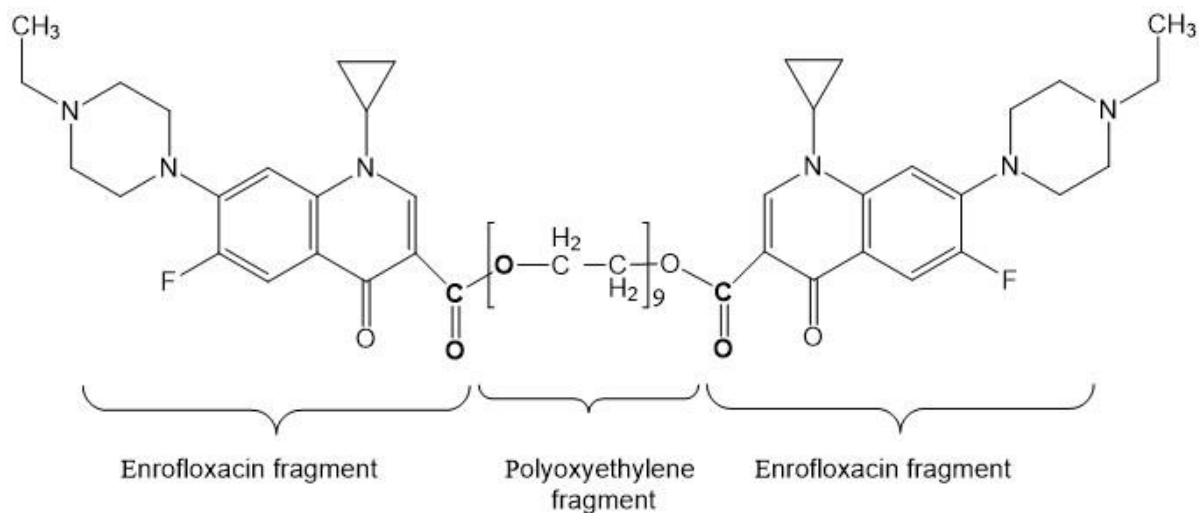


Fig. 1. 1-cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl)-1,4-dihydro-4-oxo-3-quinoline-carboxylic acid (enrofloxacin).

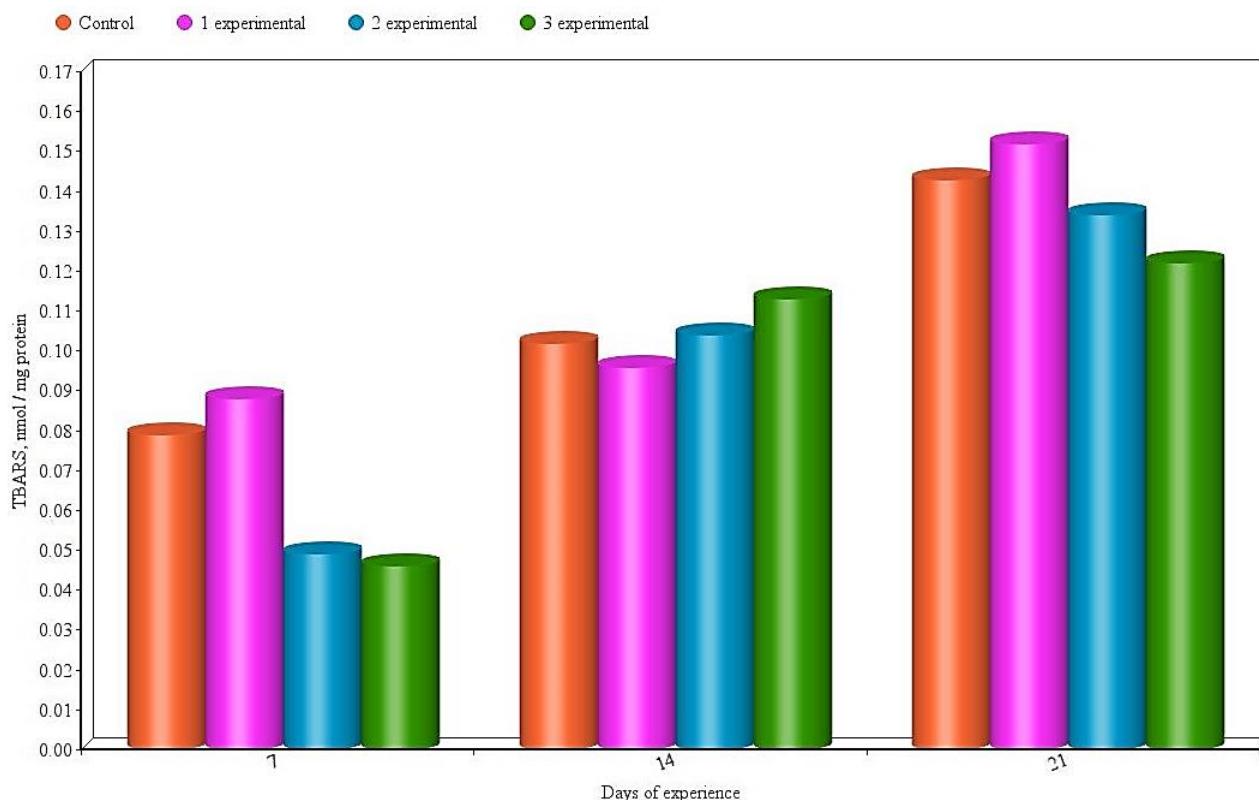
Considering this property of the antibiotic, we carried out the chemical modification of its molecules to obtain a PEGylated form. The PEGylated enrofloxacin structure (E-PEG), shown in Fig.2, has two residues of enrofloxacin molecules joined together by carboxyl groups polyoxyethylene fragment.



**Fig. 2.** The structure of PEGylated enrofloxacin (E-PEG).

Molecules of the PEGylated form of enrofloxacin have a ubiquitous nature. Their structures contain oleophilic residues of enrofloxacin and hydrophilic polyoxyethylene fragment in aqueous solutions with nanometric particle sizes of the dispersed phase. Due to these properties, E-PEG forms self-stabilized dispersions. The stabilization of these particles in an aqueous solution is caused by forming a structural-mechanical barrier of hydrated polyoxyethylene chains around the nucleus, which contains the antibiotic. According to high-performance liquid chromatography, the purity of the PEGylated product was 98–99%.

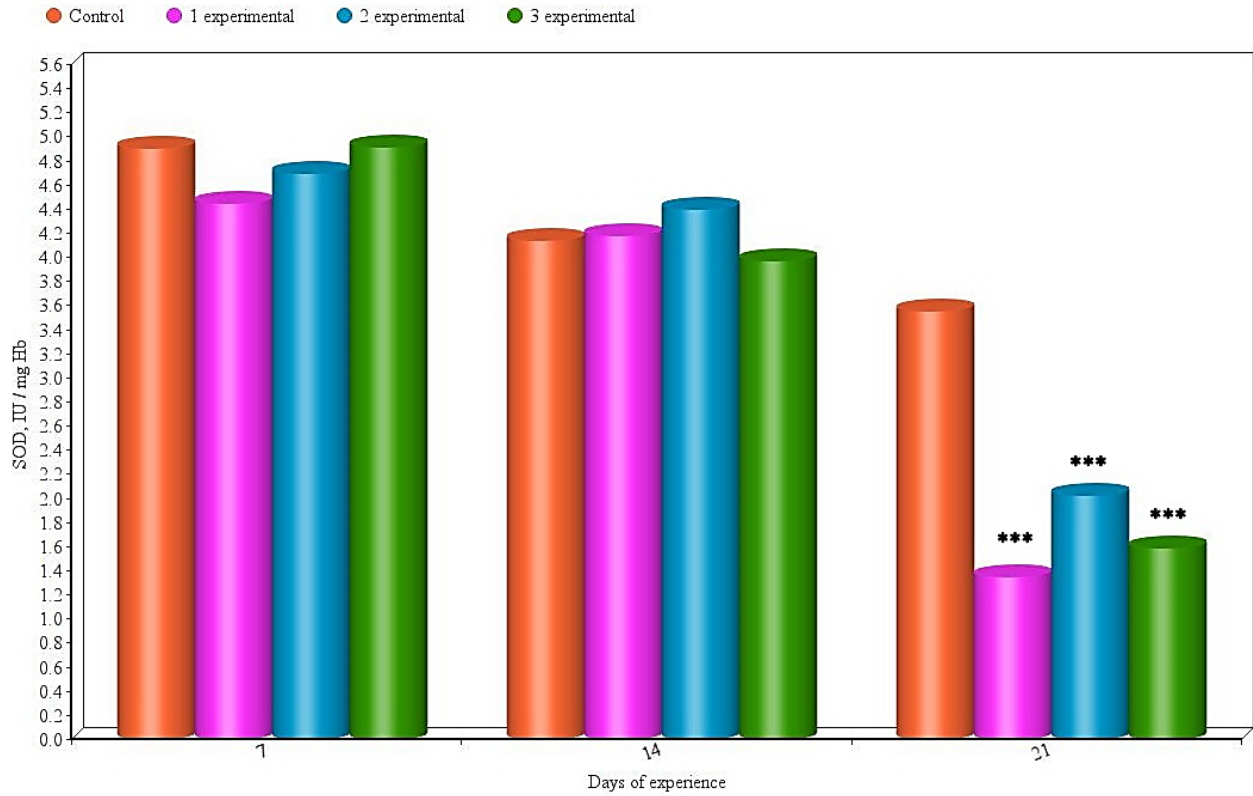
Our studies have shown that the test compounds' introduction did not cause changes in rats' clinical condition of different groups. Weight and general health conditions did not differ from control. The laboratory testing of the blood was performed seven days after four injections of the tested substances. The content of TBARS products in the plasma of animals (Fig. 3), who received 0.03 ml of nanopolymer PEG-400 (second experimental group) and those who were administered 0.03 ml of PEGylated enrofloxacin (third experimental group), was lower by 38% ( $0.049 \pm 0.018$  nmol/mg protein) and by 42% ( $0.046 \pm 0.013$  nmol/mg protein), respectively, than in the control group ( $0.079 \pm 0.015$ ). The content of TBARS increased by 11% ( $0.088 \pm 0.011$  nmol/mg protein) in the blood of animals injected with the antibiotic enrofloxacin (first experimental group).



**Fig. 3.** The concentration of TBARS, nmol/mg protein

The activity of antioxidant enzymes in experimental animals' blood varied depending on the study's time and the injected substance. In the blood of animals injected intramuscularly with PEGylated antibiotic enrofloxacin, SOD activity was at the control group level ( $4.91 \pm 0.43$  and  $4.90 \pm 0.33$  IU/mg Hb, respectively) on the 7th day after the end of the drug administrations

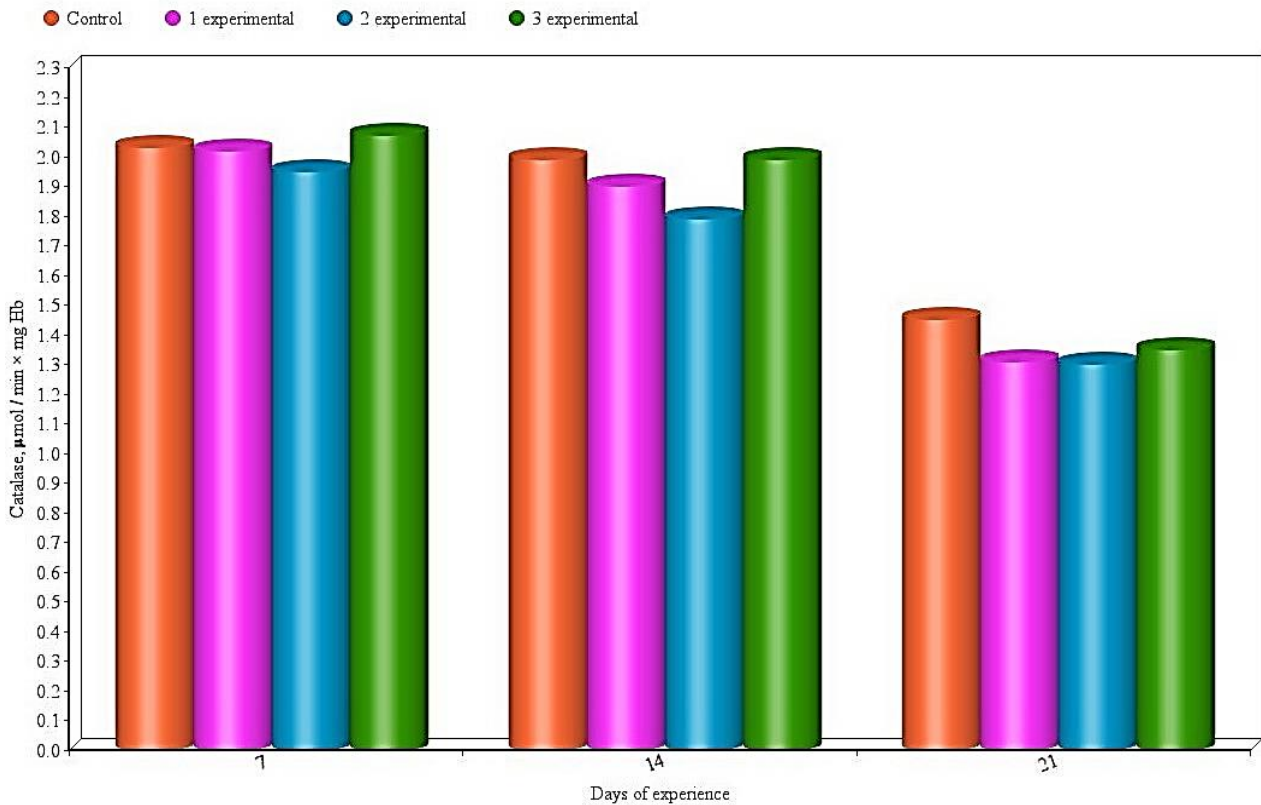
(Fig. 4). After separate administration of the antibiotic enrofloxacin and nanopolymer PEG-400, SOD activities were lower ( $4.45 \pm 0.46$  and  $4.70 \pm 0.26$  IU/mg Hb, respectively) than in control.



**Fig. 4.** SOD activity in the blood of rats, IU/mg Hb.

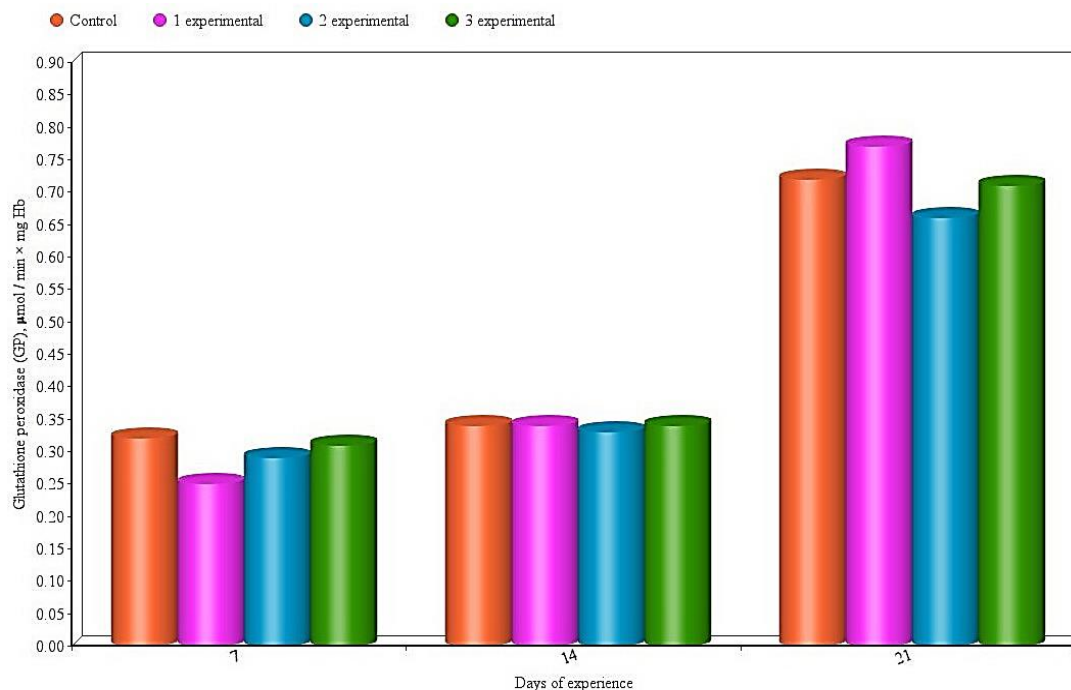
Note. In this and the following figures, the difference is statistically significant compared to the control group when \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

The blood catalase activity has differed little between experimental groups (Fig. 5) 7 days after drug administration. However, it should be noted that the highest CAT activity ( $2.07 \pm 0.23$   $\mu\text{mol}/\text{min} \times \text{mg Hb}$ ) was in animals that received our synthesized E-PEG.



**Fig. 5.** Catalase activity in the blood of rats,  $\mu\text{mol}/\text{min} \times \text{mg Hb}$ .

On the 7th day after the end of the drug introductions, the lowest GP activities were recorded in rats injected with the antibiotic ( $0.25 \pm 0.005 \mu\text{mol}/\text{min} \times \text{mg Hb}$ ). The highest GP activity was in animals injected with PEGylated enrofloxacin ( $0.31 \pm 0.011 \mu\text{mol}/\text{min} \times \text{mg Hb}$ ).



**Fig. 6.** Glutathione peroxidase (GP) activity,  $\mu\text{mol}/\text{min} \times \text{mg Hb}$

On the 14th day after the drug administrations, the content of TBARS in the blood of all experimental groups was almost at the control level (Fig. 3). SOD activities decreased on the 14th day compared with the 7th day's data in all studied animals. CAT activity in the blood of rats differed little compared with the previous study (Fig. 5). The highest activity was recorded in the third experimental group that received E-PEG. GP activity in all groups' blood increased slightly on the 14th day than the first study but remained at the same level (Fig. 6).

After 21 days of the experiment, the highest content of TBARS in the blood was observed in rats injected with enrofloxacin (Fig. 3). The content of TBARS was higher by 6% than in control ( $0.152 \pm 0.027 \mu\text{mol}/\text{mg}$  protein, against  $0.143 \pm 0.038$ , respectively). Simultaneously, the blood concentration of TBARS of rats injected with PEG-400 was lower by 7% ( $0.134 \pm 0.028$ ) than in control. In the group of animals that were injected with PEGylated enrofloxacin, blood TBARS concentration was lower by 17% ( $0.122 \pm 0.080 \mu\text{mol}/\text{mg}$  protein).

Twenty-one days after the end of drug administrations, SOD activity in rats' blood continued to decrease, especially in experimental animals (Table 4). Thus, SOD activity was lower by 62% ( $1.35 \pm 0.10 \text{ IU}/\text{mg Hb}$ ;  $p < 0.001$ ) in rats injected with the antibiotic enrofloxacin, by 43% ( $2.03 \pm 0.16$ ;  $p < 0.001$ ) after nanopolymer PEG-400 and by 55% ( $1.59 \pm 0.09$ ;  $p < 0.001$ ) after PEGylated enrofloxacin, compared to the control ( $3.55 \pm 0.23 \text{ IU}/\text{mg Hb}$ ).

The catalase analysis in the blood of rats 21 days after administering the tested drugs indicated a decrease in enzyme activity of all groups of animals (Fig. 5), compared with previous studies. The lowest catalase activities were found in the blood of animals that received the antibiotic enrofloxacin in pure form ( $1.31 \pm 0.12 \mu\text{mol}/\text{mg Hb}$ ) and nanopolymer PEG-400 ( $1.30 \pm 0.03 \mu\text{mol}/\text{mg}$ ). There was a decrease of 11% and 12% in catalase activities than the control ( $1.45 \pm 0.09 \text{ IU}/\text{mg}$ ). Simultaneously, the catalase activity level in the blood of rats injected with PEGylated enrofloxacin was higher ( $1.35 \pm 0.10 \mu\text{mol}/\text{mg Hb}$ ) than in other experimental groups (Fig. 5).

GP activity in the blood of all groups of rats was the highest on the 21st day after the administration of tested drugs (Fig. 6). During this period, the lowest GP activity of the experimental groups was in animals injected with PEG-400 ( $0.66 \pm 0.012 \mu\text{mol}/\text{min} \times \text{mg Hb}$ ) and the highest - in those who were injected with the antibiotic enrofloxacin ( $0.77 \pm 0.049 \mu\text{mol}/\text{min} \times \text{mg Hb}$ ). In the blood of animals injected with PEGylated enrofloxacin, GP activity was at the control level ( $0.71 \pm 0.061$  and  $0.72 \pm 0.049 \mu\text{mol}/\text{min} \times \text{mg Hb}$ , respectively).

## Discussion

The development of new drugs is aimed at creating a molecular structure that would promote good penetration of active substances into the cells of various organs and systems without causing adverse effects, and the components of the drug must be insensitive to the protective enzymes (Stadnyk et al., 2010; Shaker, Shaaban, 2017; Kozak et al., 2020). Antimicrobial drugs should have a targeted effect on bacterial cells, not promote cyto- and organotoxicity, and be highly effective in treating patients (Posokhova & Viktorov, 2005; Malinovskaya et al., 2017). One way to increase the effectiveness of drugs is to modify their molecules by linking one or more polyethylene glycol (PEG) chains (Nikitin et al., 2005).

Considering this, we PEGylated the antibiotic enrofloxacin using PEG-400 polymer. The carboxyl-terminal ends of enrofloxacin were attached to the polyoxyethylene hydrophilic terminal ends of PEG-400. Drug delivery systems using PEGylation and their

covalent connection with active substances play an essential role in synthesizing new drugs (Webster et al., 2007; Knop et al., 2010).

Our compounds have good solubility in water and are stable, which confirms the thesis that PEGylation promotes the solubility of newly formed substances in body fluids (Chen et al., 2008; Dron et al., 2018) that increases drug delivery efficiency to damaged cells while minimizing toxic effects on the body (Rafiei & Haddadi, 2017; Zelenina et al., 2020).

PEGylated peptides are more protected from opsonization and active phago- and endocytosis by cellular structures (Otsuka et al., 2003; Avgoustakis, 2004). Therefore, we investigated how PEGylated enrofloxacin's introduction affects the formation of TBARS and antioxidant enzymes' activity, as it is known (Posokhova & Viktorov, 2005) that antibiotics stimulate lipid peroxidation. Biochemical studies of rat blood on 7, 14, and 21 days after the last injection showed that the content of TBARS depended on the substance administered and the time of the study. The blood TBARS content increased in animals injected with the antibiotic enrofloxacin, compared to the control. It was significantly higher than in rats injected with PEGylated enrofloxacin and can indicate that the antibiotic enrofloxacin in the traditional form can induce an increase in lipid peroxidation processes. It was found (Kohanski et al., 2007) that fluoroquinolones contain carboxy- and oxy groups in the molecule, which causes the formation of bonds with phospholipids and glycoproteins and leads to disruption of cytoplasmic membrane structures, changes in its electrophysiological characteristics, inactivation of membranes ionic homeostasis and cell damage and death. The drug administration to the animal increases biological membranes' effect by activating free radical oxidation and lipid peroxidation (Janero, 1990; Alekseev et al., 2012). Therefore, probably, antioxidant enzyme activity was the lowest in the blood of animals treated with the traditional antibiotic enrofloxacin, an unfavorable sign that indicates the intensification of lipid peroxidation processes the inadequate response from the antioxidant system.

When animals were administered 0.03 ml of the PEGylated form of the antibiotic enrofloxacin, the blood concentration of TBARS was the lowest, which indicates the absence of toxic effects on the cells of the body. Simultaneously, the activities of SOD, catalase, and GP in the blood of rats treated with PEGylated enrofloxacin were stable and corresponded to the formation of lipid peroxidation products. The activity of antioxidant enzymes was the highest in rats injected with PEGylated enrofloxacin. Therefore, the intramuscular administration of the newly developed PEGylated antibiotic enrofloxacin does not cause the excessive formation of lipid peroxidation products and does not harm the body's antioxidant state.

## Conclusion

Intramuscular administration to experimental rats of the antibiotic enrofloxacin in the traditional form caused the accumulation of TBARS in the blood and reduced antioxidant enzymes' activity (superoxide dismutase, catalase, glutathione peroxidase) on 21 days. PEGylation of the antibiotic enrofloxacin with PEG-400 led to stabilization of TBARS content in the blood and the activity of SOD, catalase, and GP, which should be regarded as inhibition of lipid peroxidation and physiological course of antioxidant protection.

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