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Antimicrobial activity of the PEGylated antibiotic enrofloxacin and its functional and structural effect on the liver in rats

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The aim of the study was to PEGylate the antibiotic enrofloxacin, investigate its antimicrobial activity *in vitro*, and test the effect of intramuscular administration to Wistar rats on the functional state and structure of the liver. PEGylation of enrofloxacin was carried out using polyethylene glycol (nanopolymer PEG-400). To study the antimicrobial activity of PEGylated enrofloxacin and enrofloxacin, the minimum inhibitory concentrations (MIC) were determined using the strain from microbial culture collection: *Escherichia coli* ATCC 11105 and *Staphylococcus aureus* ATCC 6538P. It was found that the MIC of enrofloxacin against *E. coli* was 0.86 µM/l and the MIC of the PEGylated enrofloxacin 0.44 µM/l. The MIC of enrofloxacin and PEGylated enrofloxacin against *S. aureus* were 0.86 µM/l. Four groups of rats (control and three experimental) were formed to study the hepatotoxicity of the enrofloxacin in traditional and PEGylated forms. The animals of the control group were injected intramuscularly with saline in a volume of 0.03 ml. Rats of the first experimental group were injected intramuscularly with enrofloxacin (2.7 mg/kg), the second group with PEG-400 (1.5 mg/kg), and the third group with PEGylated enrofloxacin (2.7 mg/kg). Biochemical studies of blood serum and histological analysis of the liver indicated that the PEGylated enrofloxacin had lower hepatotoxicity than traditional enrofloxacin. The liver morphology of the rats injected with PEGylated enrofloxacin was unchanged and identical to the control group at the end of the experiment.

INTRODUCTION

Fluoroquinolones are considered to be the most promising group of antibacterial drugs due to a wide range of activity against a number of Gram-negative and Gram-positive bacteria (Wright *et al.,* 2000). Enrofloxacin is an antibiotic agent in the fluoroquinolone class used to treat infectious diseases of the urinary tract, pyelonephritis, sexually transmitted diseases, prostatitis, and skin and tissue infections (Tarushi *et al.,* 2010). However, many experts point to increased development of microbial resistance to enrofloxacin (Cohn *et al.,* 2003; Cummings *et al.,* 2014 ; Pereira *et al.,* 2014). In addition, the antibiotic enrofloxacin is sparingly soluble in water, is hygroscopic, and has a bitter taste which limits its use (Hewitt *et al.,* 2009). Therefore, it is important to search for new enrofloxacin compounds with improved therapeutic efficacy and minimal side effects (Dron *et al.,* 2018). Polyethylene glycol (PEG) as a carrier of the active substance is promising for the development of new antibiotic compounds (Bruce, 2001). PEG is water-soluble, biodegradable, and biocompatible because it does not form toxic metabolites and is commercially available (Mozar and Chowdhury, 2018; Wang *et al.,* 2018). The process of chemical attachment of PEG to a native drug molecule is called PEGylation. PEGylation is one of the most successful ways to improve drug delivery to the cell (Barry, 2007; Nikitin *et al.,* 2005). In addition, PEG is a hydrophilic polymer that promotes resistance to plasma protein binding and inhibits aggregation caused by salts and serum

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proteins. Due to this, PEGylated peptides are more protected from opsonization and active phago- and endocytosis of cellular structures of the macroorganism (Avgoustakis, 2004; Otsuka *et al.,* 2003).

An important step in preclinical studies is toxicity testing (Chekh *et al.,* 2017; Kozak *et al.,* 2020). Among therapeutic agents, antibacterial drugs have the most pronounced side effects, which limit their use (Kovtun *et al.,* 2011). One of the manifestations of the side effects of antibiotics is their hepatotoxicity (Polson, 2007).

The aim of the study was to PEGylate the antibiotic enrofloxacin, investigate its antimicrobial activity *in vitro*, evaluate the effect of intramuscular administration to laboratory rats of PEGylated enrofloxacin on the functional state and structure of the liver, and to assess hepatotoxicity.

MATERIALS AND METHODS

Materials

The antibiotic enrofloxacin of purity 99.5% (Sigma-Aldrich) was used for the studies. Also used were PEG-400 (Sigma-Aldrich) and PEGylated antibiotic enrofloxacin (antibiotic enrofloxacin combined with PEG-400), in which the antibiotic content was 1.0% and 1.8%, respectively.

PEGylation

PEGylation was conducted in two steps. In the first step, thionyl chloride in dimethylformamide (DMFA) was used to obtain enrofloxacin chloride by influence on the carboxyl group in the composition. The reaction was carried out for 12 hours at 45°C–50°C. In the second step, the solution of enrofloxacin chloride was added dropwise to a water-dried solution of PEG-400 for 3 hours at 20°C–25°C. The reaction scheme is shown in Figure 1.

In the first step, a 1.3–1.35 molar excess of thionyl chloride was used. For use in the second step, the acid chloride was not isolated from the solution; only partially (not more than half) was the solvent evaporated *in vacuo* to evacuate hydrochloric acid and sulfur dioxide from the solution. The second step was carried out in a 2.3–2.5 molar excess of acid chloride (based on loaded enrofloxacin). To isolate the product from the reaction mass in a vacuum, DMFA was removed, and the resulting viscous mixture was dispersed in a 5% soda solution. The dispersion product was extracted with methyl ethyl ketone. The methyl ethyl ketone was evaporated from the extract; the product was redispersed in water and reextracted with dimethyl ketone. The last procedure was repeated twice. After the last extraction and evaporation of methyl ethyl ketone, a yellowish paste-like mass of the product was obtained. The content and binding efficiency of PEG-400 to enrofloxacin were measured using high-performance liquid chromatography (HPLC).

Chromatography

Chromatography of the test products was carried out on a Waters liquid chromatograph with an Alliance 2690 separation module and a Waters 996 diode array detector. Luna C18, 250 × 4.6 mm, 5 µm was used as a chromatographic column. The mobile phase was a mixture of acetonitrile and 0.2% phosphoric acid in a ratio of 1:1. Enrofloxacin was detected (Fig. 2) at 280 nm (Anacleto, 2018). The flow rate of the mobile phase was 1 ml/minute; the injection volume was 10 µl. Standard solutions of enrofloxacin (concentration 100 µg/ml) were prepared using the mobile phase as a solvent. Samples of polymer+enrofloxacin were diluted with the same solvent to the same concentration.

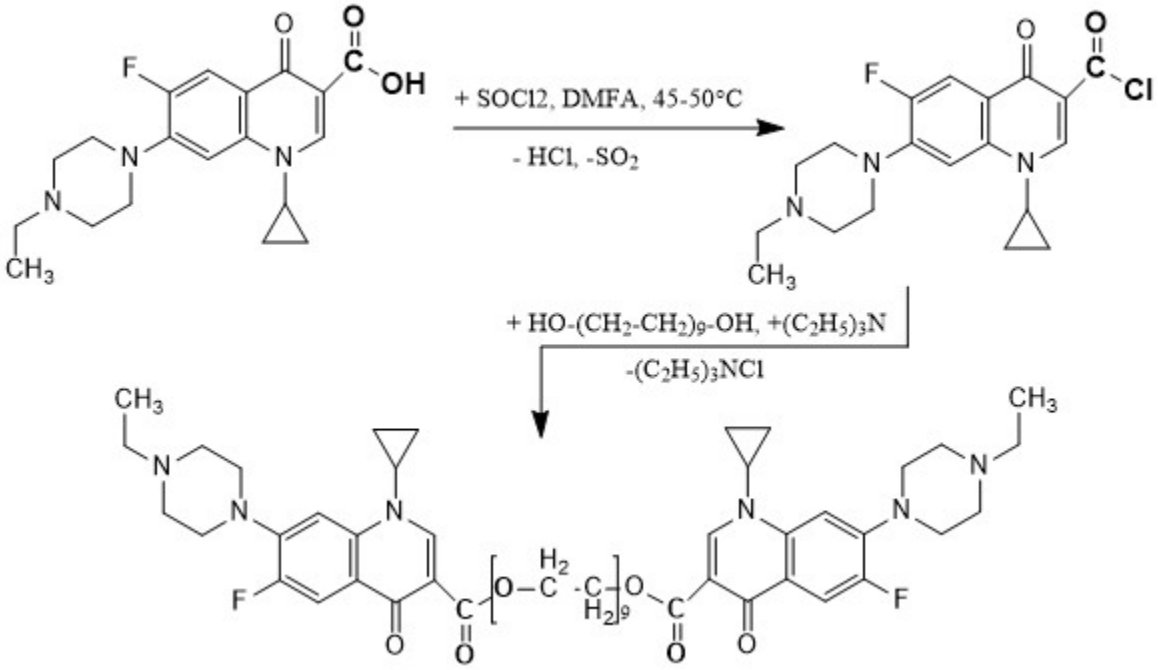

Figure 1. The scheme of the reaction of obtaining PEGylated enrofloxacin through the step of obtaining its acid chloride.

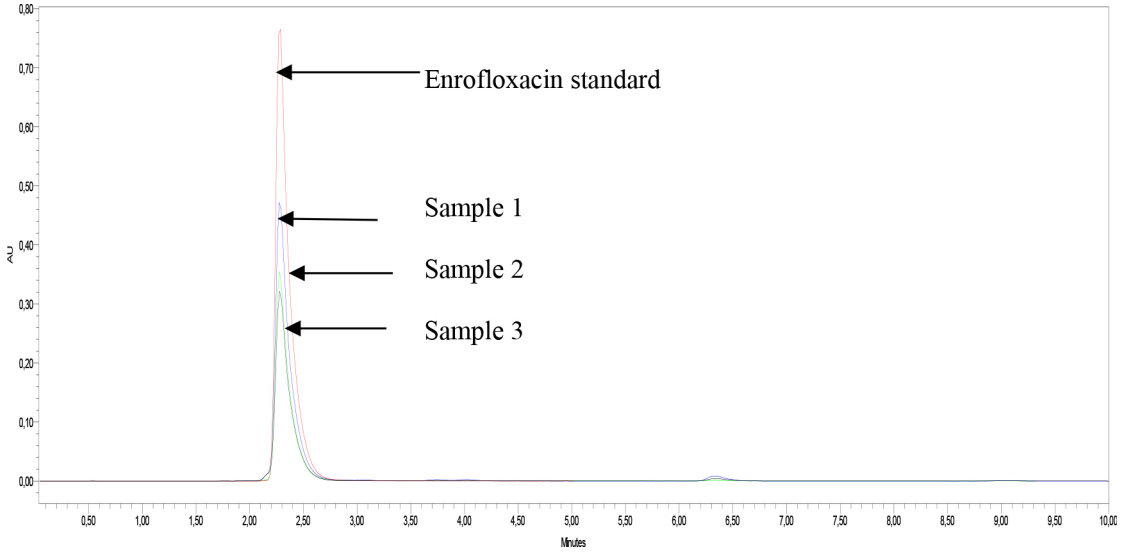

Figure 2. Chromatogram of enrofloxacin PEG-400 samples.

Antimicrobial Activity

To study the antimicrobial activity of PEGylated enrofloxacin and enrofloxacin, the minimum inhibitory concentrations (MIC) were determined using reference museum strains of *Escherichia coli* ATCC 11105 and *Staphylococcus aureus* ATCC 6538P. The MIC of enrofloxacin against *S. aureus* and *E. coli* was determined by serial dilutions in a liquid nutrient medium (meat-peptone broth). 0.1 M NaOH was used to dissolve enrofloxacin, and phosphate buffer pH 8.0 was used for dilution to 55.65 µM/l. The following dilutions of both enrofloxacin and PEGylated enrofloxacin were made from the prepared solution (55.65 µM/l): 27.8, 13.9, 7.0, 3.5, 1.75, 0.9, 0.45, 0.22, 0.11, and 0.055 µM/l. The established sensitivity criteria of *Staphylococcus* spp. and *E. coli* to enrofloxacin were considered: the MIC of enrofloxacin against *Staphylococcus* spp. ≤ 1.39 µM/l: the microorganism is sensitive, 3.59–5.56 µM/l: the microorganism is moderately sensitive, and ≥ 11.12 µM/l: the microorganism is resistant and the MIC of enrofloxacin against *E. coli* ≤ 0.70 µM/l: the microorganism is sensitive, 1.39–3.59 µM/l: the microorganism is moderately sensitive, and ≥ 5.56 µM/l: the microorganism is resistant (NCCLS document M31-A2, 2002).

Hepatotoxicity

Three-month-old healthy male Wistar rats weighing 180–200 g were randomly divided into 4 groups, 1 control and 3 experimental, 12 animals in each group. All rats were given free access to a normal diet and water.

The rats of the control group were injected intramuscularly with saline in a volume of 0.03 ml. Rats of the first experimental group were injected intramuscularly with enrofloxacin in a volume of 0.03 ml, the second group received 0.03 ml of PEG-400, and the third received 0.03 ml of PEGylated enrofloxacin. The dose of the antibiotic enrofloxacin (EMEA, 1995) in the first and third experimental groups was 2.7 mg/kg of body weight of rat per day. The dose of PEG-400 in the second and third experimental groups was 1.5 mg/kg of body weight of rat per day. The drugs were administered daily for a period of 4 days. Animals were removed from the experiment on days 7, 14, and 21 after drug administration by an overdose of thiopental anesthesia (25 mg/kg). Blood was collected from rats during anesthesia, and the liver tissue was collected immediately after death.

Laboratory Tests of the Blood

The activity of aspartate aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; EC 2.6.1.2), alkaline phosphatase (EC 3.1.3.1), and gamma-glutamyltranspeptidase (GGTP; EC 2.3.2.2.) and the contents of cholesterol, total protein, and albumin were determined in the serum collected from the rats (Vlizlo, 2012). Blood chemistry tests were carried out on the Evolution 3000, Italy.

Histology

The liver specimens were placed in a formaldehyde solution (liquid fixing agent). Subsequently, a typical dehydration sequence in ethanol and clearing in xylene was carried out (Vlizlo, 2012). Tissues were then infiltrated with histological wax and formed into blocks. Thin sections (6 µm) of the livers were mounted on glass slides and stained by Romanowsky-Giemsa's method to investigate normal and abnormal structures.

Ethical Conduct in the Care and Use of Animals

Three-month-old healthy male Wistar rats weighing 180–200 g were kindly provided by the State Scientific-Research Control Institute of Veterinary Medicinal Products and Feed Additives. All animals were maintained in a specific pathogen-free animal facility with water and commercial food provided *ad libitum*. The protocol for animal experiments was approved by the Ethical Committee of the Institute of Animal Biology of NAAS of Ukraine, and the experiments were carried out in accordance with the European Convention for the Protection of Vertebrate

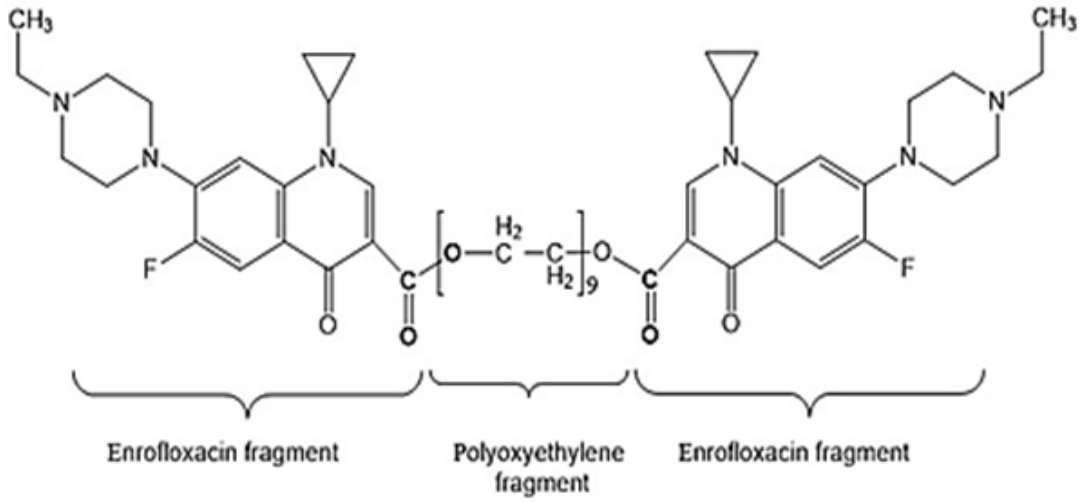

Figure 3. The connection of enrofloxacin to PEG-400 (PEGylated antibiotic enrofloxacin).

Table 1. MIC of enrofloxacin against *E. coli* ATCC 11105 and *S. aureus* ATCC 6538P.

Concentration, µM/l	*Escherichia coli* ATCC 11105		*Staphylococcus aureus* ATCC 6538P	
	Enrofloxacin	PEGylated enrofloxacin	Enrofloxacin	PEGylated enrofloxacin
27.824	—	—	—	—
13.912	—	—	—	—
6.956	—	—	—	—
1.725	—	—	—	—
0.863	+	—	+	+
0.445	+	—	+	+
0.223	+	_	+	+
0.111	+	+	+	+
0.056	+	+	+	+

"—": no growth of the microorganism (transparent broth).

"+": the growth of the microorganism is present (turbid broth).

Animals (Strasbourg, 1986), the principles of humanity set out in the European Union Directive (Directive 2010/63/EU), and the "General Ethical Principles of Animal Experiments," adopted by the First National Congress on Bioethics (Kyiv, 2001).

Statistical Analysis

Statistical calculations of results (M ± m) were carried out using Microsoft Excel 2007. The probability of differences was determined by Student's *t*-test with $p < 0.05$ accepted as statistically significant.

RESULTS AND DISCUSSION

Formation of PEGylated Enrofloxacin

The molecule of enrofloxacin (1-cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl)-1.4-dihydro-4-oxo-3-quinoline-carboxylic acid) contains reactive carboxyl groups, which are suitable for the addition of other substances. At the same time, the high surface hydrophilicity of PEG allows good conjugation with other compounds (Bunkera, 2012; Mishra *et al.,* 2016). Enrofloxacin was PEGylated by attaching to the polyoxyethylene hydrophilic ends of the carboxyl ends of enrofloxacin (Fig. 3). PEG and the antibiotic enrofloxacin were covalently linked.

In this case, a bifil- type macromolecule was modeled, which was able to form a self-stabilized dispersion with particles of the nanometric size of the dispersed phase in aqueous solutions. Stabilization of the particles in the aqueous medium is due to the formation of a structural–mechanical barrier of hydrated polyoxyethylene chains around the nucleus, which contains the antibiotic. HPLC showed that the purity of the PEGylated enrofloxacin was 98%–99%.

PEGylated antibiotic enrofloxacin has good solubility in water and is stable. Other scientists have also pointed out the positive effect of PEGylation on stability, solubility in body fluids, and increasing of the half-life (Chen *et al.,* 2008). PEGylation

facilitates the effectiveness of drug delivery to the affected organs, as well as minimizing the toxic effects on the body (Rafiei and Haddadi, 2017). Covalently linked enrofloxacin to PEG has the highest antibacterial activity. PEG has a positive impact on the cell membrane permeability, thereby increasing the uptake of the antibacterial drug by cells (Chakrabarty *et al.,* 2008; Dron *et al.,* 2018).

Study of Antimicrobial Action of "Pure" Substance and PEGylated Enrofloxacin

It was found that the MIC of enrofloxacin for *E. coli* ATCC 11105 was 0.31 μg/ml (0.86 μM/l) and the MIC of the PEGylated enrofloxacin 0.16 μg/ml (0.44 μM/l) (Table l). The test microorganism was sensitive to the PEGylated enrofloxacin and moderately sensitive to traditional enrofloxacin. The MIC both of traditional enrofloxacin and PEGylated enrofloxacin for *S. aureus* ATCC 6538P were 0.31 μg/ml (0.86 μM/l) (Table l). The test microorganism was sensitive to both types of enrofloxacin. Therefore, PEGylation of enrofloxacin increases the degree of its bacteriostatic activity in relation to *E. coli* ATCC 11105 and does not change it with regard to *S. aureus* ATCC 6538P.

Analysis of the Hepatotoxicity of Drugs

Animal studies indicated the absence of visible physiological changes in both the control and experimental groups. All rats had an appetite, and the dynamics of weight gain did not differ between the groups. However, morphological and functional changes in the organs caused by the drug are usually asymptomatic or subclinical (Babak, 2008). Hepatotoxicity is one of the most common side effects associated with the use of drugs (Chernushkin *et al.,* 2020; Polson, 2007). Liver function blood tests are recommended to monitor the side effects of certain medications known to affect the liver (Ramadori and Cameron, 2010). One of the markers of hepatic cytolysis is increased activity of the liver enzyme (Simonov and Vlizlo, 2015). ALT activity in the serum of rats gradually decreased during the experiment in all the groups (Fig. 4). When comparing the levels of the enzyme in the serum extracted from the rats of the different groups, it was found that ALT activity was significantly lower in the group injected with PEGylated enrofloxacin than in the control group and in the other experimental groups ($p < 0.01–0.001$). ALT activity in the blood of the rats injected with the antibiotic enrofloxacin remained high over three weeks, which may indicate liver cell damage.

AST activity in the serum taken from the rats during the experiment was higher in the experimental groups ($p < 0.001$) compared to that of the control group (Fig. 5). High levels of AST in the blood of the rats injected with the test substances may indicate their active penetration into cells and mitochondria, where this enzyme has high activity. Additionally, due to its branched molecular structure, PEG may slow down the active metabolism of the drug, increasing the circulation time of enrofloxacin in the blood (Bruce, 2001; Kozlowski and Harris, 2001). This may be the reason for elevated AST levels in the blood of the rats injected with PEGylated enrofloxacin and PEG-400. On days 14 and 21, AST activity in the blood of the rats injected with PEGylated enrofloxacin decreased as compared with those injected with the traditional antibiotic enrofloxacin ($p < 0.001$; 0.05).

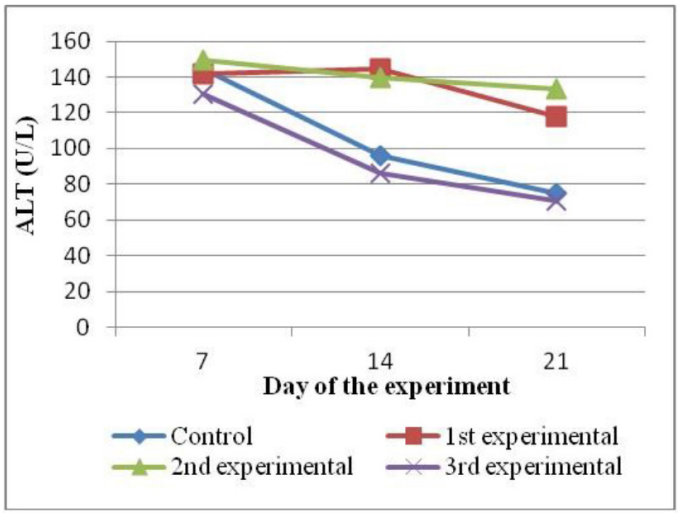

Figure 4. ALT activity in the serum of rats, U/l.

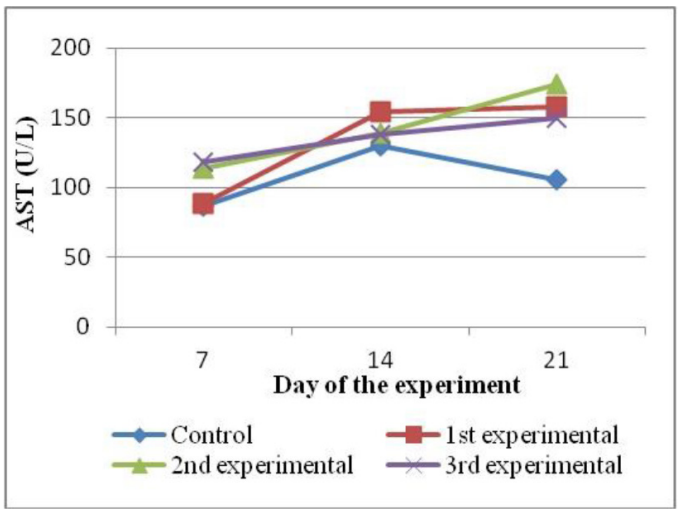

Figure 5. AST activity in the serum of rats, U/l.

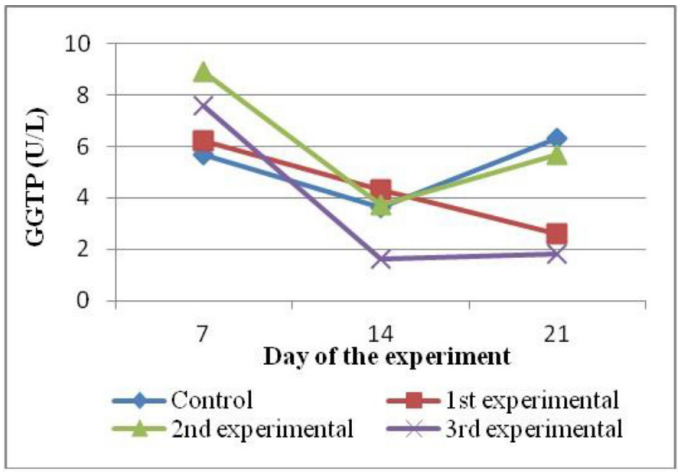

Figure 6. GGTP activity in the serum of rats, U/l.

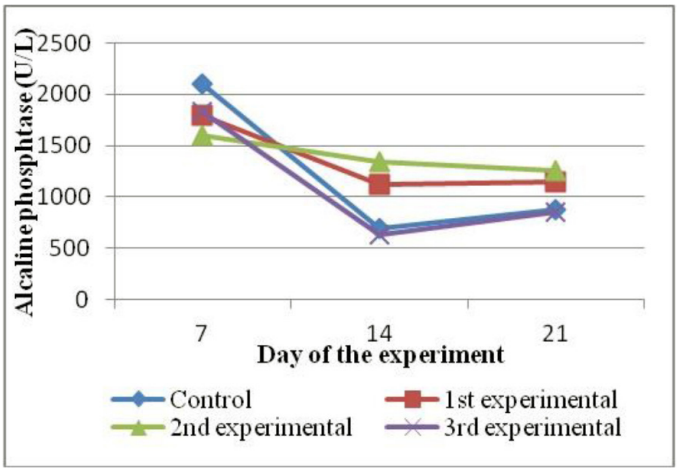

Figure 7. Alkaline phosphatase activity in the serum of rats, U/l

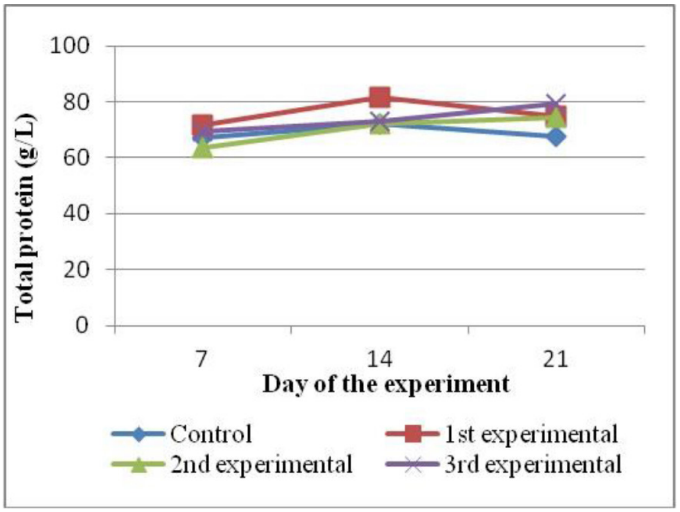

Figure 10. Plasma protein concentration of rats, g/l.

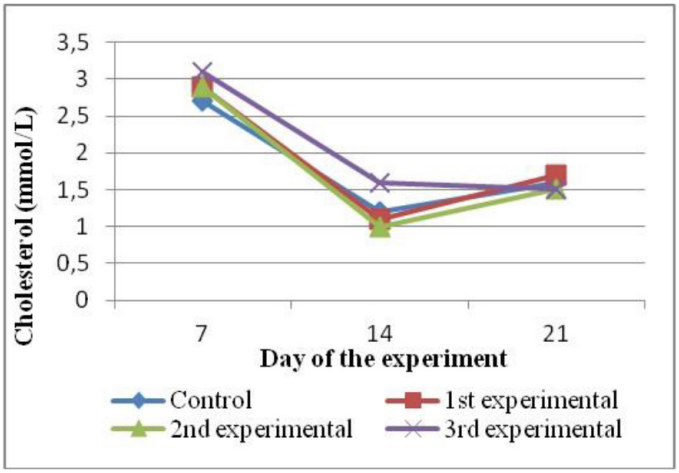

Figure 8. Blood cholesterol levels in rats, mmol/l.

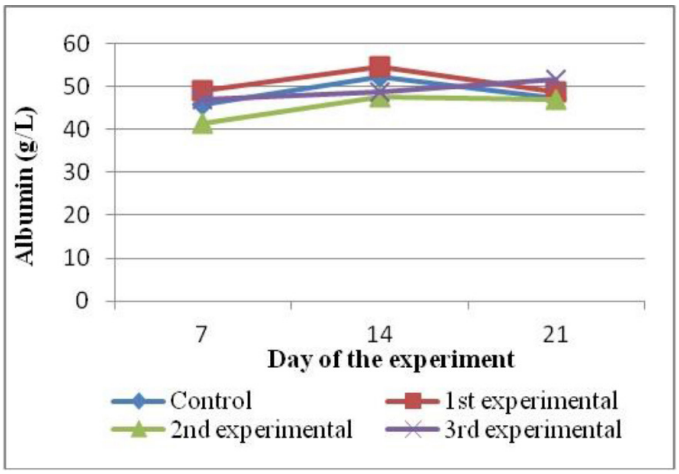

Figure 9. Levels of albumin in the blood of rats, g/l.

According to the dynamics of ALT and AST activity in the blood of animals, it can be concluded that the structure restoration of the liver cells appeared to be faster in the rats injected with PEGylated enrofloxacin as compared to the rats injected with traditional enrofloxacin.

Seven days after the last administration of drugs, the GGTP activity in the serum of the rats from all the experimental groups was higher ($p < 0.05$; 0.001) than in the control group (Fig. 6). High GGTP activity in the blood may indicate intrahepatic cholestasis and damage to the liver cells that form intrahepatic bile ducts (Simonov and Vlizlo, 2015). Fourteen days after the end of the administration of the test substances, GGTP activities in the serum collected from the rats injected with the traditional antibiotic enrofloxacin and PEGylated enrofloxacin were higher than in the control group ($p < 0.01$ and <0.001, respectively). Three weeks after the last administration, blood GGTP activity in the rats injected with the PEGylated enrofloxacin was lower than in both the control group ($p < 0.001$) and the first experimental group, which was injected with traditional enrofloxacin ($p < 0.05$).

On day 7 after the end of the administration of the test drugs, alkaline phosphatase activity was higher in the blood of the control group ($p < 0.001$) than in all three experimental groups (Fig. 7). In the next experimental periods (days 14 and 21), blood alkaline phosphatase activity in the rats treated with the traditional antibiotic enrofloxacin and PEG-400 was higher than in the rats from these two groups: control ($p < 0.001$) and the third experimental group, which was treated with PEGylated enrofloxacin.

Cholesterol content in the blood of the rats differed little between all groups (Fig. 8). Cholesterol levels were elevated in the rats injected intramuscularly with PEGylated enrofloxacin, which can be considered as the stability of its synthesis by liver cells (Janičko *et al.,* 2013).

Three weeks after treatment, albumin content in the blood of the rats was 41.5−54.5 g/l in the control and experimental groups and did not differ between either (Fig. 9). The high albumin concentration in the blood indicated the stability of the protein-synthesizing function of the liver (Carvalho and Machado, 2018).

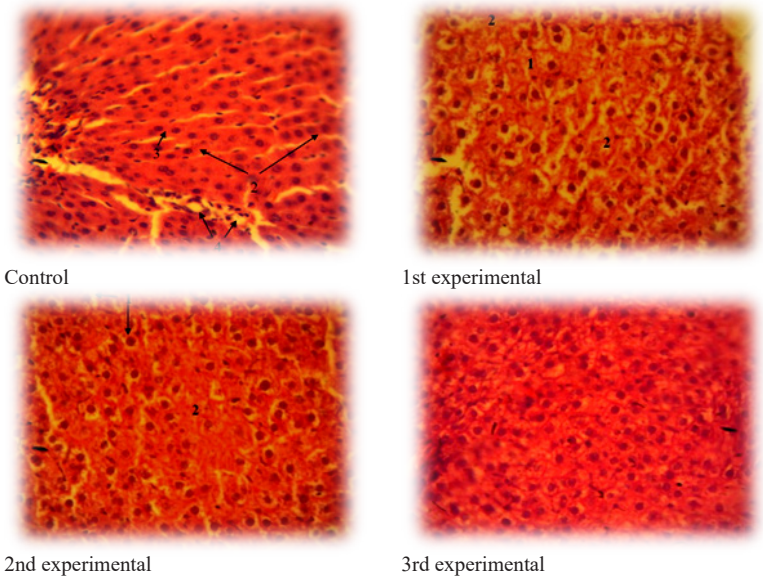

Figure 11. Sections of the liver tissue of rats (× 640).
1: hepatocytes with signs of fatty decomposition, 2: hepatocytes with signs of paranecrosis and necrosis, 3: hepatocytes, and 4: lumens of hemocapillary.

The total protein content in the serum collected from the rats differed little between all the groups (Fig. 10).

On the seventh day after the end of treatment, microscopic examination was conducted on the liver tissue from the rats injected with the traditional antibiotic enrofloxacin. The examination showed signs of granular dystrophy, lysis, and nuclear pyknosis. There were foci characteristic of paranecrosis and necrosis of liver parenchyma (Fig. 11). The livers of the rats treated with PEG-400 did not display any histological changes, but the cytoplasm of some cells was foamy and granular on days 7, 14, and 21. On the seventh day of the experiment, in the livers of rats treated with PEGylated enrofloxacin, it was found that individual liver cells were showing signs of atrophy, granular dystrophy, paranecrosis, and necrosis. On days 14 and 21 of the experiment, the liver morphology was unchanged and identical to the control group.

CONCLUSION

Enrofloxacin was PEGylated by attaching to the polyoxyethylene hydrophilic ends of carboxyl ends of enrofloxacin. The MIC of enrofloxacin against *E. coli* ATCC 11105 was 0.86 µM/l and the MIC of the PEGylated enrofloxacin 0.44 µM/l µg/ml. The MIC of traditional enrofloxacin and PEGylated enrofloxacin against *S. aureus* ATCC 6538P were 0.86 µM/l. Biochemical studies of the rat's blood and histological analysis of the liver indicated that the PEGylated enrofloxacin had lower hepatotoxicity than traditional enrofloxacin.

CONFLICT OF INTERESTS

The authors declared that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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ETHICAL APPROVALS

The protocol for animal experiments was approved by the Ethical Committee of Institute of Animal Biology of NAAS of Ukraine.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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