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ACTIVITY OF GLUTATHIONE PEROXIDASE IN LOACH EMBRYOS UNDER THE INFLUENCE OF FLUOROQUINOLON ANTIBIOTICS

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Background. In modern biology, a search test system that would allow adequate assessment of the effect of pharmacological agents on the human and animal body remains relevant. The use of embryonic objects is promising in the study of the toxicity of low-level substances, in particular, antibiotics of the fluoroquinolone class, borocin and flumiquil. They have a wide range of action and are used to treat diseases of the central nervous system, inflammatory processes, diseases of the endocrine system. Additionally, these antibiotics can serve as anticancer and antiviral drugs. However, the degree of toxicity and the mechanism of their effect on the cell have not been fully studied. Glutathione chain enzymes, particularly glutathione peroxidase activity, can serve as a sensitive indicator for assessing the body's response to external influences.

This study aimed at exploring the possibilities of using embryonic objects as a test system for testing the effects of harmful substances. In particular, we investigated the activity of glutathione peroxidase in loach embryos under the influence of flumiquil and borocin in concentrations of 2.5; 1.5 and 0.25 μg/mL during early embryogenesis (after 1, 3, and 5 hours of development).

Materials and Methods. The research was carried out on embryos of the loach *Missgurnus fossilis* L., eggs were obtained and fertilized according to A. A. Neifakh. Ovulation was stimulated by intraperitoneal injection of the human placental hormone – chorionic gonadotropin in loach females acclimatized at 18 °C. Within a period of 36–48 hours following the injection of gonadotropin, caviar was obtained and fertilized in Petri dishes with a suspension of sperm in settled tap water obtained "ex tempore" from the testes of males (caviar retains the ability to be fertilized for 5 minutes). After 1 min, the zygotes were finally washed from the excess sperm, the caviar attached to the bottom of the cup was rinsed, and in the control series they were incubated in Holtfreter's solution at a temperature of 20–22 °C. The concentration of reduced glutathione before and after the incubation was determined colorimetrically. The basis of the development of the color

reaction is the interaction of the SH-group with 5,5′ dithio-bis (2-nitrobenzoic) acid (DTNBA) with the formation of a colored product – thionitrophenyl anion (TNPA). The amount of the latter is directly proportional to the number of SH-groups that reacted with DTNBA. To find out the effect of the studied fluoroquinolones on the change in the activity of the enzymes of the antioxidant system, borocin and flumiquil at concentrations of 2.5 μg/mL, 1.5 μg/mL and 0.25 μg/mL were added to the medium in which loach embryo cells were cultivated.

Results. We investigated the influence of borocin and flumiquil at concentrations of 0.25, 1.5, and 2.5 μg/mL on the development of the embryos of *Misgurnus fossilis* L. after 1, 3, and 5 hours of exposure. It was found that borocin and flumiquil exert an active effect on the dynamic activity of glutathione peroxidase. The overall activity dynamics under the action of borocin and flumiquil in all three cases was very similar. After 1, 3, and 5 hours of development under the action of borocin, an increase in the activity of the enzyme under study was observed. The highest activating effect was manifested at a concentration of 0.25 μg/mL.

Conclusions. It was found that borocin and flumiquil have an activating effect on the dynamics of glutathione peroxidase activity. The study of enzymes of the glutathione system showed that the GPO activity of loach embryos incubated in the studied media increased the most after 3 hours of development.

Keywords: loach embryos***,*** glutathioperoxidase, antioxidant protection, borocin, flumiquil

INTRODUCTION

The use of embryonic objects is considered to be a promising accelerated method of studying the toxicity of substances influencing them, the concentration of substances inside the embryos, the speed of biochemical processes, the magnitude of cell potential, etc. Changes in the antioxidant state of cells can be used as indicators of response to external influences. These processes change under the influence of physical factors (temperature, action of magnetic and electric fields, laser radiation) and chemical agents (antibiotics, cytostatics, heavy metal ions) (Wu *et al.*, 2016; Nogueira *et al*., 2019; Mahapatra *et al*., 2022; Tryhub *et al*., 2022). Glutathione peroxidase is a key enzyme in the antioxidant protection of cells both under normal conditions and under conditions of oxygen stress. This property of the antioxidant enzyme system can be used to determine how toxic substances or a complex of substances affect the cells, and ultimately the body as a whole (Kovalenko *et al.,* 2019; Liang *et al*., 2020; Moniruzzaman & Saha, 2021; Rosas-Ramírez *et al*., 2022; Handy & Loscalzo, 2022).

The development of science and technology stimulated the widespread use of new antibacterial agents. Among the debatable problems of antimicrobial chemotherapy, the question of the possibility and contraindications of the use of fluoroquinolones in medical practice is one of the most urgent (Golomb *et al.,* 2015; Zivna *et al*., 2016; Xu *et al*., 2019; Jia & Zhao, 2021; Bhatt & Chatterjee, 2022).

The important properties of fluoroquinolones include a wide range of activity against microorganisms resistant to drugs of other classes and high clinical effectiveness in the absence of a proper therapeutic effect of other antimicrobial agents (Zhang *et al*., 2016; Michalak *et al.,* 2017; Coşkun *et al.,* 2018; Xi *et al.,* 2019; Jia *et al*., 2023). However, there are limitations, or more precisely, contraindications for the use of fluoroquinolones in a wide age range. Therefore, the use of loach embryos as a test system is relevant for researching the mechanism of fluoroquinolones.

The purpose of the present study was to investigate the possibility of using embryonic objects as test systems for testing the effects of harmful substances. To achieve the goal, we investigated the activity of glutathione peroxidase, as one of the key enzymes of the antioxidant system, under the influence of borocin and flumiquil at concentrations of 0.25, 1.5, and 2.5 μg/mL at 1, 3, and 5 h of development.

MATERIALS AND METHODS

All the manipulations with animals were conducted according to the International Convention for the Protection of Animals and the Law of Ukraine "On Protection of Animals from Cruelty" (the Minutes of the meeting of Ethics Committee of Ivan Franko National University of Lviv, Ukraine (Protocol No 42-01-2024 of 17.01.2024). In laboratory conditions, the loach were sorted by sex and kept in separate aquariums at a temperature of 4 °C. Oocytes were obtained and fertilized according to A. A. Neifakh (Bodnarchuk *et al.*, 2015). Ovulation was stimulated by intraperitoneal injection of the human placental hormone – chorionic gonadotropin in females acclimatized at 18 °C. Within a period of 36–48 hours following the introduction of gonadotropin to the females, caviar was obtained and fertilized in Petri dishes with a suspension of sperm in settled tap water, obtained "ex tempore" from the testes of males (caviar retains the ability to be fertilized for 5 minutes). After 1 min, the zygotes were thoroughly washed from excess sperm, the caviar attached to the bottom of the cup was rinsed off, and in control series they were incubated in Holtfreter's solution at a temperature of 20–22 °C, n = 100.

The rate of glutathione oxidation in the presence of tertiary butyl hydroperoxide is a measure of glutathione peroxidase enzyme activity. The concentration of reduced glutathione before and after incubation was determined colorimetrically (Bodnarchuk *et al.*, 2015). The basis for the development of the color reaction is the interaction of SH-groups with 5,5′ dithio-bis (2-nitrobenzoic) acid (DTNBA) with the formation of a colored product – thionitrophenyl anion (TNPA). The number of the latter is directly proportional to the number of SH-groups that have reacted with DTNBA.

To clarify the effect of the studied fluoroquinolones on the change in the activity of the enzymes of the antioxidant system, borocin (Borislavsk research institute "Synthesis" with research factory) and flumiquil (LLC. Vetsintez, Ukraine) were added to the medium, in which embryonic cells of loach were cultivated, at concentrations of 2.5 μg/mL, 1.5 μg/mL, and 0.25 μg/mL (Tarnovska *et al.,* 2019) (**Fig. 1** and **2**).

Statistical data processing was carried out using the Excel software package, in particular, the „Data Analysis" package, was used to calculate the main statistical indicators from direct quantitative data obtained as a result of research (arithmetic mean value – M; standard error of the arithmetic mean – m). The data were tested for normality of distribution. Student's coefficient with Bonferroni correction was calculated to assess the significance of differences between the characteristics of two alternative data sets. The difference was considered significant at a reliability index of $p > 0.99$, $p > 0.999$ (or a level of significance of $P < 0.01$, $P < 0.001$, respectively). The processed results were displayed in the form of diagrams.

RESULTS AND DISCUSSION

The research was conducted on the embryos of the loach *Missgurnus fossilis* L., which is widely used today in the study of a number of problems in embryological, biochemical, cytological and other research.

Our previous studies have shown (Bodnarchuk *et al.*, 2015; Tarnovska *&* Yatskyv, 2014; Tarnovska *et al.,* 2018) that when fluoroquinolones affect the intensity of free radical reactions and SOD activity of embryos incubated in Holtfreter's medium, the LPO and SOD systems function at a high activity level. At the stage of two blastomeres in the control, GP activity was 501±12 mmolG–SH/min mg of protein, n = 9. Under the influence of fluoroquinolones in the studied concentrations, the activity of GP embryos probably increased relative to the control (p ≥0.99). After two hours of development (stage of 36 blastomeres) in the control, the enzymatic activity increased (586±10 mmolG–SH/min mg of protein, n = 9), and at the stage of 256 blastomeres (5 hours of development) it decreased (392±17 mmolG–SH/min mg protein, n = 9). Under the influence of fluoroquinolones at these stages of the development of GP, the activity of embryos significantly increased compared to the control (p ≥0.99), (**Fig. 1**, **2**). It is likely that the increase in the enzymatic activity of GP is due to an increase in the concentration of hydrogen peroxide and organic peroxides.

After one hour of development, the greatest effect of borocin on the activity of GP (**Fig. 1**) was observed at a concentration of 0.25 μg/mL (the activity of the enzyme significantly increased to 1175±58 (p ≥0.99) mmolG–SH/min mg of protein, n = 9, compared to the control – 501±12 mmoleG–SH/min mg of protein, n = 9). A slightly lower effect of borocin on the activity of GP was detected at concentrations of 1.5 and 2.5 μg/mL (the enzyme activity significantly increased to 1051±65 (p ≥0.999) and 826±33 (p ≥0.99) mmolG–SH/min mg of protein, n = 9, respectively, compared to the control – 501±12 mmolG–SH/min mg of protein, n = 9).

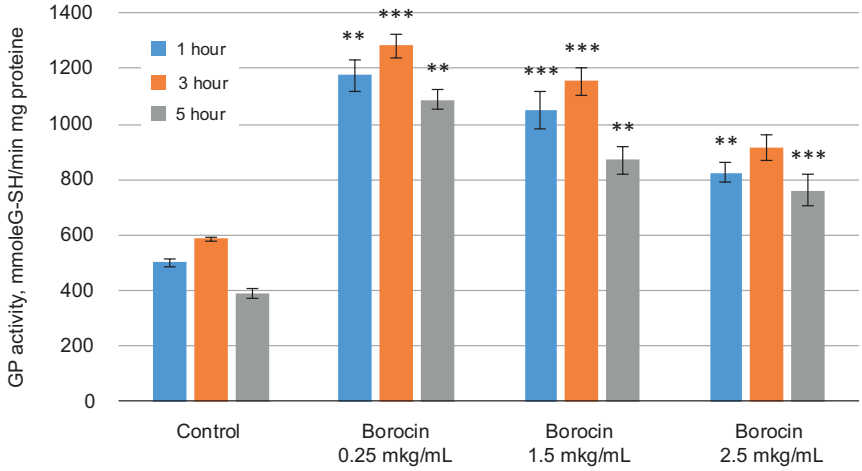

Fig. 1. Glutathione peroxidase activity under exposure to the fluoroquinolone borocin in different concentrations after 1, 3, and 5 h of development, n = 9 (** p >0.99, *** p >0.999, compared to the control)

After 3 hours of development, the greatest effect of borocin on the activity of GP (**Fig. 1**) was observed at a concentration of 0.25 μg/mL (the activity of the enzyme significantly increased to 1283±42 (p ≥0.999) mmolG–SH/min mg of protein, n = 9, compared to the control – 586±10 mmolG–SH/min mg of protein, n = 9). A slightly lower effect of borocin on GP activity was detected at concentrations of 1.5 and 2.5 μg/mL (enzyme activity significantly increased to 1153±52 (p ≥0.999) and 913±47 mmolG–SH/min mg of protein, n = 9, respectively, compared to the control – 586±10 mmolG–SH/min mg of protein, n = 9).

After 5 h of development, the activity of GP under the influence of borocin in the studied concentrations slightly reduced compared to the activity of this enzyme at other stages of development (1 and 3 h of development). The greatest effect of borocin on the activity of GP (**Fig. 1**) was also observed at a concentration of 0.25 μg/mL (the activity of the enzyme significantly increased to 1088±36 (p ≥0.99) mmolG–SH/min mg of protein, n = 9 compared to the control – 392±17 mmolG–SH/min mg of protein, n = 9). A slightly lower effect of borocin on GP activity was detected at such concentrations – 1.5 and 2.5 μg/mL (enzyme activity significantly increased to 870±50 (p≥0.99) and 760±57 (p ≥0.999) mmolG–SH/min mg of protein, n = 9, respectively, compared to the control – 392±17 mmolG–SH/min mg of protein, n = 9).

Similar changes were observed when another fluoroquinolone – flumiquil – was added to the incubation medium. Its concentration of 2.5 μg/mL significantly increases the activity of enzyme after 1 and 3 hours of development (p >0.999), compared to the control.

The lowest concentration of flumiquil – 0.25 μg/mL – led to an increase in GP activity (**Fig. 2**) after 1, 3 and 5 hours of development. All the recorded values were significantly higher compared to the control. The action of different concentrations of flumiquil did not demonstrate significantly greater differences compared with the action of the same concentrations of borocin.

After 1 hour of development, the greatest effect of flumiquil on GP activity (**Fig. 2**) was observed at a concentration of 0.25 μg/mL (enzyme activity significantly increased to 1327±43 (p ≥0.999) mmolG–SH/min mg of protein compared to the control – 501±12 mmolG–SH/min mg of protein, n = 9). A slightly lower effect of flumiquil on GP activity was detected at such concentrations – 1.5 and 2.5 μg/mL (enzyme activity significantly increased to 1153±47 (p ≥0.999), n = 9 and 911±41 mmolG–SH/min mg protein, respectively, compared to the control – 501±12 mmolG–SH/min mg protein, n = 9).

After three hours of development, the greatest effect of flumiquil on the activity of GP (**Fig. 1**) was observed at a concentration of 0.25 μg/mL (the activity of the enzyme significantly increased to 1500±56 (p ≥0.999) mmolG–SH/min mg of protein, n = 9, compared to the control – 586±10 mmolG–SH/min mg of protein, n = 9). A slightly lower effect of borocin on GP activity was detected at such concentrations – 1.5 and 2.5 μg/mL (enzyme activity significantly increased to 1327±75 (p ≥0.999) and 1066±58 (p≥0.999) mmolG–SH/min mg protein, respectively, compared to the control – 586±10 mmolG–SH/min mg protein, n = 9).

Under the effect of borocin and flumiquil on the activity of glutathione peroxidase, a pronounced peak of activity is observed after 3 hours of development.

After 5 h of development, the activity of GP under the influence of borocin in the studied concentrations was slightly lower compared to the activity of this enzyme at other stages of development (1 and 3 h of development). The greatest effect of borocin on GP activity (**Fig. 1**) was also observed at a concentration of 0.25 μg/mL (enzyme activity significantly increased to 1261±21 (p≥0.999) mmolG–SH/min mg of protein compared to the control – 392±17 mmolG–SH/min mg of protein, n = 9). A somewhat lower effect of borocin on GP activity was detected at such enzyme concentrations – 1.5 and 2.5 μg/mL (enzyme activity significantly increased to 1066±28 (p ≥0.999) and 826±75 mmolG–SH/min mg protein, respectively, compared to the control – 392±17 mmolG–SH/min mg protein, n = 9).

In general, it can be concluded that borocin, like fluoroquinolone, has the ability to increase the activity of GP in the studied concentrations. It can be assumed that borocin can act as an initiator of the formation of free radicals, in particular one of the substrates

of glutathione peroxidase – H_2O_2, which can lead to an increase in the activity of the studied glutathione-dependent enzyme.

The activity of enzymes of the "glutathione system" – glutathione peroxidase – increases significantly in samples with the studied fluoroquinolones at all stages of development. An increase in the activity of glutathione peroxidase in the studied sampes might be caused by an increase in the level of active oxygen metabolites, which stimulates the synthesis of enzymes.

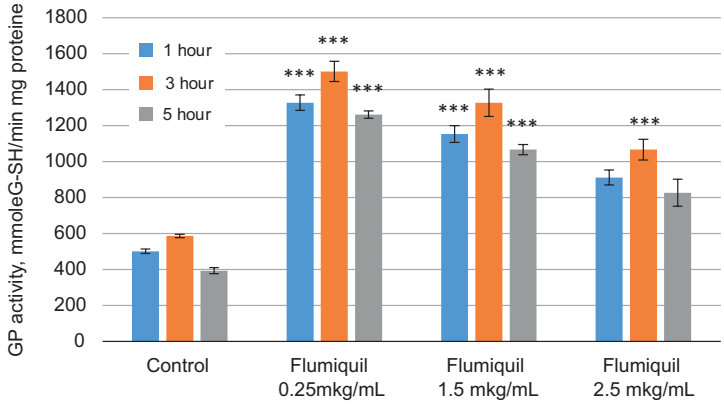

Fig. 2. Glutathione peroxidase activity under exposure to the fluoroquinolone flumiquil in different concentrations after 1, 3 and 5 hours of development, n = 9 (** p > 0.99, *** p > 0.999 compared to the control)

Under exposure to toxic compounds, peroxide accumulates in tissues. It is likely that H_2O_2 or its metabolites play the role of pro-oxidants, and therefore, their action leads to a decrease in the level of antioxidants, therefore the toxic effect is not immediately apparent (Bodnarchuk *et al.*, 2015; Handy & Loscalzo, 2022).

In general, the nature of the dynamics of activity under the action of borocin and flumiquil in all three cases is very similar (**Fig. 1–2**). After one, three and five hours of development under the influence of these antibiotics, an increase in the activity of the enzyme under study was observed. The greatest activating effect was observed at a concentration of 0.25 μg/mL.

It was found that the permeability of fluoroquinolones decreases with the simultaneous use of drugs containing magnesium, iron, calcium, zinc and aluminum ions, with which fluoroquinolones form chelate complexes (Coşkun *et al.*, 2018; Vorobets & Vorobets, 2019; Jia *et al.*, 2023). It was also known that ferric ions take part in the reactions of initiation, branching and termination of the free radical reaction chain, i.e., to manifest its pro-oxidant activity, trivalent iron must be converted into divalent iron. At concentrations of Fe^{2+} higher than critical, it acts as an antioxidant.

Therefore, the studied fluoroquinolones can be considered complex agents that change the activity of ions in reactions that regulate peroxidation. Such compounds, increasing the reaction rate constant of Fe^{2+} with molecular oxygen, accelerate the development of peroxidation due to the acceleration of the formation of $HO^{\bullet}_2$ and $HO^{\bullet}$ radicals. However, in high concentrations, they inhibit peroxidation due to rapid oxidation of Fe^{2+} to Fe^{3+}. We observed a similar reaction when exposing the embryos to borocin and flumiquil at a concentration of 0.25 μg/mL.

The activity of GP under the exposure to flumiquil slightly differs from the activity in the presence of borocin. There are two options for the development of events. The

presence of flumiquil in the medium more activates the passage of the reaction of Fe^{+} with oxygen, thereby leading to an increase in the number of reaction chains and their rapid development. When enough hydroperoxides accumulate in the system, the excessive rate of oxidation of iron by oxygen will only inhibit peroxidation with a decrease in the concentration of Fe^{+}.

According to literature data (Bodnarchuk *et al.*, 2015), in the early stages of loach embryo development, there are two characteristic points: 2–3 hours and 5–6 hours after fertilization. At these points, a change in the intensity of free radical reactions is observed: in the first case (2–3 hours) it increases, and in the second (5–6 hours) it decreases. At a point of 2 hours after fertilization, the intense division of the blastomeres of the embryo begins, which can explain the strong increase in free radical POL and, accordingly, the increase in the activity of antioxidant protection. This could be explained by the intensive membranogenesis during this period.

CONCLUSIONS

Having analyzed the dynamics of the activity of the studied enzyme of antioxidant protection, we can conclude that the nature of the influence of borocin and flumiquil on the dynamics of the activity of GP has an activating effect. It was shown that the lowest concentration of fluoroquinolones (0.25 μg/mL) leads to the greatest increase in GP activity after 1, 3, and 5 hours of development. Studies of the enzymes of the glutathione system showed that the GP activity of loach embryos incubated in the studied media, increased the most after 3 hours of development.

The results obtained suggest that embryos of the loach *Misgurnus fossilis* L., during the stages of synchronous blastomere division, provide a convenient and effective test system for studying the influence of pharmacological and chemical factors on living organisms.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: the article does not contain any experiments with humans.

Animal Rights: all international, national and institutional guidelines for the care and use of animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [A.T.; A.H.]; methodology, [A.T.; A.H.]; validation, [A.T.; A.H.]; formal analysis, [A.T.; A.H.]; investigation, [A.T.; A.H.]; resources, [A.T.; A.H.]; data curation, [A.T.; A.H.]; writing – original draft preparation, [A.T.; A.H.]; writing – review and editing, [A.T.; A.H.]; visualization, [A.T.; A.H.]; supervision, [A.T.; A.H.]; project administration, [A.T.; A.H.]; funding acquisition, [-]. All authors have read and agreed to the published version of the manuscript.

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АКТИВНІСТЬ ГЛУТАТІОНПЕРОКСИДАЗИ У ЗАРОДКІВ В'ЮНА ЗА ВПЛИВУ АНТИБІОТИКІВ КЛАСУ ФТОРХІНОЛОНІВ

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Обґрунтування. У сучасній біології залишається актуальним пошук тест-систем, які давали би змогу адекватно оцінити вплив фармакологічних засобів

на організм людини і тварин. Застосування зародкових об‘єктів є перспективним у дослідженні токсичності низки речовин, зокрема, антибіотиків класу фторхінолонів бороцину та флюміквілу. Останні мають широкий спектр дії та використовуються для лікування захворювань центральної нервової системи, запальних процесів, захворювань ендокринної системи, а також як протиракові та противірусні препарати. Проте ступінь токсичності й механізм їхнього впливу на клітину остаточно не з'ясовані. Ферменти глутатіонової ланки, зокрема, активність глутатіонпероксидази, може слугувати чутливим показником для оцінки реакції організму на зовнішні впливи.

Метою нашої роботи було з'ясувати можливості застосування зародкових об'єктів як тест-систем для перевірки впливу шкідливих речовин. Для досягнення мети ми досліджували активність глутатіонпероксидази у зародків в'юна за впливу флюміквілу та бороцину в концентраціях 2,5; 1,5 і 0,25 мкг/мл протягом раннього ембріогенезу (1-ша, 3-тя і 5-та год розвитку).

Матеріали та методи. Дослідження проводили на зародках в'юна *Missgurnus fossilis* L., яйцеклітини отримували і запліднювали за А. А. Нейфахом. Овуляцію стимулювали за допомогою внутрішньочеревного введення акліматизованим за 18 °С самкам людського плацентарного гормону – гонадотропіну хоріогонічного. Через 36–48 год після введення самкам гонадотропіну отримували ікру і запліднювали її в чашках Петрі суспензією спермієв у відстояній водопровідній воді, отриманій "ex temporе" зі сім'яників самців (час, протягом якого ікра зберігає здатність до запліднення, – 5 хв). Через 1 хв зиготи ретельно відмивали від надлишку спермієв, ополіскували ікру, що прикріпилася до дна чашки, і в контрольних серіях інкубували в розчині Ґольтфретера за температури 20–22 °С. Концентрацію відновленого глутатіону до і після інкубації визначали колориметрично. В основі розвитку кольорової реакції лежить взаємодія SH-груп із 5,5′ дитіо-біс(2-нітробензойною) кислотою (ДТНБК) з утворенням забарвленого продукту – тіонітрофенільного аніону (ТНФА). Кількість останнього прямо пропорційна кількості SH-груп, які прореагували з ДТНБК. Для з'ясування впливу досліджуваних фторхінолонів на зміну активності глутатіонпероксидази в середовище, у якому культивувалися зародкові клітини в'юна, додавали бороцин і флюміквіл у концентраціях 2,5; 1,5 і 0,25 мкг/мл.

Результати. Нами було досліджено вплив бороцину та флюміквілу в концентраціях 0,25; 1,5 і 2,5 мкг/мл на 1-шу, 3-тю і 5-ту год розвитку зародків в'юнів *Misgurnus fossilis* L. Загалом динаміка активності під дією бороцину та флуміквілу в усіх трьох випадках дуже подібна. На 1-шу, 3-тю і 5-ту год розвитку під впливом бороцину спостерігали підвищення активності досліджуваного ферменту. Найбільше активізуюча дія проявляється за концентрації 0,25 мкг/мл.

Висновки. З'ясували, що бороцин і флюміквіл мають активний вплив на динаміку активності глутатіонпероксидази. Дослідження ферментів глутатіонової системи показали, що ГПО активність зародків в'юна, інкубованих у досліджуваних середовищах, найбільше зростає на 3-тю год розвитку.

Ключові слова: зародки в'юна, ранній ембріогенез, глутатіонпероксидаза, фторхінолони

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