ORIGINAL ARTICLE

PROTECTION MECHANISM OF ERYTHROPOIETIN ON CERAMIDE-INDUCED RETINAL DAMAGE

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Abstract

This study aimed to analyse the protective effect and mechanism of erythropoietin (EPO) on ceramide-induced retinal damage. First of all, ceramide was used to induce retinal damage on rats, then EPO effect on retinal damage was studied. Annexin V-FITC/PI cell apoptosis detection kit was used to detect the apoptosis rate of retinal cells (R28) and the obtained apoptosis rates of retina R28 cells were recorded in real time. Results showed that, under the effect of different concentrations of EPO, the release of lactate dehydrogenase (LDH) in the retina had an inhibiting effect on apoptosis, concentration dependent. The staining results of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay showed that, the number of TUNEL positive cells showed a significantly increasing tendency in the ganglion cell layer of retina. In addition, the apoptosis rate of nerve cells was reduced under the effect of EPO. In conclusion, EPO can protect the ceramide-induced retinal damage to a certain extent, which provides a great perspective for further studies.

Rezumat

Obiectivul acestui studiu a fost centrat pe analiza efectului protector şi mecanismul prin care eritropoietina (EPO) protejează împotriva deteriorării retinei, induse de ceramidă. Ceramida a fost utilizată iniţial pentru a induce deteriorarea retinei la şobolani, apoi EPO a fost utilizată pentru a evalua efectul protector şi mecanismul de acţiune în deteriorarea retinei. *Kit*-ul de detecţie a apoptozei celulare Annexin V-FITC/PI a fost utilizat pentru determinarea ratei de apoptoză a celulelor retiniene R28 şi ratele de apoptoză a celulelor retiniene au fost înregistrate în timp real. Rezultatele au arătat că sub efectul diferitelor concentraţii de EPO, eliberarea de lactat dehidrogenază (LDH) la nivelul retinei are un efect antiapoptotic doză-dependent. Rezultatele colorării prin metoda transferazei terminale "*dUTP-biotin nick end labelling*" (TUNEL) au arătat că numărul de celule TUNEL pozitive prezintă o tendinţă de creştere semnificativă în stratul de celule ganglionare ale retinei. În plus, rata de apoptoză a celulelor nervoase a fost redusă sub efectul EPO. În concluzie, EPO este un factor de protecţie împotriva deteriorării retinei, induse de ceramidă.

Keywords: erythropoietin (EPO); retinal damage; ceramide; cell apoptosis

Introduction

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis and is produced in adults mostly by interstitial fibroblasts in the kidney and in the fetal and perinatal period mostly by peri-sinusoidal cells in the liver. Erythropoiesis-simulating agents that contain different types of recombinant human erythropoietin are used to treat different types of anaemia that could appear in chronic kidney disease, and in anaemia associated with antiviral or myelosuppressive chemotherapy with pegInterferon and ribavirin in chronic hepatitis C and B, with zidovudine in HIV-infected patients [6, 31]. In recent years, more and more studies have been carried out on EPO as protecting factor of blood vessels and nerves. The formation of EPO is closely related to the oxygen supply of nervous systems. In the respect of protecting nerves, EPO can effectively relieve the brain cell damage caused by ischemia and anoxia. The use of EPO in oxygen-induced cerebral injury in rodents showed that it acts as an anti-oxidant and antiapoptotic agent but also it modulates inflammatory cascades, autophagy and growth signalling [2, 18, 28, 29]. In the meantime, EPO has also a protective effect on neuronal damage in the cerebral cortex stimulated by free radicals [19, 24, 25]. Studies showed that erythropoietin protects also against CO-induced cardiac ischemia in rats by suppressing apoptosis in myocardial cells [23]. Currently, many researchers in China and abroad have carried out relevant studies. For example, in 2011, Loeliger M. M. *et al.*

[17] stated that the pre-treatment of recombinant human erythropoietin (rhEPO) could reduce the damage of intraocular hypertension on retina as well as it inhibits cell apoptosis. In 2012, Shiyong Wang *et al.* [34] carried out experiments and discovered that EPO pre-treatment had a protective effect on hypoxia damage of nerve cells; besides, the effect was concentration-dependent and time-dependent. In 2013, Chattong S. *et al.* [4] studied the influence of rhEPO on the survival and enation of cultured retinal ganglion cells (RGCs) as well as the expression of growth-associated protein-43 (GAP-43); besides, the potential mechanism of rhEPO on RGCs culture was also considered. The aim of the present study was to explore the protective effect of EPO on ceramide-induced retinal damage.

Materials and Methods

Animals. 15 male Sprague Dawley (SD) rats weighing about 250 g were purchased from Shanghai Slac Laboratory Animal Co., Ltd. The protocol was approved by the Animal Care and Use Committee of Zhengzhou University and relevant ethical approval was obtained.

Main reagents and resources. Low-glucose Dulbecco's modified eagle medium (DMEM) (American Thermo Fisher Scientific). A certain amount of sterile double distilled water was injected into a prepared conical flask. Then DMEM was added for dissolution. After that, sodium bicarbonate was added and the pH value was adjusted to 6.9 ~ 7.1 under the condition of constant volume. The final solution was sterilized. After that, the reagent was added into several 100 mL infusion bottles and each bottle neck was sealed using a kraft paper. At last, the infusion bottles were preserved in a refrigerator at 4°C.

0.25 g of Trypsin (Shanghai Shi Feng Biotechnology Co. Ltd., China) was weighed and added with 100 mL of D-Hnaks; it was dissolved by magnetic stirring and then bacteria were filtered and removed. After that, the solution was transferred to penicillin bottles on the sterile operating platform and preserved at the freezing layer of the refrigerator.

Other reagents used in this study included bovine serum albumin (Shanghai Yan Qi Biotechnology Co., Ltd.); erythropoietin (EPO), ceramide (C2) dimethyl sulfoxide (DMSO) (Shanghai Jing Ke Chemical Technology Co., Ltd.); absolute ethyl alcohol (Shanghai Zi Qi Chemical Technology Co., Ltd.); tri-chloromethane (Changsha Staherb Natural Ingredients Co., Ltd.); isopropanol (Shanghai Zi Qi Chemical Technology Co., Ltd.); glycine (Shanghai Jing Ke Chemical Technology Co., Ltd.); Annexin V-FITC/PI cell apoptosis detection kit (Shanghai Pierce Biotechnology Co. Ltd., China); phosphate buffer saline (PBS) (Shanghai Zi Qi Chemical Technology Co., Ltd.);

Main instruments and equipment. FNIR near-infrared imaging system (Beijing Upwards Teksystems Co., Ltd.), a CO_2 incubator (Guangzhou LabChina Co., Ltd.), a medical clean bench (Soochow Poxiwa Experimental Facilities Co., Ltd.) and a 4°C refrigerator (Qingdao Haier Special Electric Appliance Co., Ltd.) were also used in this study.

Experimental methods.

To develop the retinal damage model R28 cells were treated with different concentrations of ceramides (1, 2, 4, 8 and 16 μM) for 24 h and the concentration with higher apoptosis rate was taken as standard for further experiments involving the protected effect of EPO.

The protected effect of EPO on retinal damage was investigated on R28 cells treated with different concentrations of EPO (2, 4, 8, 16 and 32 U/mL) before exposure to the C2 in concentration found to be appropriate for retinal damage model.

Evaluation of the influence of different concentrations of EPO on release of lactate dehydrogenase was made according to a hypoxia control group.

Separation and culture of rats'retinal cells (R28 cells). Rats were killed by euthanasia [21]. Then the head of rats, especially eyes, were disinfected using 70% ethyl alcohol. Ophthalmology-exclusive tweezers were used to separate rats' eyeballs and the collected eyeballs were washed four times in D-Hank's buffer solution [10, 32, 39]. After that, with the help of micro-scissors, the nerve retina of rats was separated and added into 0.25% trypsin and preserved in a 37°C cell incubator. Cells were cultured using the Dulbecco's modified eagle medium (DMEM) (basic culture medium + 10% fetal bovine serum + 1% penicillin/streptomycin) with a low-sugar concentration of 1 g/L glucose.

Induction of hypoxia in the hypoxia control group. R28 cells in the hypoxia group received hypoxia treatment as following. Sugar in the culture medium was eliminated and cells were put into a sealed container which had two air holes. Nitrogen in 94% concentration and carbon dioxide in 5% concentration were mixed and input into the container through one hole in the speed of 0.5 L/h. After 3 hours, the oxygen-glucose was deprived. The gas which came out from the other hole was tested. When the concentration of oxygen was lower than 0.5%, the container was in the state of anoxia. After that, cells were put into an incubator that contained 5% carbon dioxide and 95% air and the temperature was adjusted to 37ºC for later experiment.

Detection of retina cell apoptosis (TUNEL). R28 cells were maintained into different concentrations (1, 2, 4, 8 and 16 μM) of ceramide for 24 h. Then R28 cellular morphology and activity changes were detected. Annexin V-FITC/PI cell apoptosis detection kit was used to quantify the apoptosis rate of R28 cells. Firstly, frozen sections of retina were blown dry and then cleaned with phosphate buffer saline (PBS). Then trans membrane proteins extraction was carried out for 20 min at room temperature and retina sections were

washed with PBS three times, 5 min for each time. Trans-membrane proteins solubilisation was performed using 0.1% Triton X-100. After that, frozen retina sections were incubated for 10 min as the positive control. The terminal deoxynucleotidyl transferase liquid and the labelling liquid were mixed in the ratio of 1:9 in the first group, while for the second group the labelling liquid only was added. Samples were incubated at 37°C for 1.5 h and then washed with PBS for 3 times 5 min each. Then samples were processed by 0.7 μg/mL of DAPI for half minute for nuclear staining; then they were sealed with DAKO mounting medium. Results were directly observed under a fluorescence microscope [9, 15, 38]. The excitation wavelength was 470 - 500 nm and the number of sections of each group was not fewer than 4. Using a microscope with 400 times, ten random visual fields (0.15 mm^2) were selected to obtain the number of TUNEL chromosome neurons. Then the average cell number of each group was calculated.

LDH assay was performed. according to the methods carried out by Bergmeyer, Bernt and Hess [3], the LDH value being measured using 2.5 mL of Tris-NaCl-NADH buffer solution, 150 μL of enzyme extract and 0.5 mL of Tris-NaCl-pyruvic acid. The absorbance change at 340 nm wavelength was recorded.

*Statistical methods.*SPSS.18 software was used for statistical analysis of experiment data in this study. On the basis of one-way analysis of variance, the least significant difference (LSD) method was used for the further comparison between two groups. $p < 0.05$ means there is a statistically significant difference; $p < 0.01$ means high significance.

Results and Discussion

Influence of different concentrations of ceramides upon the activity of R28 cells. Compared with cells in the normal control group, the number of R28 mature cells after treatment were reduced; moreover, most cells were nonviable. In the meantime, under the influence of different concentrations of ceramides, the observed dose-dependent manner was recorded (Figure 1).

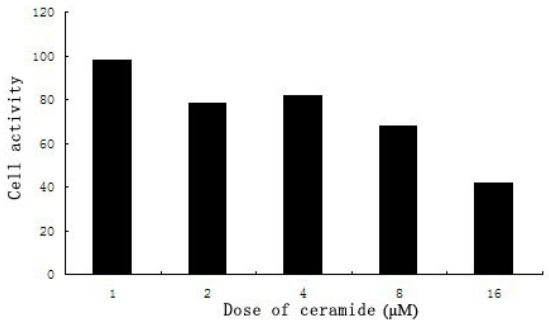

Figure 1.
Activity of R28 cells under the influence of different concentrations of ceramide

Influence of different concentrations of EPO on release of lactate dehydrogenase. The lactate dehydrogenase (LHD) release rate of the hypoxia group was considered as 100%. The LDH release rates of the hypoxia group and EPO pre-treatment group were compared and the data were recorded for drawing curves of LDH release rates. With different final concentrations of EPO (2, 4, 8, 16 and 32 U/mL) before treatment with ceramides in 16 μM added to the culture medium, the LDH release rate were 90.8%, 82.5%, 49.2%, 41.8% and 40.2% respectively. The differences between each group had statistical significance ($p < 0.01$), indicating that different concentrations of EPO could effectively inhibit the release of LDH, which was concentration-dependent. Details are shown in Figure 2.

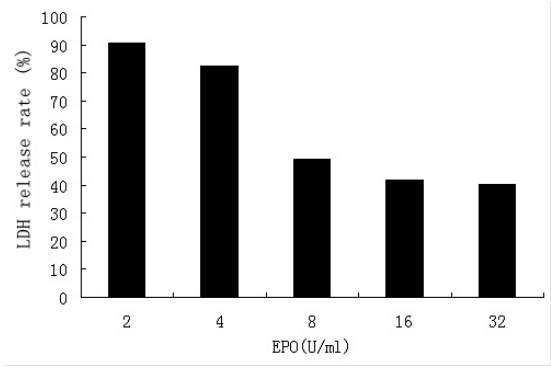

Figure 2.
Release rates of LDH under different concentrations of EPO

Influence of EPO on cell apoptosis. Ceramide plays an important role in nerve cell apoptosis in some severe diseases, which is a potential threat for human health. In recent years, many researchers have used ceramide-induced retina damage model to observe the apoptosis of retinal nerve cells [5, 16, 26, 37]. In order to verify the assumption, we used Annexin V-FITC/PI cell apoptosis detection kit to detect the apoptosis of retinal cells under the effect of ceramide. Results showed that, no cell apoptosis was found in normal control group. However, TUNEL staining results of rat recombinant retina cells after the treatment of ceramide showed that, positive cells were found in the rat retina cells processed by ceramide. In addition, such phenomenon was significant in the ganglion cell layer and the papilla of optic nerve of retina.

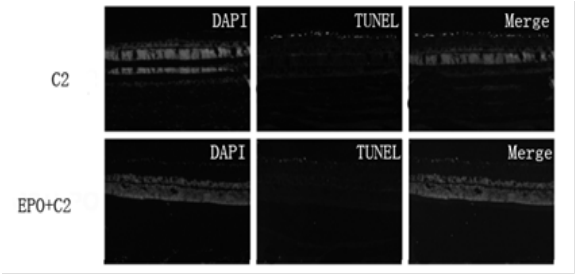

Figure 3.
TUNEL staining results of rat retina cells in different groups

Besides, many authors state in some reports that EPO had a protective effect on nerve injury [39]. Therefore, we carried out relevant experiments to verify whether the EPO pre-treatment could inhibit the ceramide-induced retinal nerve cell apoptosis. Results showed that the staining in EPO treatment group was weakened, indicating that EPO could reduce ceramide-induced nerve cell apoptosis (Figure 3). In the meantime, EPO had certain protective effect on the ceramide-induced retinal damage due to the inhibition of the apoptosis rate.

The number of TUNEL positive granules in the retinal neuron of normal group was small, only 9. However, a large number of TUNEL positive staining cells could be seen in the anoxia group. Moreover, cells began to concentrate and had a rounding tendency; yellowish brown granules were found in cell nucleus and each cell nucleus had different shapes. After the treatment using EPO in 32 U/mL, we found that the number of TUNEL positive cells was reduced.

In recent years, many researchers considered that ceramide played a vital role in the process of nerve apoptosis [12-14, 35]. Such opinion was also reflected in this study. Apoptosis of nerve cells could increase due to the addition of ceramide. Meanwhile, we found that the injection of EPO could significantly reduce the apoptosis rate of nerve cells [7, 8, 30]. EPO modulates the erythropoiesis as well as it protects nerves to a certain extent [1, 11, 27]. The biological roles of EPO are diverse, but recently it is studied in relation to the treatment of optical-damage retinal degeneration or inherited retinal pigment degeneration. Thus it was found that EPO had a significant protective effect on retina photoreceptor cells. Besides, researchers also found that an intraperitoneal injection of EPO could have pre-treatment effect 24 h before ischemia and could also recover retinal functions. However, it did not work if EPO was applied after ischemia. Therefore, EPO could protect acute retinal ischemia-reperfusion injury through an anti-apoptosis mechanism. In addition, a systemic application of EPO could rescue many injured nerve cells [20, 33]. Besides, another study also found that EPO was concentration-dependent and time-dependent during the protective process of nerve cells [36]. Through the experiments on rats' retina cells, this study found that the treatment with C2 could lead to the inflammation of rats' retina cells and the injury of optic nerve functions, while the pre-treatment with EPO could effectively treat the retinal injury caused by C2.

Conclusions

Experiments on ceramide-induced retinal nerve cell apoptosis showed that different concentrations of EPO can have different degrees of inhibiting effect on LDH release, concentration dependent. Meanwhile, the apoptosis rate of nerve cells was reduced due to EPO, the renal glycoprotein showing a protective effect on ceramide-induced retinal nerve injury.

References

1. Baradaran A., Nasri H., Rafieian-Kopaei M., Erythropoietin and renal protection. *Daru J. Fac. Pharm.*, 2013; 21(1): 1-2.
2. Bendix I., Schulze C., Haefen C., Gellhaus A., Endesfelder S., Heumann R., Felderhoff-Mueser U., Sifringer M., Erythropoietin modulates autophagy signaling in the developing rat brain in an in vivo model of Oxygen-Toxicity. *International Journal of Molecular Sciences*, 2012; 13(10): 12939-12951.
3. Bergmeyer H.U., Bernt E., Hess B., Lactated Dehydrogenase Methods. *Enzym. Anal.*, 1974; (2): 574-579.
4. Chattong S., Tanamai J., Kiatsomchai P., Nakatsu M., Sereemaspun A., Pimpha N., Praditpornsilpa K., Rojanathanes R., Sethpakadee A., Tungsanga K., Eiam-Ong S., Manotham K., Glutaraldehyde erythropoietin protects kidney in ischaemia/ reperfusion injury without increasing red blood cell production. *Brit. J. Pharmacol.*, 2013; 168(1): 189-199.
5. Di C.M., Giacomazza D., Picone P., Nuzzo D., San Biagio P.L., Are oxidative stress and mitochondrial dysfunction the key players in the neuro-degenerative diseases?. *Free Radical Res.*, 2012; 46(46): 1327-1338.
6. Docea A.O., Gofita E., Calina D., Zaharie S.I., Valcea D.I., Mitrut P., Autoimmune disorders due to double antiviral therapy with peginterferon and ribavirin in patients with hepatitis c virus infection. *Farmacia*, 2016, 64(4): 605-611.
7. Fabbro A., Sucapane A., Toma F.M., Calura E., Rizzetto L., Carrieri C., Roncaglia P., Martinelli V., Scaini D., Masten L., Turco A., Gustincich S., Prato M., Ballerini L., Adhesion to carbon nano-tube conductive scaffolds forces action-potential appearance in immature rat spinal neurons. *Plos One*, 2013; 8(8): e73621-e73621.
8. Friedmannmorvinski D., Bushong E.A., Ke E., Soda Y., Marumoto T., Singer O., Ellisman M.H., Verma I.M., Dedifferentiation of neurons and

astrocytes by oncogenes can induce gliomas in mice. *Science*, 2012; 338(6110): 1080-1084.
9. Fukuda S., Uchihashi T., Iino R., Okazaki Y., Yoshida M., Igarashi K., Ando T., High-speed atomic force microscope combined with single-molecule fluorescence microscope. *Rev. Sci. Instrum.*, 2013; 84(7): 073706-073706.
10. Godin M., Delgado F.F., Son S., Grover W.H., Bryan AK., Tzur A., Jorgensen P., Payer K., Grossman A.D., Kirschner M.W., Manalis S.R., Using buoyant mass to measure the growth of single cells. *Nat. Methods*, 2010; 7(5): 387-390.
11. Iwai M., Stetler R.A., Xing J., Hu X., Gao Y., Zhang W., Chen J., Cao G.,. Enhanced oligodendrogenesis and recovery of neurological function by erythropoietin after neonatal hypoxic/ischemic brain injury. *Stroke*, 2010; 41(5): 1032-1037.
12. James C., Connolly M., Nanomechanical responses of biopolymer composites determined by nano-indentation with a conical tip. *Eur. J. Neurosci.*, 2014; 21(9): 2311-2319.
13. Kalo D., Roth Z., Involvement of the sphingolipid ceramide in heat-shock-induced apoptosis of bovine oocytes. *Reprod. Fert. Develop.*, 2011; 23(7): 876-888.
14. Kim J.K., Kim S.H., Cho H.Y., Shin H.S., Sung H.R., Jung J.R., Quan M.L., Jiang D.H., Bae H.R., GD3 accumulation in cell surface lipid rafts prior to mitochondrial targeting contributes to amyloid-β-induced apoptosis. *J. Korean Med. Sci.*, 2010; 25(10): 1492-1498.
15. Lehman L.G., Yamadji A.L.N., Sack F.N., Bilong B.C.F., The CyScope® fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. *Am. J. Trop. Med. Hyg.*, 2010; 83(4): 906-908.
16. Li D., Zhu B., Ding L., Lu W., Xu G., Wu J., Role of the mitochondrial pathway in serum deprivation-induced apoptosis of rat endplate cells. *Biochem. Biophys. Res. Commun.*, 2014; 452(3): 354-360.
17. Loeliger M.M., Mackintosh A., De MR, Harding R., Rees S.M., Erythropoietin protects the developing retina in an ovine model of endotoxin-induced retinal injury. *Invest. Ophth. Vis. Sci.*, 2011; 52(5): 2656-2661.
18. Moallem S.A., Mohamadpour A.H., Abnous K., Sankian M., Sadeghnia HR., Tsatsakis A., Shahsavand S., Erythropoietin in the treatment of carbon monoxide neurotoxicity in rat. *Food. Chem. Toxicol.*, 2015; 86: 56-64.
19. Miljus N., Heibeck S., Jarrar M., Micke M., Ostrowski D., Ehrenreich H., Heinrich R., Erythropoietin-mediated protection of insect brain neurons involves JAK and STAT but not PI3K transduction pathways. *Neuroscience*, 2013; 258(1): 218-227.
20. Nickerson P.E.B., Mcleod M.C., Myers T., Clarke D.B., Effects of epidermal growth factor and erythropoietin on Müller glial activation and phenotypic plasticity in the adult mammalian retina. *J. Neurosci. Res.*, 2011; 89(7): 1018-1030.
21. Petra Geier, Michael Lagler, Stefan Boehm, Helmut Kubista, Dynamic interplay of excitatory and inhibitory coupling modes of neuronal L-type calcium channels. *Ajp. Cell Physiol.*, 2011; 300(4): C937-949.
22. Qu Y., Duan Z., Zhao F., Wei D., Zhang J., Tang B., Li J., Yang C., Mu D., Telomerase reverse transcriptase upregulation attenuates astrocyte proliferation and promotes neuronal survival in the hypoxic-ischemic rat brain. *Stroke*, 2011; 42(12): 3542-3550.
23. Rezaee M.A., Mohammadpour A.H., Imenshahidi M., Mahmoudi M., Sankian M., Tsarouhas K., Tsakalof A., Tsatsakis A.M., Moallem S.A., Protective effect of erythropoietin on myocardial apoptosis in rats exposed to carbon monoxide. *Life Sci.*, 2016; 148: 118-124.
24. Ryou M.G., Liu R., Ren M., Sun J.., Mallet R.T., Yang S.H., Pyruvate protects the brain against ischemia-reperfusion injury by activating the erythropoietin signaling pathway. *Stroke*, 2012; 43(4): 1101-1107.
25. Sanders L., Body & brain: Common diuretic could alleviate autism symptoms: Drug that lowers chloride levels in brain cells staves off abnormal behavior in rodents. *Sci. News*, 2014; 185(5): 10-10. -3.
26. Seif S., Malekshahian D., Shamsi M., Prevalence of primary ethambutol resistance in new smear-positive pulmonary TB cases. *Int. J. Mycobacteriol.*, 2015; 4(1): 1-12.
27. Shin T., Ahn M., Moon C., Kim S., Erythropoietin and autoimmune neuroinflammation: lessons from experimental autoimmune encephalomyelitis and experimental autoimmune neuritis. *Anat. Cell Biol.*, 2012; 45(4): 215-220.
28. Sifringer M., Brait D., Weichelt U., Zimmerman G., Endesfelder S., Brehmer F., von Haefen C., Friedman A., Soreq H., Bendix I., Gerstner B., Felderhoff-Mueser U., Erythropoietin attenuates hyperoxia-induced oxidative stress in the developing rat brain. *Brain, Behavior, and Immunity*, 2010; 24(5): 792-799.
29. Sifringer M., Genz K., Brait D., Brehmer F., Löber R., Weichelt U., Kaindl A.M., Gerstner B., Felderhoff-Mueser U., Erythropoietin attenuates hyperoxia-induced cell death by modulation of inflammatory mediators and matrix metalloproteinases. *Develop. Neuroscience*, 2009; 31(5): 394-402.
30. Stanke J., Moose H.E., El-Hodiri H.M., Fisher A.J., Comparative study of Pax2 expression in glial cells in the retina and optic nerve of birds and mammals. *J. Comp. Neurol.*, 2010; 518(12): 2316-2333.
31. Taisescu C.I., Iliescu G., Taisescu O., Predescu A., Andrei A.M., Bogdan C., Siloşi C., Biciuşcă V., Haematological adverse effects of interferon and ribavirin treatment in patients with chronic C virus hepatitis. *Farmacia*, 2015; 63(3): 465-469.
32. Taniguchi Y., Choi P.J., Li G.W., Chen H., Babu M., Hearn J., Emili A., Xie X.S., QuantifyingE. coliProteome and Transcriptome with Single-Molecule Sensitivity in Single *Science*, 2010; 329(5991): 533-538.
33. Teste I.S., Tamos Y.M., Cruz Y.R., Cernada A.M., Rodríguez J.C., Martínez N.S., Antich R.M., González-Quevedo A., Rodríguez J.C., Dose effect evaluation and therapeutic window of the neuro-EPO nasal application for the treatment of the focal

ischemia model in the Mongolian gerbil. *Sci. World J.*, 2012; 2012(2): 223-229.
34. Wang S.Y., Wu Z.T., Chiang P.P., Fink D.J., Mata M., Vector-mediated expression of erythropoietin improves functional outcome after cervical spinal cord contusion injury. *Gene Ther.*, 2012; 19(9): 907-914.
35. Wesley U.V., Hatcher J.F., Dempsey R.J., Sphingomyelin Synthase 1 Regulates Neuro-2a Cell Proliferation and Cell Cycle Progression Through Modulation of p27 Expression and Akt Signaling. *Perspect. Biol. Med.*, 2014; 51(3): 1530-1541.
36. Yin H., Pan X., Song Z., Wang S., Yang L., Sun G., Protective effect of wheat peptides against indomethacin-induced oxidative stress in IEC-6 cells. *Nutrients*, 2014; 6(2): 564-574.
37. Yoshimura S.H., Khan S., Maruyama H., Nakayama Y., Takeyasu K., Fluorescence labeling of carbon nanotubes and visualization of a nanotube-protein hybrid under fluorescence microscope. *Biomacromolecules*, 2011; 12(4): 1200-1204.
38. Zhang K., Martiny A.C., Reppas N.B., Barry K.W., Malek J., Chisholm S.W., Church G.M., Sequencing genomes from single cells by polymerase cloning. *Nat. Biotechnol.*, 2006; 24(6): 680-686.
39. Zhang Y.H., Zhang X.M., Wei X.Y., Protective effect of intravitreal injection of EPO on retinal neuronal apoptosis of rats with diabetes. *Today Health*, 2015; (8): 77-78.